THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 1, Issue of January 5, pp. 458–465, 1996

Printed in U.S.A.

Biochemical Characterization and Molecular Cloning of Cardiac Triadin*

(Received for publication, August 3, 1995, and in revised form, September 22, 1995)

Wei Guo‡, Annelise O. Jorgensen§, Larry R. Jones¶, and Kevin P. Campbell‖

From the Howard Hughes Medical Institute and the Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242, the §Department of Anatomy and Cell Biology, University of Toronto, Toronto, Ontario M5S 1A8, Canada, and the ¶Krannert Institute of Cardiology and the Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana 46202

Triadin is an intrinsic membrane protein first identified in the skeletal muscle junctional sarcoplasmic reticulum and is considered to play an important role in excitation-contraction coupling. Using polyclonal antibodies to skeletal muscle triadin, we have identified and characterized three isoforms in rabbit cardiac muscle. The cDNAs encoding these three isoforms of triadin have been isolated by reverse transcription-polymerase chain reaction and cDNA library screening. The deduced amino acid sequences show that these proteins are identical in their N-terminal sequences, whereas the C-terminal sequences are distinct from each other and from that of skeletal muscle triadin. Based upon both the amino acid sequences and biochemical analysis, all three triadin isoforms share similar membrane topology with skeletal muscle triadin. Immunofluorescence staining of rabbit cardiac muscle with antibodies purified from the homologous region of triadin shows that cardiac triadin is primarily confined to the I-band region of cardiac myocytes, where the junctional and corbular sarcoplasmic reticulum is located. Furthermore, we demonstrate that the conserved region of the luminal domain of triadin is able to bind both the ryanodine receptor and calsequestrin in cardiac muscle. These results suggest that triadin colocalizes with and binds to the ryanodine receptor and calsequestrin and carries out a function in the lumen of the junctional sarcoplasmic reticulum that is important for both skeletal and cardiac muscle excitation-contraction coupling.

Ca^{2+} release from the sarcoplasmic reticulum of skeletal and cardiac muscle is regulated by similar but distinct mechanisms (1–3). In cardiac muscle, depolarization leads to the opening of voltage-gated Ca^{2+} channels. Ca^{2+} influx through L-type Ca^{2+} channels (the dihydropyridine receptor) triggers the opening of the ryanodine receptor/Ca^{2+} release channel in the sarcoplasmic reticulum. However, in skeletal muscle, entry of external Ca^{2+} is not needed for this signal transduction process. The skeletal muscle dihydropyridine receptor interacts either directly or indirectly with the ryanodine receptor, thereby activating the Ca^{2+} release channel without a requirement for extracellular calcium. Despite this difference, many factors that modulate the channel properties of the skeletal muscle ryanodine receptor also affect the cardiac ryanodine receptor in a similar fashion (4, 5). Identification of protein components in the junctional sarcoplasmic reticulum is fundamental to our understanding of the mechanisms of Ca^{2+} storage and release in muscle cells. So far, the major components of the excitation-contraction coupling, such as the ryanodine receptor, the dihydropyridine receptor, and calsequestrin, have been extensively studied in skeletal muscle, and their counterparts have later been identified and characterized in cardiac muscle.

Triadin is an intrinsic membrane protein originally identified in skeletal muscle (6–8). It is specifically enriched in the junctional sarcoplasmic reticulum, where it colocalizes with the ryanodine receptor/Ca^{2+} release channel (6, 8). In addition, triadin probably forms multimers in the lumen of the sarcoplasmic reticulum through disulfide bonds (6–8). Triadin was previously proposed to bind the ryanodine receptor and the dihydropyridine receptor and to serve as the "linking protein" that mediates the signal transduction process between these two Ca^{2+} channels in skeletal muscle (8). Recently, triadin has been found to interact with both the ryanodine receptor and calsequestrin in the lumen of the skeletal muscle sarcoplasmic reticulum (9). These results (9) along with membrane topology analysis (7, 23) suggest that triadin anchors calsequestrin to the junctional face membrane near the sarcoplasmic reticulum "foot" ryanodine receptor and is probably involved in the functional coupling between the Ca^{2+} release channel and the intraluminal calcium-binding protein calsequestrin (10–13).

Recent reports have described the possible existence of triadin in cardiac muscle (14–17). To gain further insight into the function of triadin and to examine the molecular differences between skeletal and cardiac muscle triadin, we have identified and biochemically characterized three cardiac triadin isoforms and subsequently cloned the cDNAs encoding these proteins. Immunofluorescence staining indicates that these cardiac triadin isoforms are primarily confined to the junctional and corbular sarcoplasmic reticulum. In addition, using an affinity binding assay, we found that the conserved region of the luminal domain of triadin is able to interact with both the ryanodine receptor and calsequestrin in the lumen of the cardiac muscle junctional sarcoplasmic reticulum. These results suggest that triadin does not carry out a skeletal muscle-specific function. Instead, it probably plays an important role in both skeletal and cardiac muscle excitation-contraction coupling.

* This work was supported in part by a grant-in-aid from the Heart and Stroke Foundation of Ontario (to A. O. J.) and by National Institutes of Health Grant HL28556 (to L. R. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U31540, U31555, and U34201.

‡ Supported by a predoctoral fellowship from the American Heart Association, Iowa Affiliate.

‖ Investigator of the Howard Hughes Medical Institute. To whom correspondence should be addressed: Howard Hughes Medical Institute/University of Iowa, Dept. of Physiology and Biophysics, 400 Eckstein Medical Research Bldg., Iowa City, IA 52242. Tel.: 319-335-7867; Fax: 319-335-6957; E-mail: kevin-campbell@uiowa.edu.

EXPERIMENTAL PROCEDURES

Generation of Anti-triadin Polyclonal Antibodies—Skeletal muscle triadin luminal domain-GST[1] fusion protein (L-triadin) (9) was used to generate polyclonal antibodies in sheep (Sh33). Also, full-length skeletal muscle triadin was purified from SDS-polyacrylamide gel slices and was used to generate polyclonal antibodies in guinea pigs (GP57 and GP58).

Isolation of Cardiac Microsomes—Fresh whole adult rabbit hearts were rapidly frozen using liquid nitrogen. Cardiac muscle microsomes were prepared from the frozen hearts as described previously (7). Protein samples were analyzed by SDS-PAGE using the buffer system of Laemmli (18) and transferred to nitrocellulose according to Towbin *et al.* (19). The polyclonal antibodies were used for immunoblot assay as described previously (6).

Vesicle Protection Assay of Rabbit Cardiac Muscle Triadin—Trypsin was used at a 1:50 (w/w) ratio to digest rabbit cardiac muscle microsomes in the presence or absence of 0.3% CHAPS for 15 min at 37 °C. The reactions were quenched with 2 mM phenylmethylsulfonyl fluoride and 3% SDS in Laemmli sample buffer (18). The protein samples were separated using 3–15% gradient SDS-PAGE and transferred to nitrocellulose. Polyclonal antibody GP58 was used at a 1:1000 dilution for the immunoblot analysis.

Isolation of cDNAs Encoding Three Isoforms of Cardiac Triadin—Rabbit cardiac muscle total RNA was isolated by homogenization in RNAzol according to the protocol of Cinna/Biotecx followed by chloroform extraction. cDNA was synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase (Promega). A DNA forward primer corresponding to skeletal muscle triadin nucleotides 1–23 (F1) and reverse primers corresponding to nucleotides 818–845 (R2) and 2002–2124 (R1) were used to amplify cardiac triadin sequence from the reverse-transcribed cDNA using polymerase chain reaction (RT-PCR). A DNA fragment of 845 bp was generated by primers F1 and R2. This RT-PCR product was subcloned into pBluescript SK(−), sequenced, and further used to synthesize γ-^{32}P-labeled probe to screen a random-primed adult rabbit cardiac muscle λZAPII library constructed using a predigested λZAPII/*Eco*RI/CIAP cloning kit (Stratagene). Positive clones were plaque-purified and subcloned into pBluescript SK(−) vector for sequencing. The sequence was analyzed using PC/GENE software from IntelliGenetics, Inc. (Mountain View, CA).

Affinity Purification of Polyclonal Antibodies—The DNA fragment corresponding to the conserved region of the luminal domain of triadin (amino acids 69–264) was subcloned into pGEX-2T vector by the *Eco*RI site and expressed as a GST fusion protein (H-triadin). Purified H-triadin was used to purify antibodies GP58 and Sh33 as described previously (24). After purification, anti-GST antibodies were removed with GST fusion protein-nitrocellulose strips.

Immunofluorescence Labeling of Papillary Muscle Cryosections—Dissection, cryofixation, and cryosectioning (6–8 μm) of atrial and ventricular muscle tissues of anesthetized rabbits and dogs were performed as described previously (24, 26). Indirect immunofluorescence labeling of cardiac muscle was carried out as described previously (26). Affinity-purified GP58 antibodies to H-triadin were used for primary labeling. Affinity-purified F(ab′)$_2$ fragments of goat anti-guinea pig immunoglobulin conjugated to fluorescein were used as the secondary reagent. Confocal microscopy was carried out with a photomicroscope (Nikon Inc., Garden City, NY) provided with a confocal fluorescence imaging system (Lasersharp MRC-600, Bio-Rad) using a krypton-argon laser for illumination (29).

Solubilization of Cardiac Muscle Microsomes and Fusion Protein-Sepharose Affinity Binding Assay—Cardiac muscle microsomes were solubilized at a final concentration of 2.5 mg/ml in buffer containing 20 mM Tris-HCl, pH 7.5, 1 M NaCl, 1.5% CHAPS, and protease inhibitors (aprotinin (76.8 nM), benzamidine (0.83 mM), iodoacetamide (1 mM), leupeptin (1.1 μM), pepstatin A (0.7 μM), and phenylmethylsulfonyl fluoride (0.23 mM)) at 4 °C for 1 h. The mixture was then centrifuged using a Beckman TL-100 ultracentrifuge at 100,000 rpm for 15 min. The supernatant was collected and diluted 10-fold in a buffer containing 20 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, and the protease inhibitors as described above (9). The diluted material was either labeled with [^{3}H]ryanodine for subsequent ryanodine receptor binding assay as described previously (9) or directly incubated with triadin-GST fusion protein-glutathione-Sepharose to examine the interaction of triadin with calsequestrin as described previously (9).

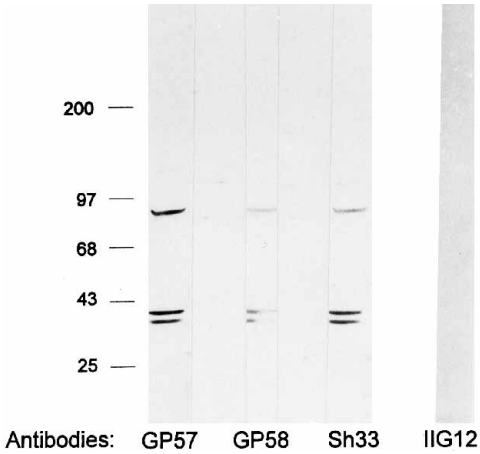

FIG. 1. **Identification of cardiac triadin isoforms by Western blot analysis.** Polyclonal antibodies generated from guinea pigs (GP57 and GP58) and sheep (Sh33) and a monoclonal antibody (IIG12) were used to detect triadin in 100 μg of rabbit cardiac microsomes using Western blot analysis. The molecular mass standards (in kilodaltons) are indicated on the left.

RESULTS

Identification of Three Triadin Isoforms in Cardiac Muscle—Polyclonal antibodies generated from skeletal muscle L-triadin-GST fusion protein (Sh33) and polyclonal antibodies generated from native full-length 95-kDa skeletal muscle triadin (GP57 and GP58) all recognized three proteins in rabbit cardiac muscle microsomes (Fig. 1). One of the three proteins has a molecular mass of 92 kDa. The other two have molecular masses of 35 and 40 kDa, respectively. Monoclonal antibody IIG12, which was previously used to characterize skeletal muscle triadin (7), does not recognize any protein in cardiac muscle microsomes (Fig. 1).

Vesicle Protection Assay of Cardiac Triadin—The membrane topology of cardiac triadin was probed using the vesicle protection assay (Fig. 2). After trypsin was applied to cardiac microsomes, all three proteins exhibited a molecular mass shift of ~4 kDa on SDS-PAGE in the absence of the detergent CHAPS. However, in the presence of CHAPS, all the fragments were completely digested. The protease-resistant fragments were probably located in the lumen of the microsomal vesicles and were protected by the sealed lipid membrane. The portion of ~4 kDa digested in the absence of CHAPS represents the cytoplasmic domain of these three cardiac triadin isoforms. This result suggests that the three isoforms of cardiac triadin share a similar membrane topology with their skeletal muscle counterpart (7).

Effects of Sulfhydryl Reagents on Cardiac Triadin—One distinct feature of skeletal muscle triadin is that it migrates as a single band of 95 kDa on SDS-PAGE only in the presence of sulfhydryl-reducing agents. In the presence of *N*-ethylmaleimide, triadin migrates as multimers on SDS-PAGE. Here, we also examined the effects of the sulfhydryl reagents on the migration of the three cardiac triadin isoforms. As shown in Fig. 3, there is no major difference in the migration pattern of cardiac triadin in the presence of either 0.1% β-mercaptoethanol or 5 mM *N*-ethylmaleimide. This is a major difference between triadins in the two different tissues. Brandt *et al.* (16) reported earlier that reducing reagents did not alter the mobility of the large cardiac triadin isoform. Our result is consist-

[1] The abbreviations used are: GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); L-triadin, luminal domain triadin fusion protein; H-triadin, homologous region of the luminal domain triadin fusion protein; C-triadin, cytoplasmic domain of triadin fusion protein.

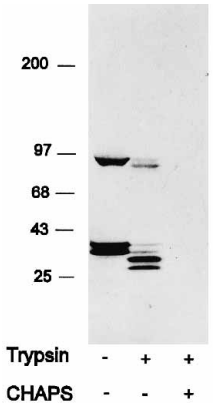

FIG. 2. **Vesicle protection assay of cardiac triadin isoforms.** Rabbit cardiac muscle microsomes were treated with a 1:50 ratio of trypsin in the presence or absence of 0.3% CHAPS for 15 min at 37 °C. The reactions were stopped with 2 mM phenylmethylsulfonyl fluoride. The protein samples were separated by 3–15% SDS-PAGE and transferred to nitrocellulose. The immunoblot was stained with polyclonal antibody GP58. The molecular mass standards (in kilodaltons) are indicated on the left.

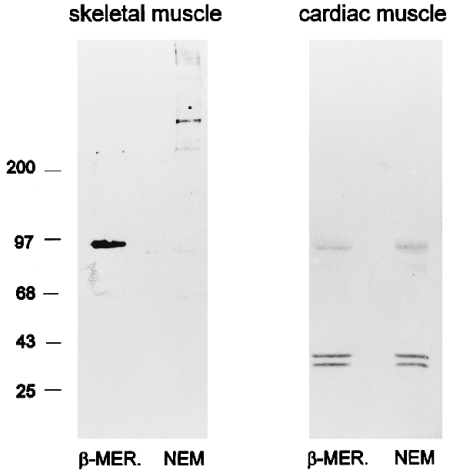

FIG. 3. **Effects of sulfhydryl agents on the migration of triadin on SDS-PAGE.** Rabbit skeletal muscle triads (*left*) and cardiac muscle microsomes (*right*) were run on 3–15% SDS-polyacrylamide gels in the presence of either 0.1% β-mercaptoethanol (*β-MER.*) or 5 mM *N*-ethylmaleimide (*NEM*). After transferring to nitrocellulose, the blots were stained with antibody IIG12 (*left*) or GP58 (*right*) at a 1:1000 dilution. The molecular masses (in kilodaltons) are indicated on the left.

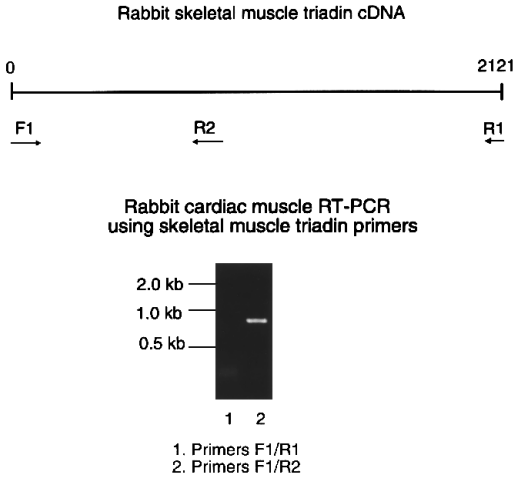

FIG. 4. **Isolation of cardiac muscle triadin DNA sequence by RT-PCR.** Primers corresponding to the skeletal muscle triadin sequences were used to amplify the cardiac triadin sequence from rabbit cardiac muscle RNA (see "Experimental Procedures"). A fragment of 845 base pairs were generated by primers F1 and R2 (*lane 2*). However, no fragment was amplified using primers F1 and R1 (*lane 1*).

ent with their finding. This difference might be explained by the sequence analysis of cardiac triadin (see "Discussion").

cDNA Cloning of Cardiac Triadin Isoforms—Using primers F1 and R2 (see "Experimental Procedures"), a fragment of 845 bp was amplified by RT-PCR from rabbit cardiac muscle poly(A) RNA. However, using primers F1 and R1, which corresponds to the C-terminal sequence of skeletal muscle triadin, no fragment was amplified (Fig. 4). The amplified fragment was subcloned into pBluescript SK(−) vector and sequenced. Interestingly, the nucleotide sequence of this fragment is the same as skeletal muscle sequence 1–845.

This 845-bp fragment was then used as probe for hybridization screening of a λZAPII cDNA library constructed from poly(A) RNA isolated from an adult rabbit cardiac muscle. Two clones that contain open reading frames were named cardiac triadin 1 (CT1) and cardiac triadin 2 (CT2). Their nucleotide sequences and translations are shown in Fig. 5 (*A* and *B*). Both clones share the same 5′-sequences with skeletal muscle triadin until nucleotide 793 and then diverge with the remainder of their sequences. Because of their identity at the 5′-sequences, the predictions for the initiation codons of the cardiac isoforms are the same as those for the skeletal sequence (7). The predicted molecular masses of CT1 and CT2 are 32,097 and 34,596 Da, respectively. These probably correspond to the 35- and 40-kDa doublets detected on the Western blots (Fig. 1). The 35-kDa isoform was solubilized with 2% Triton X-100 and purified from the cardiac sarcoplasmic reticulum using a phosphocellulose column. Three peptide fragments were generated by endoproteinase Asp-N digestion and isolated by high pressure liquid chromatography. The obtained peptide sequences EGNASTTTTV, DPLKLV, and EKPERKI totally match the deduced triadin sequence (Fig. 5*A*).

The homologous region of the small triadin isoforms ends at nucleotide 793. However, the RT-PCR product is 845 bp. The 52-bp sequence at the 3′-end is identical to that of the skeletal sequence, but differs from CT1 and CT2. This sequence probably represents part of the 92-kDa cardiac triadin (CT3) sequence. A forward primer corresponding to nucleotides 795–815 was synthesized (TCAGTATGCATTCTGTCGATA). Using this primer together with pBluescript primer KS, we were able to amplify the 3′-sequence from the λZAPII library to obtain the full-length open reading frame of CT3. Fig. 5*C* shows the nucleotide sequence of CT3 and its translation. In comparison with skeletal muscle triadin (Fig. 6), except for the deletion of GCA from positions 991 to 993, CT3 has the same sequence from nucleotides 1 to 1932. The rest of the 3′-sequence is totally different. The translated protein therefore has a unique C-terminal sequence of 23 amino acids. Fig. 6 shows the alignment of the three cardiac triadin isoforms together with the skeletal muscle sequence. Since the conserved regions of these triadin sequences are the same at the nucleotide level, these isoforms are probably the result of alternative splicing of the same gene. Hydrophobicity analysis of the proteins was conducted according to the method of Kyte and Doolittle (20) using PC/GENE software (Fig. 7). The hydrophobicity plots suggest that all three proteins share similar membrane topology with their skeletal muscle counterpart, although their luminal segments have different lengths.

Cellular and Subcellular Distribution of Triadin in Ventricular Tissue—Since each of the three triadin isoforms in cardiac muscle contains a common homologous luminal domain (H-triadin; amino acids 69–264), the cellular distribution of triadin was probed using two affinity-purified antibodies against H-triadin (Sh33 and GP58). Confocal imaging showed that

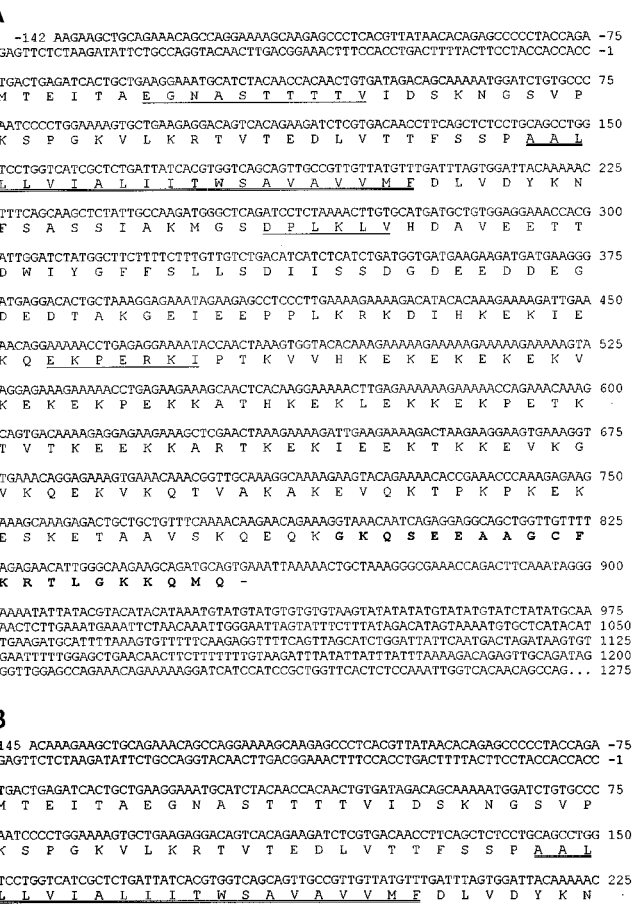

FIG. 5. **cDNA sequences and protein translations of cardiac triadin 1 (A), cardiac triadin 2 (B), and cardiac triadin 3 (C).** The *double underlined* sequences denote the putative transmembrane-spanning domain. The *single underlined* amino acids were confirmed by peptide sequencing. The *boldface* letters indicate the amino acid residues that are unique to the cardiac triadin isoforms.

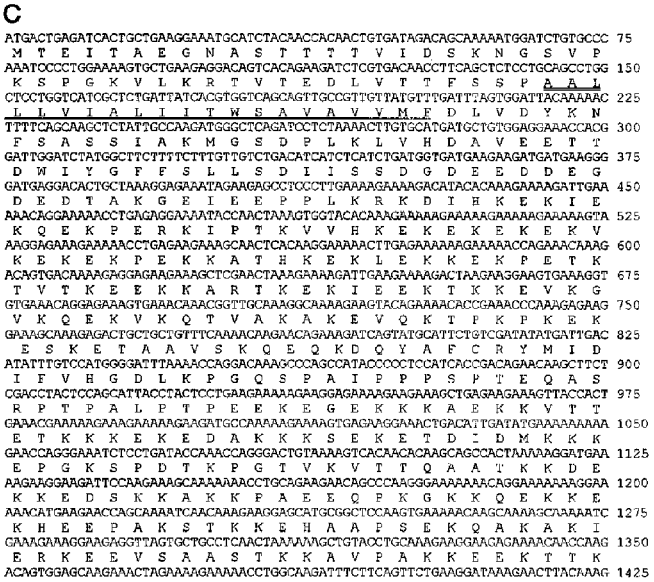

FIG 5—*continued*

specific labeling for H-triadin was densely distributed in rabbit atrial (Fig. 8*a*) and ventricular (Fig. 8, *b* and *c*) myofibers, where it was localized to discrete foci. In contrast, the intensity of labeling of vascular smooth muscle cells (*sm*; Fig. 8*a*), endothelial cells (*E*; Fig. 8*a*), and fibroblasts (not shown) present in the same sections was only marginally higher than that of the background. Since the marginal increase in the labeling intensity was diffusely distributed throughout these cells, it seems unlikely that it represents specific labeling for triadin in these subcellular organelles. Furthermore, as expected, specific labeling of atrial and ventricular myocytes as well as of nonmuscle cells was not detected with monoclonal antibody IIG12 (6, 7), which is specific for the C terminus of skeletal muscle triadin (not shown).

Regarding the subcellular distribution of triadin, confocal imaging of longitudinal cryosections from rabbit papillary muscle shows that specific labeling was mainly confined to parallel strands oriented transversely to the longitudinal axis of the fibers (Fig. 8, *b* and *c*). These strands were frequently resolved into discrete foci (Fig. 8*c*, *white arrows*). Phase-contrast imaging of the same field (Fig. 8*d*) shows that the discrete fluorescent foci were primarily localized to the center of the I-band region (Fig. 8*d*, *white arrows*), where most of the internal junctional and corbular sarcoplasmic reticulum is localized in mammalian myofibers.

Binding of the Triadin Luminal Domain Homologous Region (H-triadin) to the Cardiac Muscle Ryanodine Receptor and Calsequestrin—Previous experiments (9) demonstrated that the luminal domain of skeletal muscle triadin (L-triadin), but not the cytoplasmic domain of triadin (C-triadin), binds both the ryanodine receptor and calsequestrin in the lumen of the sarcoplasmic reticulum. Since the cardiac muscle triadin isoforms have the same luminal sequences from amino acids 69 to 264, we examined whether this homologous region of triadin (H-triadin) was able to bind the cardiac muscle ryanodine receptor and calsequestrin. The solubilized cardiac muscle microsomes were incubated with triadin-GST fusion protein-glutathione-Sepharose, and the bound cardiac muscle calsequestrin was detected by Western blot analysis using anti-calsequestrin polyclonal antibody (21). As shown in Fig. 9*A*, both H-triadin and L-triadin were able to bind to and remove calsequestrin from solubilized cardiac microsomes. As a control, C-triadin was not able to bind calsequestrin.

To examine whether H-triadin interacts with the ryanodine receptor, we prelabeled the solubilized cardiac muscle microsomes with [^{3}H]ryanodine. Triadin-GST fusion protein-Sepha-

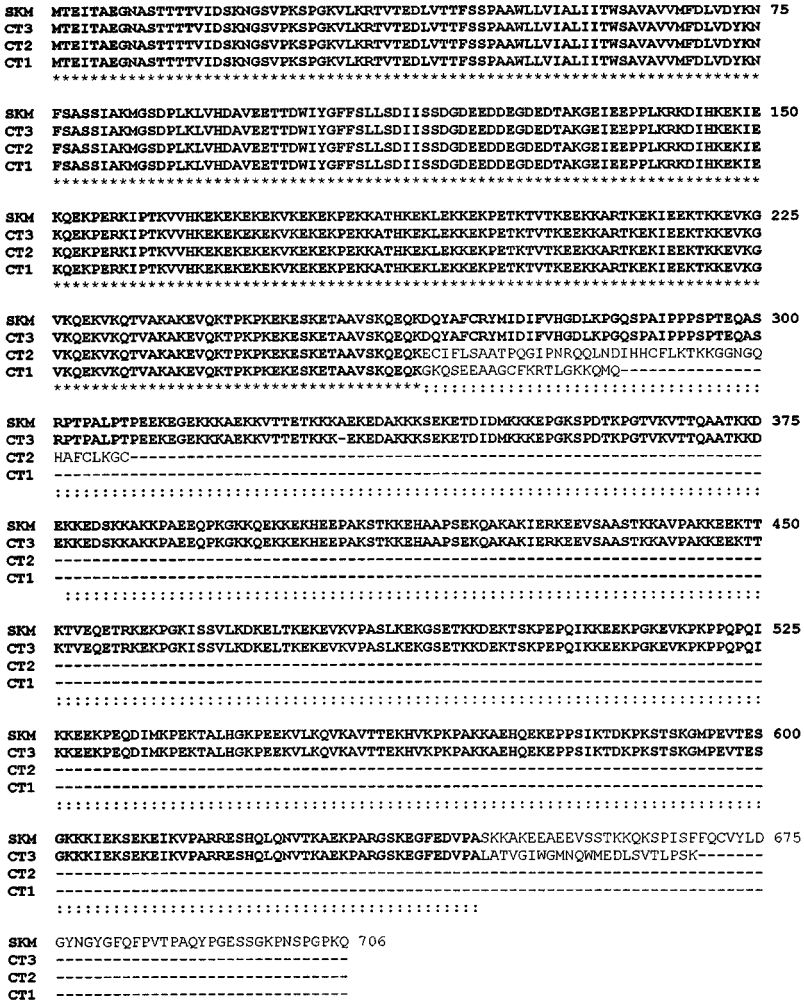

FIG. 6. **Protein sequence comparison of skeletal and cardiac muscle triadin isoforms.** Amino acid sequences of skeletal muscle triadin (*SKM*) and cardiac triadin isoforms CT1, CT2, and CT3 are aligned. The residues that are identical in all the isoforms are indicated by *asterisks*; the conserved sequences between skeletal muscle and CT3 are denoted by *colons*.

rose was incubated with the radiolabeled material, and the amount of bound receptor was determined by counting the [^{3}H]ryanodine on the Sepharose. As shown in Fig. 9*B*, both L-triadin and H-triadin bound the labeled receptor, whereas C-triadin did not bind to the receptor. As a positive control, the ryanodine receptor polyclonal sheep antibody (6) Sepharose was capable of binding to the solubilized ryanodine receptor.

DISCUSSION

We have previously cloned skeletal muscle triadin and found that triadin was present only in skeletal muscle by Northern and Western blot analyses (7). This suggested that triadin carries out a function that is specific to skeletal muscle excitation-contraction coupling. Recently, however, several reports have suggested the existence of triadin in cardiac muscle (14–17). Peng *et al.* (15) were able to detect several messages of triadin in cardiac muscle using Northern blot analysis. Notably, the messages could be identified using a probe corresponding to the 5′-fragment (but not the 3′-fragment) of skeletal muscle triadin. This result suggests that skeletal and cardiac muscle triadin isoforms share similar sequences at the N-terminal region, but differ at their C termini.

Using polyclonal antibodies, we demonstrate the existence of three isoforms of triadin in cardiac muscle microsomes (Fig. 1). One migrates at a molecular mass of 92 kDa, slightly smaller than the skeletal muscle counterpart of 95 kDa. The other two form doublets of 35 and 40 kDa, respectively. Interestingly, none of the proteins could be detected by monoclonal antibody IIG12, which was used to isolate the skeletal muscle triadin cDNA (7). In fact, none of the monoclonal antibodies that were used by Knudson *et al.* (6, 7) to characterize skeletal muscle triadin were able to recognize the cardiac muscle triadin isoforms by either Western blot analysis or immunocytochemical staining (data not shown). The antigenic epitopes for these monoclonal antibodies have now been mapped to the C-terminal region of skeletal muscle triadin (data not shown), further supporting the prediction that the triadin isoforms differ at their C termini.

By RT-PCR and library screening, we have obtained cardiac triadin sequences (Fig. 5). The alignment of triadin sequences is shown in Fig. 6. These sequences are the same until nucleotide 793. For the long cardiac triadin isoform CT3 (Fig. 5*C*), except for the deletion of the trinucleotide GCA at positions 991–993, the nucleotide sequence is identical to that of skeletal muscle triadin from nucleotides 1 to 1932. The rest of the sequence is different. As a result of the GCA deletion, an alanine residue at position 331 is missing. The translated protein is therefore basically homologous to skeletal muscle triadin amino acids 1–665, but has a unique C terminus of 23 amino acids. The cardiac triadin isoform CT3 is 39 amino acids shorter than its skeletal counterpart, which explains why it has a slightly smaller molecular mass than skeletal muscle triadin as detected by Western blot analysis.

In skeletal muscle triadin, there are two cysteine residues at positions 270 and 671 in the proposed luminal segment. These two residues are believed to be responsible for the disulfide-linked homomultimeric structure of triadin (7). Interestingly, for CT3, there is only one cysteine residue (position 270) in the luminal segment. Fig. 3 demonstrates that CT3 only migrates

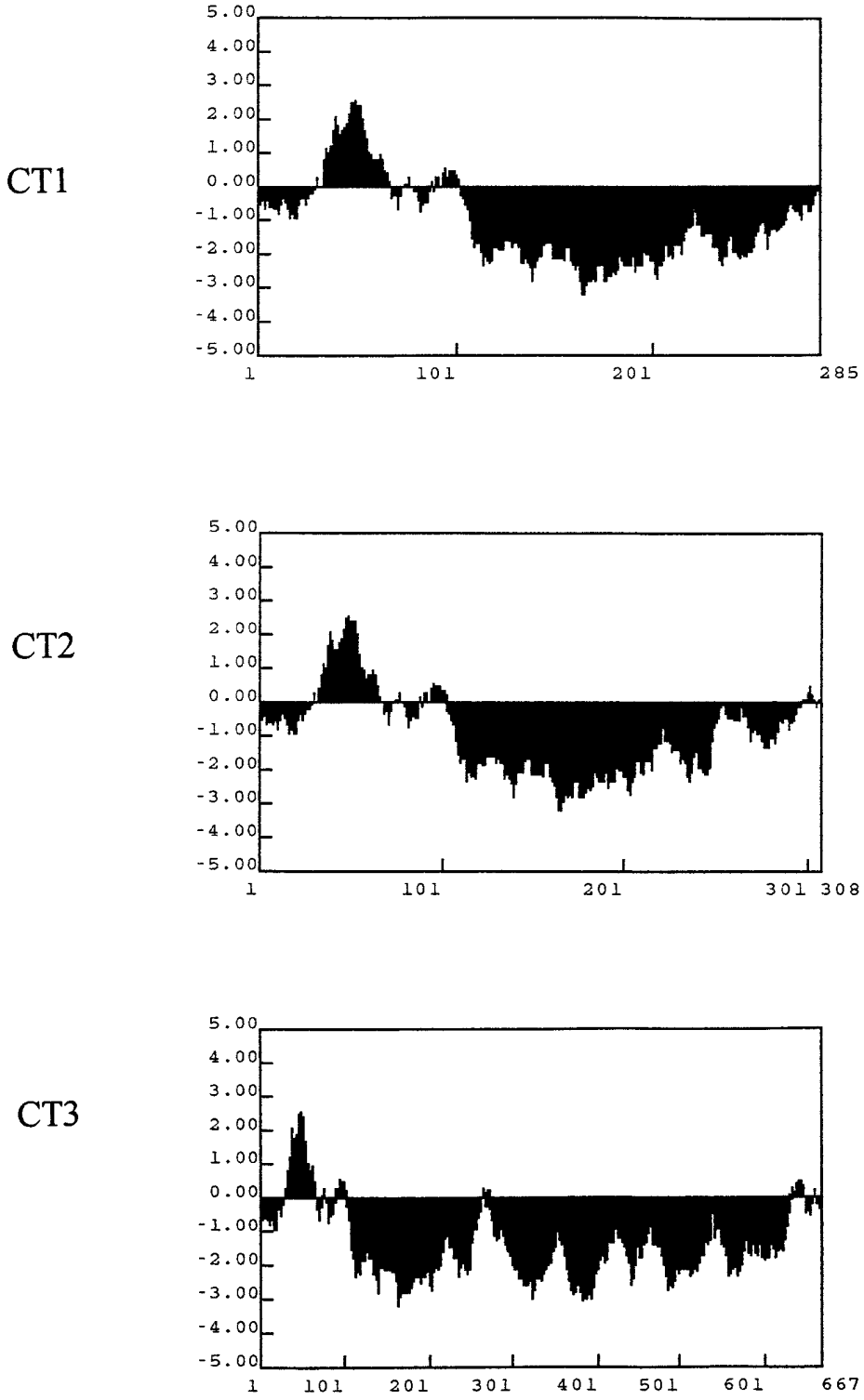

FIG. 7. **Hydrophobicity analysis of cardiac triadin isoforms.** The hydrophobicities of the three cardiac triadin isoforms (CT1, CT2, and CT3) were analyzed by the method of Kyte and Doolittle (20) using a window size of 19 residues. *Abscissa* values represent amino acid numbers, while *ordinate* values indicate the relative hydrophobicity.

as a single band of ~92 kDa in the presence of *N*-ethylmaleimide. Therefore, CT3 does not form multimeric complexes through disulfide bonds. The lack of a cysteine residue might affect the formation of multimeric structures of cardiac triadin.

Based on hydrophobicity analysis by the method of Kyte and Doolittle (20), each of the three cardiac triadin isoforms contains a single transmembrane domain that separates it into cytoplasmic and luminal segments (Fig. 7). Since cardiac triadin isoforms are homologous to skeletal muscle triadin at their N-terminal portions, previous analysis of this portion of the sequences (7) applies to these cardiac proteins. Like their skeletal muscle counterpart, only the N-terminal 47 amino acids are cytoplasmic, with the bulk of the proteins located in the lumen of the sarcoplasmic reticulum. This prediction is consistent with the results of the vesicle protection assay (Fig. 2).

The cellular and subcellular distribution of triadin in cardiac muscle was also examined by immunofluorescence staining (Fig. 8). Specific staining was primarily detected in cardiac myocytes where the labeling of triadin corresponds very closely to that of the ryanodine receptor and calsequestrin, which were previously demonstrated to be localized to the junctional and corbular sarcoplasmic reticulum in cardiac myocytes (24, 25, 27, 28). The subcellular distribution of triadin in cardiac muscle in this study is very similar to that reported by Brandt *et al.*

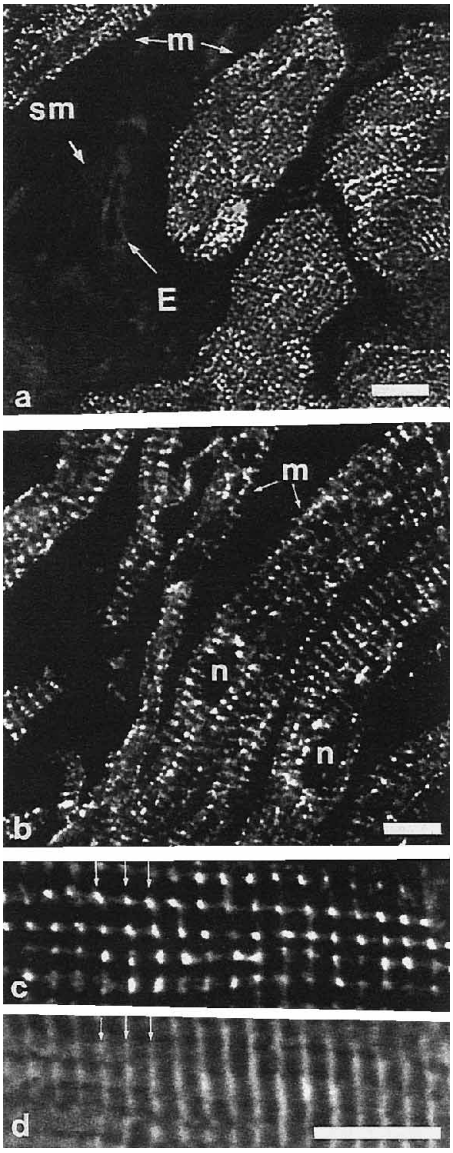

FIG. 8. **Localization of triadin in cryosections of adult rabbit atrial and ventricular muscle by immunofluorescence labeling.** Cryosections of atrial (*a*) and ventricular (*b* and *c*) muscle tissues were immunofluorescently labeled with affinity-purified antibodies to H-triadin (GP58) and imaged by confocal microscopy. Comparison of the immunofluorescence staining pattern in a longitudinal section of rabbit papillary muscle (*c*) with the position of the A- and I-bands in the same field imaged by phase-contrast microscopy (*d*) showed that transversely oriented rows of discrete fluorescent foci (*c*, *white arrows*) were localized at the center of the I-band region (*d*, *white arrows*), where most of the junctional and corbular sarcoplasmic reticulum is localized in mammalian papillary myofibers. Note that the intensity of labeling for triadin was very high in atrial (*m*; *a*) and ventricular (*m*; *b* and *c*) myofibers, while the intensity of labeling in endothelial cells (*E*; *a*) and arterial smooth muscle cells (*sm*; *a*) was only marginally higher than that of the background.

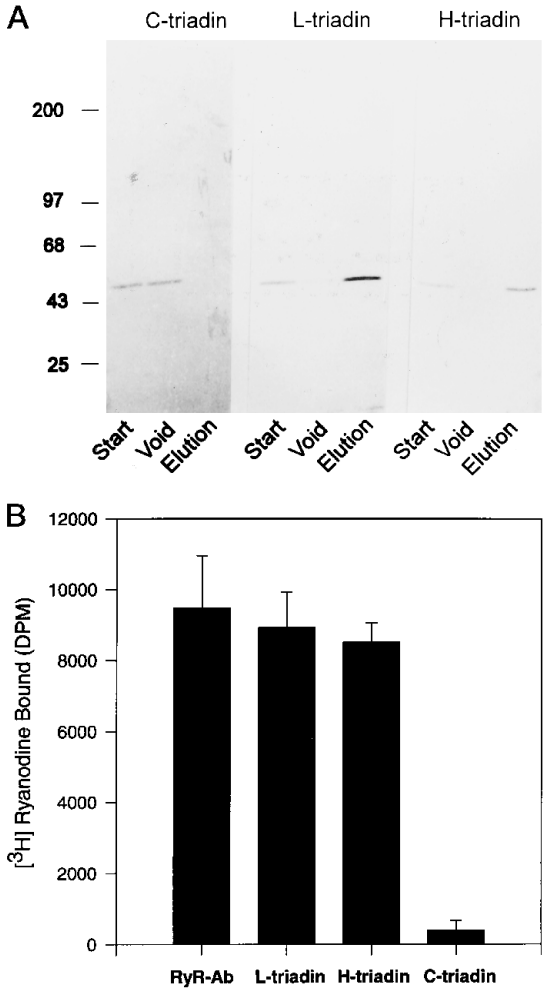

FIG. 9. **The homologous region of cardiac triadin binds calsequestrin and the ryanodine receptor in cardiac muscle microsomes.** *A*, binding of cardiac muscle calsequestrin to triadin fusion proteins. The calsequestrin in the starting material (*Start*), in the flow-through material (*Void*), and eluted from the fusion protein-Sepharose column (*Elution*) was detected using polyclonal antibody Rabbit H at a 1:100 dilution. *B*, binding of triadin fusion proteins to [^{3}H]ryanodine-labeled receptors in rabbit cardiac muscle microsomes. As a positive control, anti-ryanodine receptor polyclonal antibody (*RyR-Ab*)-Sepharose was also used in the binding assay.

(16) and Carl *et al.* (17) using a monoclonal antibody that recognizes both skeletal and cardiac muscle triadin.

We also examined the possible interaction of triadin with the ryanodine receptor and calsequestrin in cardiac muscle. As shown in Fig. 9, the homologous region of the triadin luminal segment (H-triadin) was able to bind [^{3}H]ryanodine-labeled cardiac receptor and cardiac calsequestrin.

Triadin was originally found only in skeletal muscle (6, 7). It was therefore proposed that triadin carries out a function that is specific to skeletal muscle excitation-contraction coupling (6, 7). Recently, we found that triadin interacts with the ryanodine receptor and calsequestrin in the lumen of the skeletal muscle sarcoplasmic reticulum (9). Triadin may be the transmembrane protein that anchors calsequestrin to the junctional sarcoplasmic reticulum near the "sarcoplasmic reticulum foot" ryanodine receptor and thus may be involved in the functional coupling between these two proteins (10–13). In cardiac muscle, calsequestrin and the ryanodine receptor are localized to the junctional and corbular sarcoplasmic reticulum membrane. If triadin is the anchoring protein for calsequestrin in skeletal muscle, there should be triadin homologs in cardiac muscle. In this study, we report the identification, molecular cloning, immunofluorescent localization, and biochemical characterization of three isoforms of triadin in rabbit cardiac muscle. We find that cardiac triadin isoforms share a large region of sequence identity with their skeletal muscle counterpart. In addition, their similar membrane topologies and patterns of molecular interactions suggest that triadin plays a role that is important to both skeletal and cardiac muscle excitation-contraction coupling.

Acknowledgment—We thank Wayne Arnold for expert technical assistance.

REFERENCES

1. McPherson, P. S., and Campbell, K. P. (1993) *J. Biol. Chem.* **268,** 13765–13768
2. Adams, B. A., and Beam, K. G. (1990) *FASEB Lett.* **4,** 2809–2816
3. Stem, M. D., and Lakatta, E. G. (1992) *FASEB Lett.* **6,** 3092–3100
4. Coronado, R., Morrissette, J., Sukhareva, M., and Vaughan, D. M. (1994) *Am. J. Physiol.* **266,** C1485–C1504
5. Meissner, G. (1994) *Annu. Rev. Physiol.* **56,** 485–508
6. Knudson, C. M., Stang, K. K., Jorgensen, A. O., and Campbell, K. P. (1993) *J. Biol. Chem.* **268,** 12637–12645
7. Knudson, C. M., Stang, K. K., Moomaw, C. R., Slaughter, C., and Campbell, K. P. (1993) *J. Biol. Chem.* **268,** 12646–12654
8. Caswell, A. H., Brandt, N. R., Brunschwig, J. P., and Purkerson, S. (1991) *Biochemistry* **30,** 7507–7513
9. Guo, W., and Campbell, K. P. (1995) *J. Biol. Chem.* **270,** 9027–9030
10. Ikemoto, N., Ronjat, M., Meszaros, L. G., and Koshita, M. (1989) *Biochemistry* **28,** 6764–6771
11. Ikemoto, N., Antoniu, B., Kang, J. J., Meszaros, L. G., and Ronjat, M. (1991) *Biochemistry* **30,** 5230–5237
12. Gilchrist, J. S., Belcastro, A. N., and Katz, S. (1992) *J. Biol. Chem.* **267,** 20850–20856
13. Kawasaki, T., and Kassai, M. (1994) *Biochem. Biophys. Res. Commun.* **199,** 1120–1127
14. Sun, X.-H., Protasi, F., Takahashi, M., Takeshima, H., Ferguson, D. G., and Franzini-Armstrong, C. (1995) *J. Cell Biol.* **129,** 659–671
15. Peng, M., Fan, H., Kirley, T. L., Caswell, A. H., and Schwartz, A. (1994) *FEBS Lett.* **348,** 17–20
16. Brandt, N. R., Caswell, A. H., Carl, S. A. L., Ferguson, D. G., Brandt, T., Brunschwig, J.-P., and Bassett, A. L. (1993) *J. Membr. Biol.* **131,** 219–228
17. Carl, S. L., Felix, K., Caswell, A. H., Brandt, N. R., Ball, W. J., Jr., Vaghy, P. L., Meissner, G., and Ferguson, D. G. (1995) *J. Cell Biol.* **129,** 673–682
18. Laemmli, U. K. (1970) *Nature* **227,** 680–685
19. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
20. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157,** 105–132
21. Parys, J. B., Sernett, S. W., DeLisle, S., Snyder, P. M., Welsh, M. J., and Campbell, K. P. (1992) *J. Biol. Chem.* **267,** 18776–18782
22. Deleted in proof
23. Marty, I., Robert, M., Ronjat, M., Bally, I., Arlaud, G., and Villaz, M. (1995) *Biochem. J.* **307,** 769–774
24. Jorgensen, A. O., Shen, A. C.-Y., Arnold, W., McPherson, P. S., and Campbell, K. P. (1993) *J. Cell Biol.* **120,** 969–980
25. Jorgensen, A. O., and Campbell, K. P. (1984) *J. Cell Biol.* **98,** 1597–1602
26. Jorgensen, A. O., and Jones, L. R. (1987) *J. Cell Biol.* **104,** 1343–1352
27. Jorgensen, A. O., Mcleod, A. G., Campbell, K. P., and Denney, G. H. (1984) *Circ. Res.* **55,** 267–270
28. Jorgensen, A. O., Shen, A. C.-Y., and Campbell, K. P. (1985) *J. Cell Biol.* **101,** 257–268
29. White, J. G., Arnos, W. B., and Fordham, M. (1987) *J. Cell Biol.* **105,** 41–48