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Characterization and Ultrastructural Localization of a Novel 90-kDa Protein Unique to Skeletal Muscle Junctional Sarcoplasmic Reticulum*

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Monoclonal antibodies were used to identify and characterize a novel 90 kDa protein that was specifically localized to the junctional sarcoplasmic reticulum of rabbit skeletal muscle. Biochemical experiments show that the 90 kDa protein is an integral membrane protein of the junctional face membrane and is a substrate for the intrinsic protein kinase in triads. Immunofluorescence staining of serial transverse sections of skeletal muscle with a monoclonal antibody to the 90 kDa protein showed preferential staining of type II "fast" fibers. Specific labeling was confined to the interphase between the A- and I-bands, where the triad structure is localized. Immunoelectron microscopical labeling further indicates that the 90 kDa protein, like the ryanodine receptor/Ca^{2+}-release channel and triadin, is confined to the terminal cistemae of the sarcoplasmic reticulum. Western blot analysis with a combination of monoclonal antibodies against the 90 kDa protein shows that it is specifically expressed in skeletal muscle but not in cardiac muscle or brain. Similarly, specific immunofluorescence labeling to the 90 kDa protein was not detected in ventricular myocytes or vascular smooth muscle cells. The junctional localization and phosphorylation of this protein suggest that it may play an important regulatory or structural role in the skeletal muscle triad junction.

Excitation-contraction coupling (E-C coupling) is the signal transduction process by which depolarization of the muscle cell membrane triggers the release of calcium from sarcoplasmic reticulum, and the elevation of the cytosolic calcium leads to muscle contraction. In skeletal muscle, E-C coupling is mediated by morphologically specialized structures known as triads, which are formed by contacts between the transverse tubules (T-tubules) and terminal cisternae of the sarcoplasmic reticulum. Several proteins localized to triads play important roles in E-C coupling (for recent review, see Refs. 1 and 2). The T-tubular dihydropyridine receptor is believed to be the voltage "sensor" that detects the membrane depolarization and transmits the signal to the sarcoplasmic reticulum (3-6). The ryanodine receptor is the actual calcium release channel of sarcoplasmic reticulum (8-10). It forms the foot structure in the triad junction and is believed to mediate the association of the T-tubule and the terminal cistemae of sarcoplasmic reticulum (7, 11, 12). Calsequestrin is a moderate affinity, high capacity Ca^{2+}-binding protein that resides in the lumen of sarcoplasmic reticulum (13-16). It is anchored to the junctional face membrane through an integral membrane protein (16) and is thought to sequester and concentrate Ca^{2+} near the site of Ca^{2+} release. Triadin is a 95-kDa transmembrane glycoprotein located in the junctional sarcoplasmic reticulum, and is believed to form disulfide-linked polymers in the lumen of sarcoplasmic reticulum (17,18). Triadin was originally proposed to bind both the dihydropyridine receptor and ryanodine receptor (19, 20). However, the molecular cloning and membrane topology analysis of triadin indicate that the bulk of the protein is intraluminal (21). Therefore, the interaction of triadin with the dihydropyridine receptor still remains controversial. Instead, triadin may be the membrane-anchoring protein for calsequestrin and thus mediate the coupling between calsequestrin and ryanodine receptor in the lumen of sarcoplasmic reticulum (1, 21).

Using purified skeletal muscle triads, we have generated a library of monoclonal antibodies against the junctional-specific proteins (22). These antibodies have been successfully used in the identification and/or characterization of the ryanodine receptor, dihydropyridine receptor, triadin, and TS-28 (8, 18, 21-23, 28, 37, 45). Here, we report the identification, characterization, and subcellular localization of a novel 90 kDa sarcoplasmic reticulum protein with the monoclonal antibodies. Using a combination of biochemical and immunocytochemical techniques, we show that the 90 kDa protein is a membrane protein specifically localized to the terminal cistemae of the skeletal muscle sarcoplasmic reticulum, and is a substrate for the endogenous kinase of the triads. The localization and phosphorylation of this protein suggest that it may play an important role in skeletal muscle triad junction.

EXPERIMENTAL PROCEDURES

Membrane Preparation and Characterization—Adult rabbit skeletal muscle light and heavy sarcoplasmic reticulum (LSR, HSR)[1] and T-tubules were isolated as described previously (24-26). Triads were prepared by a modification of the method of Mitchell *et al.* (27) as described previously (28). Junctional face membranes were prepared from heavy sarcoplasmic reticulum by treatment with 1% Triton X-100 followed by extraction with a buffer containing 0.8 M NaCI (29). Protein samples were analyzed by 3-15% polyacrylamide gel electrophoresis (SDS-PAGE) in the absence or presence of reducing agents using the buffer

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[1] The abbreviations used are: LSR, light sarcoplasmic reticulum; HSR, heavy sarcoplasmic reticulum; SR, sarcoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid.

system of Laemmli (30), and either stained with Coomassie Blue or transferred to nitrocellulose for immunostaining as described previously (23).

Antibody Production—Monoclonal antibodies were prepared using spleens from mice immunized with isolated triads (22). Junctional specific monoclonal antibodies were selected by immunodot assay using the hybridoma supematants as described previously (22). The monoclonal antibodies VF1c, VIIC12$_3$G$_4$, and VIG7-1 react with the 90 kDa protein in the Junctional SR membrane preparations.

KCl Washing and Alkaline Extraction of Triads—For KCl washing, heavy sarcoplasmic reticulum vesicles were diluted in a 3 mg/ml concentration in the buffer containing 50 nM Tris-HCl (pH 7.4), 0.8 M KCl 5% sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 0.75 mM benzamidine, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, and 0.5 μg/ml pepstatin A. After incubation at 4°C for 1 h with constant mixing, the mixture was centrifuged for 30 min at 100,000 x *g*. The supematants and pellets were collected for immunoblot analysis. For alkaline treatment, the vesicles were diluted in a buffer containing 20 mM Tris-HCl, 200 mM NaCl, 5% sucrose, and the protease inhibitors. The mixture was titrated to pH 11.5 with 1 M NaOH. After a 1-h incubation at room temperature with constant stirring, the samples were centrifuged for 30 min at 100,000 x *g*. The proteins of the supematants and pellets were separated on SDS-PAGE and transferred to nitrocellulose for immunostaining.

Phosphorylation of Triads and Immunoprecipitation of the Phosphorylated 90 kDa Protein—The isolated triads were incubated with 50 μM [γ-^{32}P]ATP (2000 cpm/pmol) at 30°C in 50 mM Tris-PIPES buffer (pH 6.8) containing 15 nM $MgCl_2$, 100 mM KCl, and 10 mM EGTA. After incubation for 30 s, the reaction was stopped by Laemmli sample buffer (15% SDS, 0.575 M sucrose, 5% β-mercaptoethanol, 0.325 M Tris-HCl, pH 6.8) and the phosphorylated triads were analyzed by SDS-PAGE and autoradiography. For immunoprecipitation, the phosphorylated triads were solubilized by a buffer containing 50 mM Tris-HCl (pH 6.2), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 nM NaF, and 100 kalikrein-inactivating units/ml aprotinin at 4°C for 1 h. The mixture was centrifuged for 30 min at 100,000 x *g* to remove insoluble material. The supernatant was incubated at 4°C with 50 μg of monoclonal antibody VFIc and 50 μl of goat anti-mouse IgG-Sepharose 4B with gentle mixing for 4 h. The immunoprecipitates were collected by centrifugation and washed three times with 1 ml each of 100 mM Tris-HCl (pH 7.4), 0.2 M NaCl, and 20 nM NaF. The immunoprecipitates were boiled for 1 min and then separated on SDS-PAGE. The gel was stained with Coomassie Blue, dried, and subjected to autoradiography using Kodak X-Omat AR film.

Dissection, Fixation, and Sectioning of Adult Skeletal Muscle—For immunofluorescence studies, fixed and unfixed bundles of rabbit skeletal muscle (psoas and diaphragm) were prepared as described previously (5). Briefly, bundles of myofibers were dissected and quickly frozen in liquid nitrogen-cooled isopentane. Psoas muscle fibers were immobilized on an applicator stick using sutures at 100-120% of the resting length. The fibers were allowed to recover for 30 min in a modified Krebs-Henseleit buffer (145 mM NaCl, 2.6 HIM KC1, 5.9 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 nun $NaHCO_3$, and 10 mM glucose saturated with a mixture of 95% O_2, and 5% CO_2,). The samples were washed three times for 10 min with 0.1 M sodium cacodylate buffer for 30 min and frozen onto corkboard using Tissue Tek and liquid nitrogen. Cryosectioning was performed as described previously (5).

For immunoelectron microscopical studies, the psoas muscle samples were cryofixed, freeze-dried, and low temperature embedded in Lowicryl K4M as described previously (5). Thin sections (60-80 nm) were collected on nickel grids coated with formvar.

Immunofluorescence Labeling—Immunofluorescence staining of 6-8-um thick transverse cryosections from unfixed adult rabbit psoas or diaphragm muscle was carried out as described previously (31). Adjacent sections of unfixed tissues were labeled with monoclonal antibody that specifically recognize the fast (Type II) fiber form of the Ca^{2+}-ATPase (monoclonal antibody IIH11) or the 90 kDa protein (monoclonal antibody VFIc). The secondary antibody was F(ab)'$_2$ fragments of affinity-purified goat anti-mouse IgG conjugated to fluorescein isothiocyanate used at 1:50 dilution. Confocal fluorescence microscopy was carried out with a photo microscope (Nikon, Inc., Garden City, NY) provided with a confocal fluorescence imaging system laser sharp MRC 600 (Bio-Rad) using a Krypton-argon laser for illumination.

Immunocolloidal Gold Labeling—Thin sections of Lowicryl K4M-embedded rabbit psoas muscle were first labeled with monoclonal antibody VFIc and subsequently labeled with affinity-purified rabbit anti-mouse (Fc fragment) γ-globulin (Jackson ImmunoResearch Laboratory Inc.) used at 25 μg/ml in phosphate-buffered saline containing 3% bovine serum albumin. Finally, the sections were incubated with affinity-purified goat anti-rabbit γ-globulin-colloidal gold (3-7 nm) conjugate used at 0.5 mg/ml phosphate-buffered saline containing 3% bovine serum albumin. After immunolabeling the sections were first stained for 15 s in saturated uranyl acetate in 50% ethanol and then for 15 s in lead citrate. The sections were examined in Hitachi 7000 transmission electron microscope.

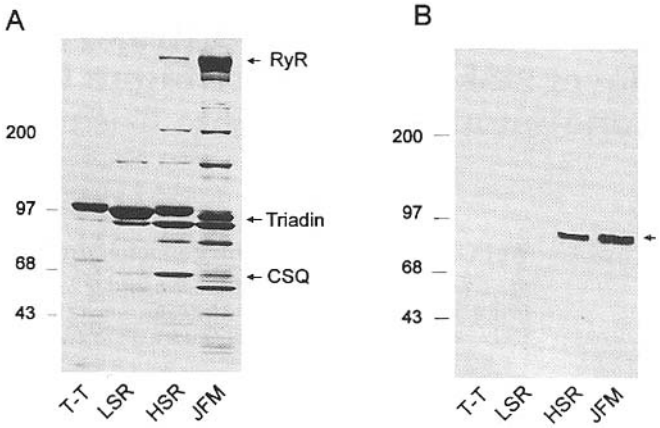

FIG. 1. **Localization of the 90 kDa protein to the junctional sarcoplasmic reticulum.** T-tubules (*T-T*), LSR, HSR, and junctional face membrane (*JFM*) were prepared as described under "Experimental Procedures." The samples were analyzed by SDS-PAGE and either stained with Coomassie Blue (*A*) or transferred to nitrocellulose and stained with monoclonal antibody VF1c against the 90 kDa protein (*B*). The *arrow* indicates the 90 kDa protein on the immunoblot. Molecular weight standards (kDa) are indicated on the *left*.

RESULTS

Biochemical Localization—Isolated sarcoplasmic reticulum vesicles from rabbit skeletal muscle have been found to be heterogeneous with respect to structure and function (32). Vesicles of different buoyant densities isolated from sucrose gradient centrifugation differ in protein composition (32). The HSR is enriched with ryanodine receptor, calsequestrin, and triadin (15, 21, 22). Isolated skeletal muscle triad preparations have been used to generate and screen monoclonal antibodies against junctional specific proteins (22). Monoclonal antibodies that were immunoreactive to HSR but not LSR or T-tubules by immunodot assay (data not shown) were selected. Immunoblotting of proteins of various membrane preparations separated by SDS-PAGE showed that monoclonal antibody VF1c recognized a protein with an apparent molecular mass of 90 kDa. This protein is enriched in junctional SR fractions but not detected in LSR or T-tubules (Fig. 1*B*). Junctional face membranes were prepared by sequential treatment of HSR with Triton X-100 and 0.8 M NaCl (29). This treatment preferentially solubilizedATPase and extracted peripheral proteins including calsequestrin while leaving the junctional sarcoplasmic reticulum membrane proteins including the ryanodine receptor and triadin intact (18, 22). As shown in Fig. LB, the 90 kDa protein is enriched in the junctional face membrane.

KCl Washing and Alkaline Extraction of Heavy Sarcoplasmic Reticulum—To determine whether the 90 kDa protein is an integral membrane protein or a peripheral protein that is attached to the junctional face membrane, heavy SR vesicles were subjected to 0.8 M KCl washing or alkaline extraction. As shown in Fig. 2, the 90 kDa protein remained in the insoluble membrane fraction and was not detected in the soluble supernatant after the vesicles were washed with 0.8 M KCl (Fig. 2*A*) or extracted by a buffer of pH 11.5 (Fig. 2*B*). Since these conventionally used treatments have been shown to remove peripheral membrane proteins while leaving the transmembrane proteins intact (29, 34), these results suggest that the association of the 90 kDa protein to membrane is unlikely to be me-

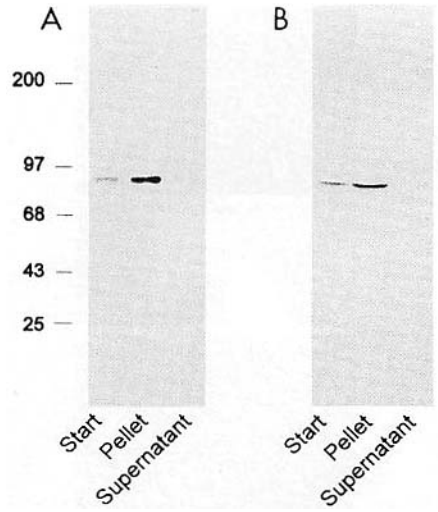

FIG. 2. **KC1 washing and alkaline extraction of the 90 kDa protein from heavy sarcoplasmic reticulum membrane.** The treatment of the heavy sarcoplasmic reticulum with KC1 (A) and alkaline (B) are described under "Experimental Procedures." The starting material (Start), resuspended pellets (Pellet), and supernatant were analyzed by Western blot using the antibody against the 90 kDa protein. The molecular weight standards (kDa) are indicated on the left.

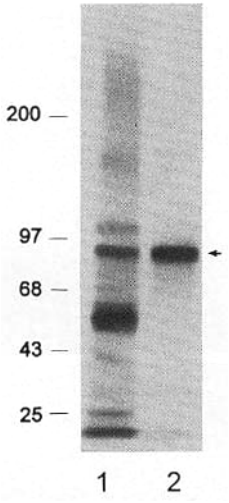

FIG. 3. **Phosphorylation of the 90 kDa protein in the isolated triads.** Phosphorylation of the isolated triads and immunoprecipitation of the 90 kDa protein were performed as described under "Experimental Procedures." The phosphorylated triads (*lane 1*) and the immunoprecipitates (*lane 2*) were analyzed on SDS-PAGE and autoradiography. The *arrow* indicates the 90 kDa protein. Molecular weights (kDa) are indicated on the *left*.

diated by an anchoring protein. In fact, the 90 kDa protein is not solubilized by 1% Triton X-100 during the preparation of junctional face membrane (Fig. 1*B*). It can only be removed from membrane by Zwittergent 3-14, a zwitterionic detergent (data not shown). These characteristics suggest that the 90 kDa protein is an integral membrane protein of the junctional face membrane.

Phosphorylation of the 90 kDa Protein in the Triads—Phosphorylation of the isolated triads was performed by adding [γ-^{32}ATP] for 30 s at 30°C. The phosphorylated proteins were analyzed by SDS-PAGE and autoradiography. The major phosphorylated proteins exhibited apparent molecular masses of 170,000,100,000, 90,000,60,000, and 22,000 Da (Fig. 3, *lane 1*). The phosphorylated triads were solubilized by a buffer containing 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS, and the 90 kDa protein was immunoprecipitated using monoclonal antibody VFlc. The immunoprecipitate was then separated on SDS-PAGE and subjected to autoradiography. As shown in Fig. 3, the antibody precipitated the 90 kDa protein, which was phosphorylated in the isolated triads in the presence of EGTA.

Effect of Sulfhydryl Reagents on the Migration of the 90 kDa Protein—During the study of this protein, it was found that the monoclonal antibodies were sometimes reactive to a band of an apparent molecular mass of approximately 170 kDa in addition to the 90-kDa band. This high molecular mass band probably represents a protein complex containing the disulfide-linked 90 kDa proteins. The junctional face membrane was separated on SDS-PAGE in the presence of 1% β-mercaptoethanol or 10 mM AT-ethylmaleimide, and stained with monoclonal antibody VFlc (Fig. 4*A*). In the presence of the reducing agent β-mercaptoethanol, the antibody identified a single band that migrated at an apparent molecular mass of 90 kDa. In the absence of the reducing agent and presence of *N*-ethylmaleimide, a band with an apparent molecular mass of approximately 170 kDa was also detected (Fig. 4*A*). The molecular mass of the band suggests that it is probably composed of dimers of the 90 kDa protein. As control, *panel B* shows the effect of these sulfhydryl reagents on the migration of triadin, which is believed to form a multimeric protein complex by disulfide bonds in the lumen of sarcoplasmic reticulum (18). In the presence of 10 mM *N*-ethylmaleimide, all of the triadin molecules existed as multimeric forms. However, under the same condition, a portion of the 90 kDa proteins were still present as monomers. So far, we have not been able to find a condition under which the 90 kDa protein is completely converted to a dimeric complex.

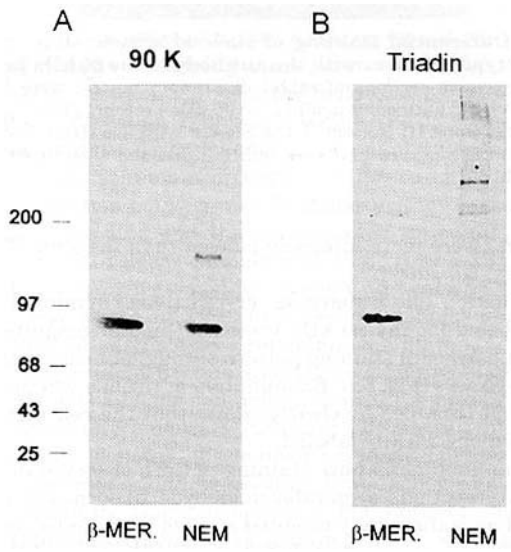

FIG. 4. **Effect of sulfhydryl reagents on the migration of the 90 kDa protein on SDS-PAGE.** Junctional face membranes were run on SDS-PAGE in the presence of 1% of β-mercaptoethanol (*β-MER*), or 10 mm *N*-ethylmaleimide (*NEM*). The samples were transferred to nitrocellulose and immunostained with monoclonal antibody VF1c against the 90 kDa protein (*panel A*) or IIG12 against triadin (*panel B*). Molecular weight standards (kDa) are indicated on the *left*.

Immun.ofluorescen.ce Localization of the 90 kDa Protein—To determine the fiber type specificity of the 90 kDa protein, serial transverse sections of rabbit diaphragm muscle were immunofluorescently labeled with monoclonal antibody VF1c (Fig. 5*a*). The staining pattern was compared with the distribution of "fast' (Type II) and "slow" (Type I) fibers identified by labeling with monoclonal antibody IIH11 to the sarcoplasmic reticulum Ca^{2+}-ATPase that specifically labels the fast (Fig. 5*b, white dots*) but not the slow fibers (Fig. 5*b, stars*). The results showed that the monoclonal antibody to the 90 kDa protein labeled all myofibers (Fig. 5*a*); however, the fast (Type II) fibers (Fig. 5*a*,

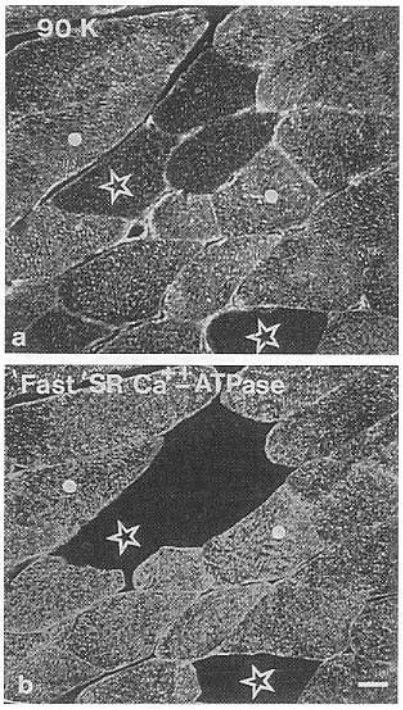

FIG. 5. **Differential staining of skeletal muscle slow (type I) and fast (type n) fibers with the antibody to the 90 hDa protein.** Serial transverse sections of rabbit diaphragm muscle were labeled with monoclonal antibodies against the 90 kDa protein (VFIc) (*a*, 90 *k*), and the fast (type II) isoform of the SR Ca^{2+}-ATPase (IIH11) (*b*). The *white dots* and *stars*, respectively, indicate fast and slow fibers. *Scale bar*, 20 μm.

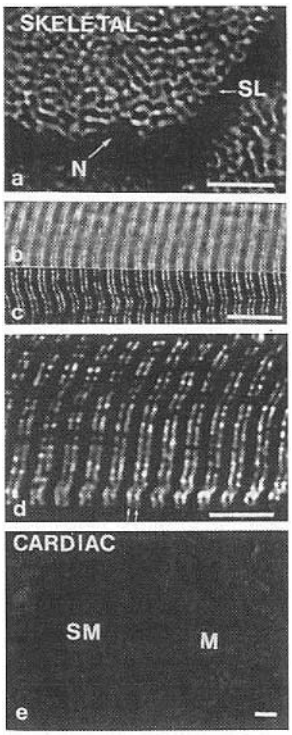

FIG. 6. **Immunofluorescent labeling of cryosections of rabbit skeletal muscle and ventricular muscle with the antibody to the 90 kDa protein.** Transverse (*a*) and longitudinal (*b-d*) cryosections of rabbit psoas muscle and rabbit ventricular muscle (*e*) were immunofluorescently labeled with monoclonal antibody VF1c against the 90 kDa protein (*a-e*). The staining pattern of the 90 kDa in panel c was compared with the position of the A- and I-bands in the same respective field as imaged by phase-contrast microscopy (*panel b*). Immunofluorescence labeling in longitudinal sections (*c and d*) was distributed in transversely oriented rows of small discrete foci (*d, arrows*) located in the interphase between A- and I-bands (*6 and c*) where triads are present. Specific labeling was not detected in either cardiac muscle cells (*e, M*) or vascular smooth muscle cells (*e, SM*) present in cryosections of rabbit ventricular muscle. *SL*, cell periphery. *N*, nucleus. *Scale bar*, 10 μm.

white dots) were more intensely labeled than the slow (Type I) fibers (Fig. 5*a, stars*).

Examination of transverse cryosections immunofluorescently labeled for the 90 kDa protein at higher magnification showed a polygonal staining pattern throughout the cytoplasm of the myofibers (Fig. 6*a*). Examination of regions where nuclei are present (Fig. 6*a, N*) clearly shows that the cell periphery (*SL*) is not specifically labeled.

The immunofluorescence staining pattern observed in longitudinal cryosections of paraformaldehyde fixed psoas muscle appeared as transversely oriented rows of discrete foci (Fig. 6, *c* and *d, small arrows*). The distribution of the rows of fluorescent foci (Fig. 6*c*) corresponded to the interphase between the A-bands and the I-bands as determined by imaging the same field by phase-contrast microscopy (Fig. 6*b*). Since the junctional complexes between junctional SR and the T-tubules (triads) are localized in the interfibrillar spaces at the level of the interphase between A- and I- bands, these results are consistent with the idea that the 90 kDa protein is localized to the junctional sarcoplasmic reticulum. The finding that the labeling for the 90 kDa protein was not detected in paraformaldehyde fixed tissue unless the sections were treated with 0.5% Triton X-100 suggests that the epitope on the 90 kDa protein labeled by the monoclonal antibody VF1c is close to if not embedded in the lipid bilayer of the junctional sarcoplasmic reticulum.

Cryosections from rabbit ventricular tissue were also examined. No specific labeling of ventricular myocytes (Fig. *6e, M*) or of vascular smooth muscle (Fig. 6*e, SM*) was detected.

Immunoelectron Microscopical Localization of the 90 kDa Protein—To determine more precisely whether the 90 kDa protein is indeed confined to the terminal cistemae its subcellular distribution was examined in rabbit psoas muscle by immunoelectron microscopical labeling of cryofixed, freeze-dried, and Lowicryl K4M-embedded tissue (23, 33).

Examination of longitudinal thin sections showed that a majority of the immunolabeling for the 90 kDa protein is confined to the triad region (Fig. 7, *a-d, arrowheads*) and not detected over the light sarcoplasmic reticulum (Fig. 7*b, LSR*), the sarcolemma (Fig. l*a, SL*), myofibers (Fig. 7, *a-d*), and mitochondria (not shown). Within the triad region, small clusters of colloidal gold particles were generally located in close vicinity to terminal cistemae. However, the frequency and intensity of labeling of the terminal cisternae varied considerably from triad to triad as well as from region to region of a particular terminal cisternae of a triad visualized in an electronmicrograph (Fig. 7, *a-d*). On average approximately 20-30% of the terminal cisternae were labeled (Fig. 7). Furthermore, it generally appeared that dense labeling was observed where junctional regions of terminal cisternae were not well delineated and presumably obliquely cut (Figs. 7*c* and 7*d, asterisks*) while well delineated junctional regions of the terminal cisternae were sparsely labeled (Fig. 7, *a* and *b*).

Tissue Distribution of the 90 kDa Protein—The tissue-specific expression of the 90 kDa protein was examined by Western blot analysis of microsomes isolated from rabbit skeletal muscle, cardiac muscle, and brain. A combination of the 90 kDa monoclonal antibodies, VF1c, $VIIC12_3G_4$, and VIG7-1, recognized this protein in the skeletal muscle but not in either cardiac muscle or brain microsomes (Fig. 8). Consistent with this observation, examination of cryosections from rabbit ventricular tissue after immunofluorescence labeling using the monoclonal antibody VF1c did not detect any specific labeling of

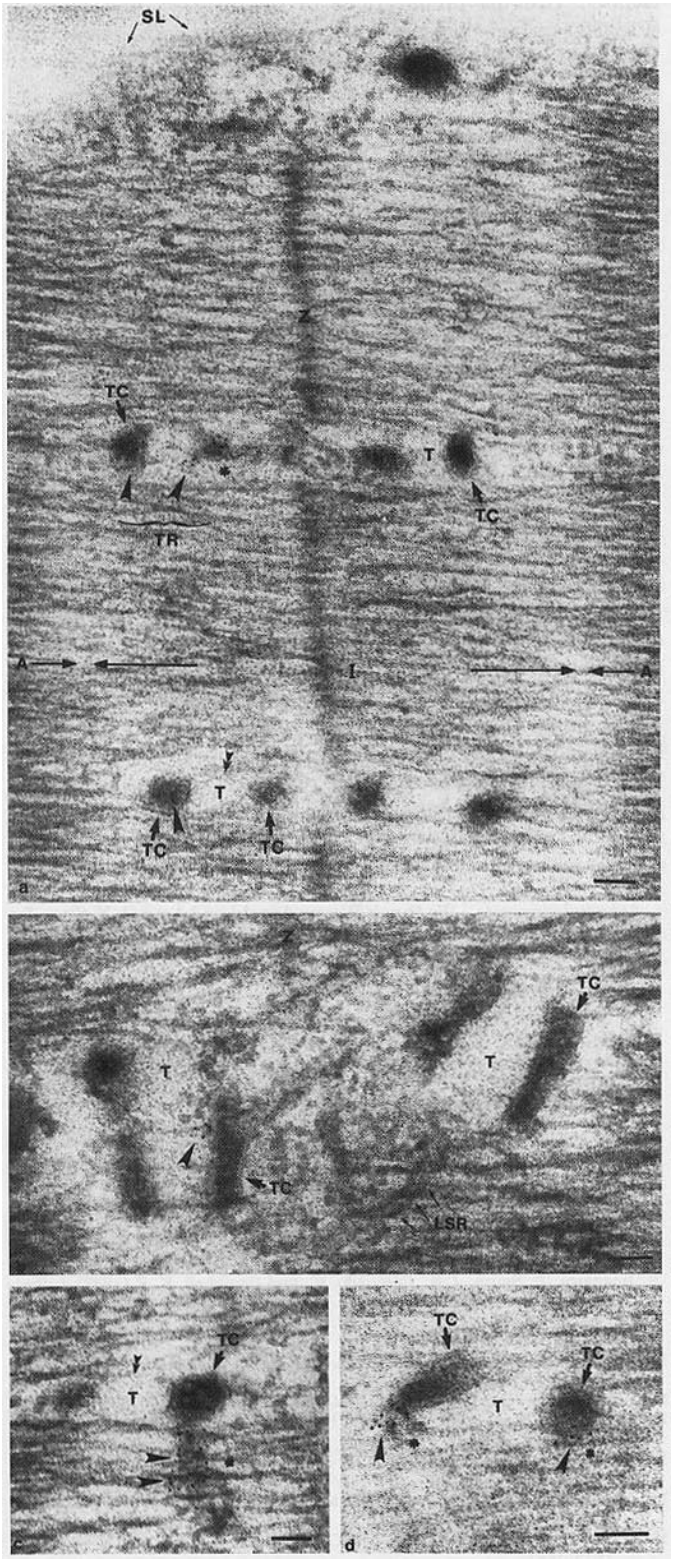

FIG. 7. **Subcellular distribution of the 90 kDa protein in rabbit psoas muscle.** Electronmicrographs of longitudinal thin sections of cryofixed, freeze-dried, and Lowicryl K4M-embedded rabbit psoas muscle labeled with monoclonal antibody VF1c against the 90 kDa protein by the immunocolloidal gold labeling procedure. Specific labeling is confined to the triad region (*a, TR*) where colloidal gold particles are occasionally observed in close vicinity to the interface of some (*a-d, arrowheads*) but not all junctional complexes between T-tubules (*T*) and terminal cisternae (*TC*). Generally, dense immunolabeling is observed to be associated with obliquely cut domains of terminal cisternae (*a, c,* and *d; asterisk*). Specific labeling was not detected over either nonjunctional T-tubular membrane (*c, double arrowheads*), longitudinal sarcoplasmic reticulum (*b, LSR*), or the sarcolemma (*a, SL*). A, A-bands; *Z*, Z-line; *I*, I-bands. *Scale bar*, 0.1 μm.

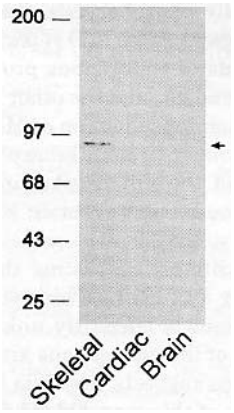

FIG. 8. **Tissue distribution of the 90 kDa protein.** A combination of the 90 kDa monoclonal antibodies (VF1c, $VIIC12_3G_4$, and VIG7-1) were used to probe 100 μg of microsomes prepared from the skeletal muscle, cardiac muscle, and brain. The *arrow* indicates the 90 kDa protein in the skeletal muscle preparation. The molecular weight standards (kDa) are shown on the *left*.

ventricular myocytes or ventricular vascular smooth muscle cells (Fig. 6*e*).

DISCUSSION

Protein components in skeletal muscle triads play essential roles in excitation-contraction coupling (see Refs. 1 and 2 for recent reviews). Studying these junctional proteins is important to the understanding of the molecular mechanisms of excitation-contraction coupling. Despite the identification of a number of critical components in E-C coupling, several elementary questions remain unanswered. For example, how is the communication between the T-tubular voltage sensor dihydropyridine receptor and the sarcoplasmic reticulum ryanodine receptor/Ca^{2+}-release channel mediated? How is the functional coupling between ryanodine receptor and calsequestrin mediated? How is the interaction between T-tubules and terminal cistemae of sarcoplasmic reticulum maintained? We believe that further identification and characterization of protein components of the triads can help answer these questions.

In this study, a novel 90 kDa sarcoplasmic reticulum protein from rabbit skeletal muscle has been identified and characterized using monoclonal antibodies. Immunoblot analysis of various membrane fractions localized this protein to the terminal cistemae of sarcoplasmic reticulum (Fig. 1). Especially, the 90 kDa protein is enriched in the junctional face membrane, a membrane preparation enriched with junctional specific proteins such as the ryanodine receptor/Ca^{2+}-release channel and triadin (18, 22). This protein is probably not a peripheral membrane protein of the junctional face membrane because it cannot be removed from the membrane by either high concentration salt washing or alkaline extraction (Fig. 2), which are conventionally used methods to remove peripheral membrane proteins such as calsequestrin and spectrin (29, 34, 35). Since the 90 kDa protein can only be solubilized by some detergents, we believe it is an integral membrane protein of the junctional face membrane.

The 90 kDa protein is a substrate for an endogenous kinase in skeletal muscle triads (Fig. 3). In the presence of [γ-^{32}P]ATP, the 90 kDa protein is rapidly phosphorylated. The phosphorylation of this protein was confirmed by immunoprecipitation of the detergent-solubilized phosphorylated triads using the monoclonal antibody VF1c against the 90 kDa protein. It is known that the isolated skeletal muscle triads contain kinase

systems that specially phosphorylate the junctional proteins (37-41, 46-48). Imagawa *et al*. (37) reported that isolated triads contain two kinds of endogenous protein kinases. One is Ca^{2+}/calmodulin-dependent, and the other is neither dependent on Ca^{2+}/calmodulin nor dependent on cAMP. The latter one was referred to as an intrinsic protein kinase (37). The α_1 subunit and the β subunit of the dihydropyridine receptor could be phosphorylated by the intrinsic protein kinase in the isolated triads (37). Similarly, the 90 kDa protein is phosphorylated under the same conditions, suggesting that the phosphorylation was mediated by the intrinsic kinase. The function of the phosphorylation system is currently unknown. However, the selective association of the endogenous kinase system with the junctional membranes suggests that it is involved in the regulation of the function of this morphologically specialized structure. The phosphorylation of the 90 kDa protein indicates that the 90 kDa protein contains an active domain in the junctional region, where excitation-contraction coupling occurs.

The migration of the 90 kDa protein on SDS-PAGE is affected by sulfhydryl reagents (Fig. 4). Under reducing condition, it migrates as a single 90 kDa band. In the presence of *N*-ethylmaleimide, a major band of ~ 170 kDa appears in addition of the 90 kDa band. The molecular mass suggests that it is probably composed of dimers of the 90 kDa protein. Similarly, triadin is also affected by sulfhydryl reagents (22). However, under the same nonreducing condition, triadin forms multimers that consist of variable numbers of molecules in the protein complexes. The physiological consequence and function of the 90 kDa dimeric complex are currently unknown. However, this unique property provides a distinguishing characteristic for this protein.

Immunocytochemical labeling of muscle cryosections with monoclonal antibody VF1c against the 90 kDa phosphoprotein was performed at both the light and electron microscopic levels of resolution. Staining of serial sections shows that the fast (Type II) fibers are more intensely labeled than the slow (Type I) fibers (Fig. 5). This pattern of staining is similar to those of dihydropyridine receptor, calsequestrin, and triadin (5, 18, 49). This is probably due to the greater abundance ofT-tubules and junctional sarcoplasmic reticulum found in Type II fibers (36). However, the presence of fiber type-specific isoforms of 90 kDa protein cannot be excluded. Subcellular distribution of the 90 kDa protein was examined using both transverse and longitudinal cryosections (Fig. 6). The staining pattern of the longitudinal cryosections corresponded to the interphase between the A- and I-band by imaging the same field by phase-contrast microscopy, suggesting that the 90 kDa protein is a junctional protein confined to the triad region.

Immunoelectron microscopy further confirmed the junctional localization of the 90 kDa protein (Fig. 7). The labeling intensities were variable with clusters of labeling in close vicinity of some terminal cistemae and little or no labeling of others. We believe that the specimen preparation leads to dilatation of the T-tubules and reorientation of the triad junctions, which lead to diverse exposure of the triad junction after sectioning (18). The low frequency of labeling of the terminal cistemae is likely due to that the epitope on the 90 kDa protein is not readily exposed to the surface of the thin section. This possibility is supported by the finding that immunofluorescent labeling of the 90 kDa protein on cryosections of paraformaldehyde fixed muscle tissue was not detected unless the buffer used for the labeling procedure contained Triton X-100. Because of the dilation of the T-tubules, it was clear that the labeling was not detected over the nonjunctional portion of the T-tubular membrane. In addition, the absence of the labeling over the transverse tubules and the lack of this protein in isolated T-tubule preparations in the Western blot analysis (Fig. 1*B*) strongly suggest that the 90 kDa protein is associated with the terminal cistemae of the sarcoplasmic reticulum and not with the T-tubules.

The 90 kDa protein was detected only in skeletal muscle, but not in cardiac muscle or brain in the immunoblot assay using a combination of 90 kDa monoclonal antibodies (Fig. 8). Consistent with this result, specific immunofluorescence labeling was not detected in rabbit ventricular myocytes or vascular smooth muscles cells (Fig. 6*e*). These data do not exclude the possibility that other tissues express low levels of the protein or that isoforms of the 90 kDa protein exist but do not contain the epitopes recognized by these monoclonal antibodies used in this study. However, since several monoclonal antibodies were used in this assay, it is more likely that the 90 kDa protein is unique to skeletal muscle, and therefore is involved in a skeletal muscle-specific function.

The 90 kDa protein described here has a similar molecular mass to a protein called calnexin (42). Calnexin is an endoplasmic reticulum protein, and has been shown to be phosphorylated by casein kinase II (42, 43). However, these two proteins do not seem to share the same identity. The 90 kDa protein is a skeletal muscle junctional specific protein while calnexin is a protein detected in many tissues. Furthermore, unlike the 90 kDa protein, calnexin is distributed in both terminal ristemae and free sarcoplasmic reticulum membrane fractions (43, 44).

The function of the 90 kDa protein is presently unknown. However, the distinct distribution of this protein to the junctional region of the sarcoplasmic reticulum and its ability to be phosphorylated by a kinase intrinsic to the isolated triads suggest that it may play an important regulatory role in the skeletal muscle excitation-contraction coupling.

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