

Biochemical Characterization and Ultrastructural Localization of a Major Junctional Sarcoplasmic Reticulum Glycoprotein (Triadin)*

(Received for publication, January 15, 1993)

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Monoclonal antibodies were used to identify a 94-kDa protein that was greatly enriched in triads and junctional face membranes ($9.3 \pm 0.2\%$) but not detected in the transverse tubular and nonjunctional sarcoplasmic reticulum membranes. The 94-kDa protein is a hydrophobic glycoprotein based on endoglycosidase H sensitivity, concanavalin A binding, and labeling with a hydrophobic probe. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the absence and presence of reducing agents suggests that this protein is present as a population of multimeric structures containing a variable number of the 94-kDa subunits. Immunofluorescent staining of serial transverse sections of skeletal muscle shows staining of all fiber types with preferential staining of type II fast fibers. Specific immunofluorescence staining in longitudinal sections of skeletal muscle is confined to the interface between the A- and I-bands where the triad structures are localized. Immunocolloidal gold labeling revealed the 94-kDa glycoprotein to be localized over a region of the junctional sarcoplasmic reticulum where the ryanodine receptor/ Ca^{2+} release channel is localized. The distribution and high abundance of the 94-kDa glycoprotein in the junctional membrane suggest that it performs a structural or functional role in the storage or release of calcium from the junctional sarcoplasmic reticulum in skeletal muscle.

which depolarization of the transverse tubules results in calcium release from the junctional sarcoplasmic reticulum has been termed excitation-contraction coupling. Several proteins have been localized to the triad junction and are thought to play important roles in excitation-contraction coupling (Fill *et al.*, 1989). The T-tubular dihydropyridine receptor (Fosset *et al.*, 1983; Jorgensen *et al.*, 1989; Flucher *et al.*, 1990) is thought to "sense" the voltage across the T-system membrane (Rios and Brum, 1987) and signal calcium release from the sarcoplasmic reticulum. The ryanodine receptor is localized to the junctional sarcoplasmic reticulum (Kawamoto *et al.*, 1986) and is thought to be responsible for the physiological release of calcium from the junctional sarcoplasmic reticulum (Imagawa *et al.*, 1987; Lai *et al.*, 1988; Smith *et al.*, 1988). Further evidence suggests that the ryanodine receptor forms the foot structure (Inui and Fleischer, 1988; Lai *et al.*, 1988; Block *et al.*, 1988), an electron-dense bridge between the T-system and the junctional sarcoplasmic reticulum (Franzini-Armstrong, 1970). Calsequestrin is an intralumenal, high capacity, moderate affinity, Ca^{2+} -binding protein (MacLennan and Wong, 1971; MacLennan *et al.*, 1983) that is localized to the junctional sarcoplasmic reticulum (Jorgensen *et al.*, 1983). It is thought to sequester and buffer calcium near the site of physiological release. Although these proteins were first identified and characterized in skeletal muscle, homologous proteins have subsequently been identified and studied in other tissues including the heart and the brain.

Recent research has focused on the mechanism of calcium regulation in skeletal muscle. A combination of electron microscopy and membrane fractionation has identified the junction between the transverse tubular membrane (T-system) and the terminal cisternae of the sarcoplasmic reticulum as the site of calcium release (Franzini-Armstrong, 1970; Campbell *et al.*, 1980). This junction has been named the triad junction because of the characteristic presence of two terminal cisternae on either side of a transverse tubule. The process by

Despite the identification and characterization of these critical components of excitation-contraction coupling, several elementary questions remain concerning the function and regulation of these proteins at the triad junction. First, what is the mechanism of signal transduction between the dihydropyridine receptor and the ryanodine receptor? Second, how is the interaction between the T-system and the terminal cisternae of the sarcoplasmic reticulum maintained? Finally, what prevents the passive migration of the junctional proteins away from the triad junction? We have tried to address these questions by identifying and characterizing additional junctional T-system or junctional sarcoplasmic reticulum proteins. It is hoped that identification and characterization of additional proteins will provide insight into the mechanism of sarcoplasmic reticulum calcium regulation and the maintenance of the architecture of the triad junction. We have previously characterized a 28-kDa protein that was localized to the T-system but not to the sarcolemma or the sarcoplasmic reticulum (Jorgensen *et al.*, 1990). In this study a unique 94-kDa protein has been identified and shown to be a highly abundant glycoprotein that may exist as a high molecular weight multimeric complex composed of 94-kDa "monomers" joined covalently by disulfide bonds. Using a combination of biochemical and immunocytochemical techniques, we show

* This work was supported in part by National Institutes of Health Grants HL14388 and HL39265 (to K. P. C.) and Medical Research Council of Canada Grant-in-aid MT6463 (to A. O. 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.

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that this protein is localized to the terminal cisternae of the sarcoplasmic reticulum. The high abundance and localization of this protein are consistent with the possibility that it plays an important regulatory or structural role at the triad junction.

EXPERIMENTAL PROCEDURES

Membrane Isolation and Biochemical Analysis—Junctional face membranes were prepared from adult rabbit skeletal muscle triads by extraction with 0.5% Triton X-100 in the presence of 1 mM CaCl_2 followed by extraction with 2 mM EDTA buffer as described previously (Costello *et al.*, 1986). Adult rabbit skeletal muscle triads were isolated by a modification of Mitchell *et al.* (1983) as described previously (Sharp *et al.*, 1987). Light sarcoplasmic reticulum vesicles (Campbell *et al.*, 1980) and T-system vesicles (Roseblatt *et al.*, 1981) were isolated as described previously (Knudson and Campbell, 1989). Protein was determined by the method of Lowry *et al.* (1951) as modified by Peterson (1977). Protein samples were analyzed by 3–12% SDS-PAGE¹ either in the absence or presence of reducing agents using the buffer system of Laemmli (1970) and either stained with Coomassie Blue or transferred to nitrocellulose according to Towbin *et al.* (1979). Densitometric analysis was performed on three preparations of junctional face membranes to determine the enrichment for the 94-kDa glycoprotein and the ryanodine receptor. Densitometric scanning was performed using a Molecular Dynamics 300S computing densitometer. Analysis was performed using ImageQuant software provided by the manufacturer. Indirect immunoperoxidase staining of nitrocellulose blots was performed using nonfat dry milk as a blocking agent as described previously (Leung *et al.*, 1987). ConA-peroxidase staining was performed using Tris-buffered saline-Tween as a blocking agent. The immunoblot was stained for 1 h at room temperature with a 1:2,000 dilution of ConA-peroxidase. The blot was washed with Tris-buffered saline-Tween buffer and developed using 4-chloro-1-naphthol as the substrate.

The glycoprotein nature of the 94-kDa protein was assessed by endo H treatment. Junctional face membrane samples were diluted into a buffer containing a final concentration of 50 mM sodium citrate, pH 5.5, boiled for 3 min, and incubated for 30 min at 37 °C in the absence or presence of endo H (0.0015 unit). Junctional face membranes were labeled with 3-(trifluoromethyl)-3-(*m*-[¹²⁵I]iodophenyl)diazirine ([¹²⁵I]TID) as described (Jay *et al.*, 1991). The labeled junctional face membranes were then treated with endo H as described above. Incorporation of radiolabeled probe was detected by autoradiography after separation of the membranes by SDS-PAGE.

Antibody Production—Monoclonal antibodies against the 94-kDa glycoprotein were previously prepared as described (Campbell *et al.*, 1987). Sheep antiserum to highly purified ryanodine receptor (Imagawa *et al.*, 1987) was prepared and characterized as described previously (Knudson *et al.*, 1990). Affinity-purified antibodies to the rabbit skeletal ryanodine receptor were prepared and characterized as described previously (Yuan *et al.*, 1991).

Dissection, Fixation, and Sectioning of Adult Skeletal Muscle—Fixed and unfixed bundles of skeletal muscle from rabbit gracilis and psoas muscle were prepared as described previously (Jorgensen *et al.*, 1989). Briefly, bundles of gracilis and diaphragm myofibers were dissected and quickly frozen in liquid nitrogen-cooled isopentane (Jorgensen *et al.*, 1989). Narrow longitudinal bundles of the gracilis and psoas muscle fibers from adult female rabbits were immobilized on an applicator stick using sutures at 100–120% of the resting length (Jorgensen *et al.*, 1989). The fibers were allowed to recover for 30 min in a modified Krebs-Henseleit buffer (145 mM NaCl, 2.6 mM KCl, 5.9 mM CaCl_2 , 1.2 mM MgSO_4 , 25 mM NaHCO_3 , and 10 mM glucose saturated with a mixture of 95% O_2 and 5% CO_2). The gracilis muscle samples to be used for immunofluorescence labeling were immersed and fixed in ice-cold sodium cacodylate (0.1 M, pH 7.4) solution containing 2.5% paraformaldehyde for 3 h. The samples were washed three \times 10 min with 0.1 M sodium cacodylate buffer alone. The samples were infused with 0.6 M sucrose in sodium cacodylate buffer for 30 min and frozen onto corkboard using Tissue Tek and liquid nitrogen. Cryosectioning was performed as described previously (Jorgensen *et al.*, 1989).

The psoas muscle samples to be used for immunoelectron micros-

copy were cryofixed, freeze-dried, and low temperature embedded in Lowicryl K4M (Jorgensen and McGuffee, 1987; procedure II) as modified by Jorgensen *et al.* (1988b). After ultramicrotomy, thin sections (60–80 nm) were collected on nickel grids coated with Formvar.

Immunofluorescence Labeling—Immunofluorescence staining of 6–8- μm thick transverse cryosections from unfixed adult rabbit psoas or diaphragm muscle was carried out as described previously (Ohlndieck *et al.*, 1991). Adjacent transverse sections of unfixed tissue were first labeled with mouse monoclonal antibodies that recognize either the cardiac or slow form of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase (monoclonal antibody IID8) or the fast form of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase (monoclonal antibody IIH11) or the 94-kDa glycoprotein (XIIH11₂). The secondary antibody was a 1:50 dilution of affinity-purified fluorescein isothiocyanate-labeled goat F(ab')₂ anti-mouse IgG (Boehringer Mannheim). Immunofluorescence staining of 6–8- μm thick longitudinal cryosections of paraformaldehyde-fixed gracilis muscle were first labeled with monoclonal antibody IIG12 to the 94-kDa glycoprotein. The secondary antibody was F(ab')₂ fragments of affinity-purified goat anti-mouse IgG₁ conjugated to Texas Red (Sera, Coptorne, United Kingdom). It was used at a dilution of 1:80. Conventional fluorescence microscopy was carried out with a photomicroscope (Carl Zeiss, Inc., Thornwood, NY) provided with an epifluorescence attachment and a phase-contrast condenser.

Immunocolloidal Gold Labeling—Immunocolloidal gold labeling of thin sections from Lowicryl-embedded rabbit psoas muscle was carried out as described previously (Jorgensen *et al.*, 1989; Jorgensen and McGuffee, 1987; Chiovetti *et al.*, 1985). To immunolocalize the ryanodine receptor, thin sections were first labeled with affinity-purified sheep antibodies to rabbit skeletal ryanodine receptor (20 $\mu\text{g}/\text{ml}$) and subsequently labeled with affinity-purified rabbit anti-sheep γ -globulin-colloidal gold conjugate (3–7 nm used at 0.5 mg/ml in phosphate-buffered saline containing 3% bovine serum albumin (Janssen Pharmaceutical, Beerse, Belgium).

To immunolocalize the 94-kDa glycoprotein thin sections of Lowicryl K4M-embedded tissue were first labeled with monoclonal antibody IIG12 and subsequently labeled with affinity-purified rabbit anti-mouse (Fc fragment) γ -globulin (Jackson Immuno Research Laboratories Inc.) used at 25 $\mu\text{g}/\text{ml}$ 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 in phosphate-buffered saline containing 3% bovine serum albumin (Janssen Pharmaceutical). 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 a Hitachi 7000 transmission electron microscope.

Materials—Endoglycosidase H was from Boehringer Mannheim and [¹²⁵I]TID was from Amersham Corp. All other reagents were of reagent grade quality.

RESULTS

Biochemical Localization—Junctional face membranes were prepared by sequential treatment of triads with Triton X-100 and EDTA. Triton X-100 extraction has been shown to preferentially solubilize the nonjunctional sarcoplasmic reticulum including the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase while leaving the junctional sarcoplasmic reticulum including the specific “feet” structures intact (Campbell *et al.*, 1980; Costello *et al.*, 1986). We have used this membrane preparation in the production and selection of monoclonal antibodies against junctional proteins (Campbell *et al.*, 1987). One of these antibodies recognize a 94-kDa protein that is enriched in triads (Fig. 1B, lane 3) and junctional face membranes (Fig. 1B, lane 4) but not detected in light sarcoplasmic reticulum (Fig. 1B, lane 1) or T-system (Fig. 1B, lane 2). The 94-kDa protein was not detected in skeletal muscle sarcolemma (Ohlndieck *et al.*, 1991) (data not shown). Thus, the 94-kDa protein is clearly distinct from the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase based on the absence in light sarcoplasmic reticulum and on the glycoprotein nature of the 94-kDa protein (Fig. 2). The 94-kDa protein migrates just slightly faster than the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase as illustrated by the slight downward deflection and curvature of the

¹ The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; endo H, endoglycosidase H; [¹²⁵I]-TID, 3-(trifluoromethyl)-3-(*m*-[¹²⁵I]iodophenyl)diazirine; ConA, concanavalin A.

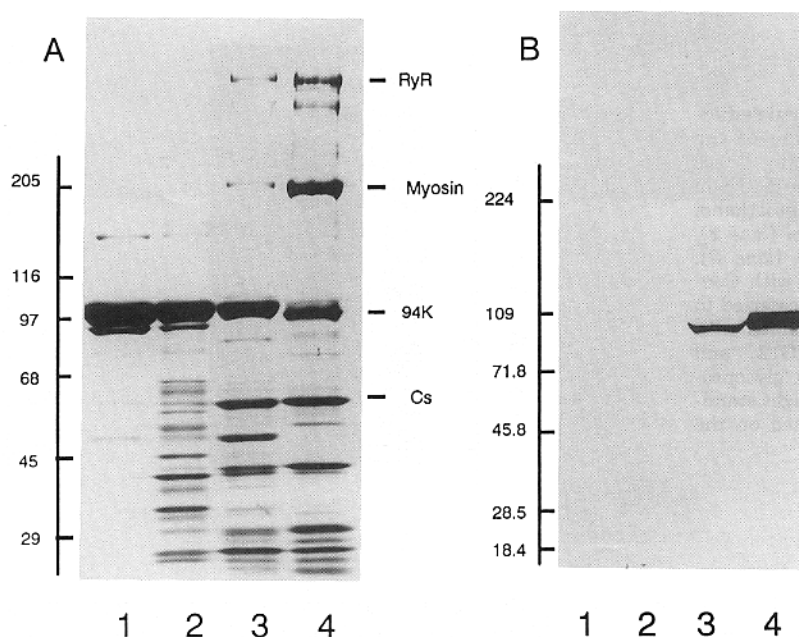


FIG. 1. Localization of the 94-kDa glycoprotein to the junctional sarcoplasmic reticulum. Light sarcoplasmic reticulum (*lane 1*), T-system (*lane 2*), triads (*lane 3*), and junctional face membranes (*lane 4*) were prepared as described under "Experimental Procedures." The samples were analyzed by SDS-PAGE and either stained with Coomassie Blue (*panel A*) or transferred to nitrocellulose and stained with monoclonal antibodies XIIIH11₂ and IIG12 against the 94-kDa glycoprotein (*panel B*). Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left.

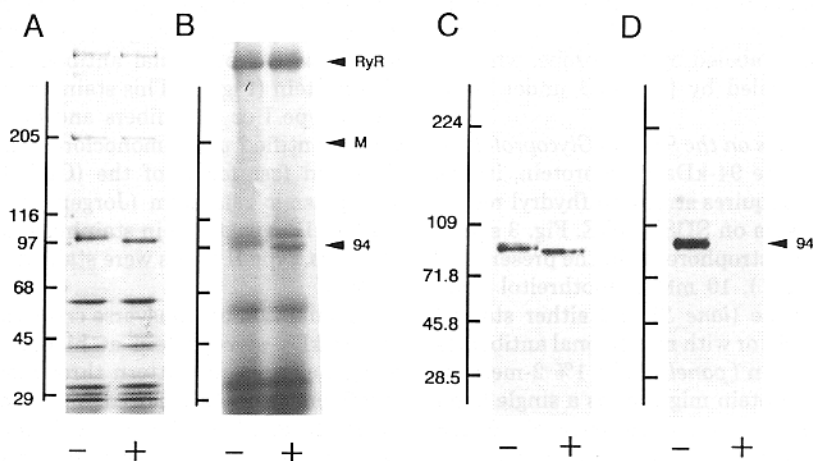


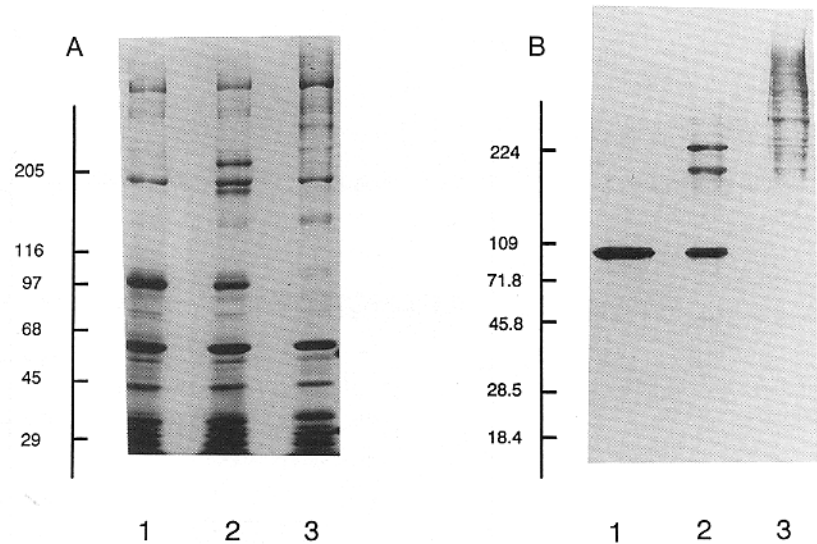
FIG. 2. Biochemical characterization of the 94-kDa glycoprotein. Each lane contains 100 μ g of junctional face membranes treated in the absence (-) or presence (+) of endo H. Samples were analyzed by 3-12% SDS-PAGE as described under "Experimental Procedures." *Panel A* was stained by Coomassie Blue and illustrates a shift of approximately 2 kDa in the relative molecular mass of the 94-kDa glycoprotein. *Panel B* shows junctional face membranes treated with [¹²⁵I]TID, followed by endo H treatment, analyzed by SDS-PAGE, dried, and placed on Kodak XAR-5 film as described under "Experimental Procedures." *Panel C* shows staining of a nitrocellulose transfer by monoclonal antibodies XIIIH11₂ and IIG12 against the 94-kDa glycoprotein. *Panel D* shows staining of a nitrocellulose transfer of junctional face membranes with ConA peroxidase as described under "Experimental Procedures." The molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left.

immunoblot staining of isolated triads (Fig. 1*B*, *lane 3*). The similar mobilities of the 94-kDa protein and the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase prevent the identification by Coomassie Blue staining of the 94-kDa protein in preparations which contain $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase (Fig. 1*A*, *lane 3*). Therefore, the relative abundance of the 94-kDa glycoprotein was determined by densitometric scans of three preparations of junctional face membranes which show the 94-kDa protein to be highly enriched in this preparation and constituted $9.3 \pm 0.2\%$ of the total protein. This value is unlikely to include residual $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase as nearly the entire Coomassie Blue staining band at 94 kDa changes migration either upon endo H treatment (Fig. 2, *A* and *C*) or in the absence of reducing

agents (Fig. 3, *A* and *B*). The $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase shows no such shift under these conditions. The highly abundant ryanodine receptor constituted $4.9 \pm 0.5\%$ of the protein in these same preparations. However, it is difficult to compare the relative stoichiometric or molar ratio between these proteins as the ryanodine receptor is partially solubilized by Triton X-100 while the 94-kDa glycoprotein is not (data not shown).

Biochemical Characterization—The mobility on SDS-PAGE gels of the 94-kDa protein was shown to shift approximately 2 kDa upon treatment with endo H as illustrated by Coomassie Blue and immunoblot staining with monoclonal antibodies (Fig. 2, *A* and *C*). Consistent with the glycoprotein

FIG. 3. Effect of sulfhydryl reducing agents on the migration of the 94-kDa glycoprotein. Junctional face membranes were run on SDS-PAGE in the presence of 0.1% 2-mercaptoethanol (lane 1), 10 mM dithiothreitol (lane 2), or 5 mM *N*-ethylmaleimide (lane 3). Samples were either stained with Coomassie Blue (panel A) or transferred to nitrocellulose and immunostained with monoclonal antibodies IIG12 and XIIH11₂ against the 94-kDa glycoprotein (panel B). Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left.



nature of the 94-kDa protein, it is recognized by the lectin ConA in an endo H-dependent manner (Fig. 2D). The protein was further characterized by labeling with [¹²⁵I]TID (Fig. 2B), a hydrophobic probe that has been used to identify intrinsic membrane proteins (Brunner and Semenza, 1981). The protein labeled by the [¹²⁵I]TID is clearly the 94-kDa glycoprotein as the radiolabeled band shifts upon endo H treatment to the same extent as is seen with antibody staining. The ryanodine receptor, an intrinsic membrane protein (Takeshima *et al.*, 1989; Lai *et al.*, 1988) is also labeled by this probe, whereas myosin (205 kDa) is not labeled by [¹²⁵I]TID under these conditions.

Effects of Sulfhydryl Reagents on the 94-kDa Glycoprotein—During the examination of the 94-kDa glycoprotein, it was discovered that this protein requires strong sulfhydryl reducing agents to migrate at 94 kDa on SDS-PAGE. Fig. 3 shows junctional face membranes electrophoresed in the presence of 1% 2-mercaptoethanol (lane 1), 10 mM dithiothreitol (lane 2), or 5 mM *N*-ethylmaleimide (lane 3) and either stained with Coomassie Blue (panel A) or with monoclonal antibodies against the 94-kDa glycoprotein (panel B). In 1% 2-mercaptoethanol, the 94-kDa glycoprotein migrates as a single band based on immunoblot staining. In the presence of 10 mM dithiothreitol, the antibodies recognize the 94-kDa band and in addition recognize two prominent bands at about 180 and 225 kDa. These two higher molecular mass bands are relatively abundant as they can easily be identified with Coomassie Blue staining (Fig. 3A, lane 2). In the absence of reducing agent and presence of *N*-ethylmaleimide the Coomassie Blue staining band at 94 kDa disappeared, and the antibodies recognized a large number of discrete bands ranging in molecular mass between 180 and in excess of 500 kDa (Fig. 3B, lane 3). In samples that were treated with iodoacetamide, greater concentrations of *N*-ethylmaleimide or no sulfhydryl modifying reagent showed staining indistinguishable from 5 mM *N*-ethylmaleimide (data not shown). In addition, the presence of 10 mM *N*-ethylmaleimide or iodoacetamide in initial homogenization buffers did not alter the migration pattern of the 94-kDa glycoprotein (data not shown). Thus, we have been unable to find conditions that simplified the immunostaining pattern of the 94-kDa glycoprotein when analyzed in the absence of sulfhydryl reducing agents. These data suggest that the complex contains a heterogeneous number of 94-kDa glycoprotein subunits. Although these results are consistent with a homomultimeric

complex composed of a variable number of 94-kDa subunits, additional components to the complex have not been ruled out. Finally, although the consequence and function of this complex are not clear, this unique property provides a distinguishing characteristic of the 94-kDa glycoprotein which allows the protein to be readily identified on SDS-PAGE.

Immunofluorescent Localization of the 94-kDa Glycoprotein—The fiber type specificity of the 94-kDa glycoprotein was explored by staining serial transverse sections of psoas muscle with monoclonal antibody XIIH11₂ against the 94-kDa protein (Fig. 4). This staining was compared with staining of type I or slow fibers and type II or fast fibers which were identified using monoclonal antibodies specific for the slow and fast forms of the (Ca²⁺ + Mg²⁺)-ATPase of the sarcoplasmic reticulum (Jorgensen *et al.*, 1988a). Although the 94-kDa glycoprotein staining showed internal staining of all fibers, type II fibers were stained more intensely than type I fibers.

Examination of transverse cryosections immunolabeled for the 94-kDa glycoprotein at higher magnification showed a polygonal staining pattern throughout the cytoplasm of the myofibers (Fig. 5a). The diameter of neighboring polygons ranged from 1.1 to 1.5 μm. The immunofluorescence staining pattern observed in longitudinal cryosections of fixed psoas muscle appeared as transversely oriented rows of discrete bright foci (Fig. 5, b and d). The distribution of the rows of foci (Fig. 5b) corresponded to the interface between the A-band and the I-band as determined by imaging the same field by phase-contrast microscopy (Fig. 5c). The intensity of labeling of the cell periphery was generally indistinguishable from that of the extracellular space (Fig. 5a). This is particularly apparent in the A-band region of the cell periphery. The distribution of the immunofluorescent staining for the 94-kDa glycoprotein corresponds to the location of triads in rabbit psoas muscle.

Immunoelectron Microscopical Localization of the 94-kDa Glycoprotein—To determine more precisely whether the 94-kDa glycoprotein is confined to the terminal cisternae, its subcellular distribution in rabbit skeletal muscle was determined using immunocolloidal gold labeling and compared with labeling with anti-ryanodine receptor antibodies. To optimize the preservation of the antigenicity of the 94-kDa glycoprotein, the muscle tissue was cryofixed, freeze dried, and low temperature embedded in Lowicryl K4M (Jorgensen and McGuffee, 1987). We have demonstrated previously that

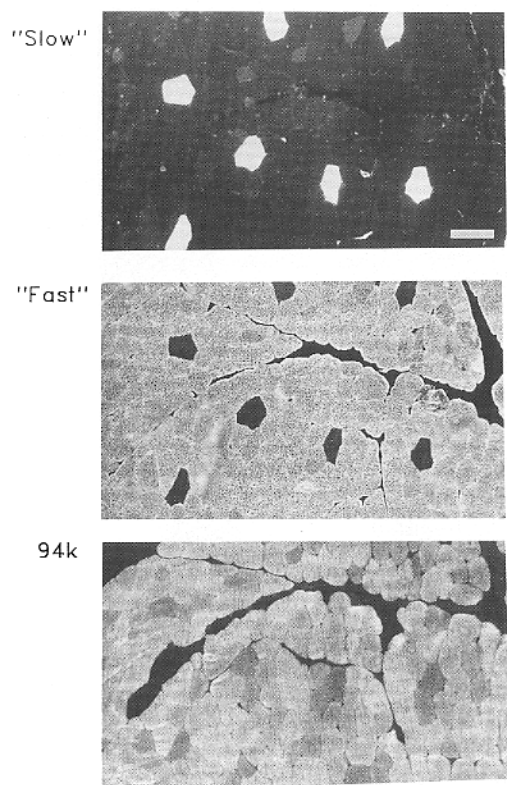


FIG. 4. Differential staining of type I and II fibers with antibodies to the 94-kDa glycoprotein. Serial transverse sections of rabbit skeletal muscle were immunofluorescently stained with monoclonal antibodies against the 94-kDa glycoprotein' (XIIH11₂) (bottom panel, 94k), the fast isoform (type II) of the (Ca²⁺ + Mg²⁺)-ATPase (IIH11) (middle panel), and the slow or cardiac (type I) isoform of the (Ca²⁺ + Mg²⁺)-ATPase (IID8) (top panel). Scale bar = 70 μ m for each.

cellular membranes are well preserved and readily visualized in cryofixed and freeze-dried cardiac (Jorgensen and McGuffee, 1987) and skeletal muscle tissue (Jorgensen *et al.*, 1989) when the tissue is vapor osmicated before embedding in Spurr.

Although the osmication step is compatible with the immunolocalization of protein present in the lumen of subcellular organelles including cardiac calsequestrin (Jorgensen *et al.*, 1988b), this step unfortunately blocks immunoelectron microscopical localization of the 94-kDa glycoprotein and the ryanodine receptor. Since it is not feasible to embed osmicated tissue in Lowicryl K4M, the visualization of membranes in sections of Lowicryl K4M-embedded tissue is variable and less than optimal. However, terminal cisternae (Figs. 6 and 7, TC and thick arrows) and transverse tubules (T, Figs. 6 and 7) can be identified. In contrast, longitudinal sarcoplasmic reticulum is rarely visualized. The degree of dilation of transverse tubules (T, Figs. 6 and 7) is variable and most likely caused by the differential effect of the cryoprotectant polyvinylpyrrolidone (Jorgensen *et al.*, 1988b) on the osmolarity of the cytosol and the lumen of the transverse tubules. Dilation of T-tubules is frequently associated with a rotation of the normally transversely oriented junctional complex between T-tubules and the junctional face of the terminal cisternae (Fig. 6c, TC) to an oblique or even longitudinal orientation (Fig. 6, a and b, double arrowheads). The observed structural rearrangement of some of the triad junctions (Fig. 6, a and b) predicts that the longitudinal sections of skeletal muscle fibers used in this study would sometimes result in tangential cuts through the junctional complex of the triad. This would

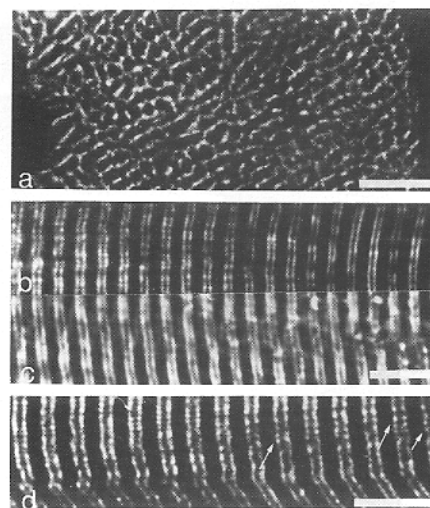


FIG. 5. Immunofluorescent staining of transverse and longitudinal sections of rabbit skeletal muscle with antibodies to the 94-kDa glycoprotein. Transverse (panel a) and longitudinal (panels b-d) cryosections of rabbit psoas muscle were immunofluorescently stained with monoclonal antibody IIG12 against the 94-kDa glycoprotein (panels a, b, and d). The immunofluorescent staining pattern in panel b was compared with the position of the A- and I-bands in the same respective field (mirror image, panel c) as viewed by phase-contrast microscopy. Regular fluorescent staining with monoclonal antibodies IIG12 appeared as small bright foci (arrows) in the interface between the A- and I-bands (panels b and d) where triads are located in rabbit skeletal muscle. Magnification: panels a and d, $\times 1,600$; panels b and c, $\times 1,350$. Scale bar = 9.25 μ m.

expose areas of the junctional face of the terminal cisternae to the surface of a longitudinal section and thereby to junctional sarcoplasmic reticulum proteins. This variable degree of reorientation of the triad junction results in a variable amount of exposure of components of the triad junction to the surface of Lowicryl-embedded sections.

94-kDa Glycoprotein and Ryanodine Receptor—Examination of longitudinal sections of Lowicryl K4M-embedded tissue showed most of the immunolabeling for the 94-kDa glycoprotein to be confined to the triad region (Figs. 6 and 7A) and absent from interfibrillar regions where longitudinal sarcoplasmic reticulum is present. However, the intensity of labeling varied considerably from triad to triad. Generally the intensity of labeling was observed to be higher over triad regions with very dilated T-tubules (Fig. 7A). Within the triad region, clusters of colloidal gold particles were mostly located in close vicinity to the terminal cisternae (Figs. 6c and 7A, arrowheads) and only occasionally present over the lumen of less well visualized regions of the terminal cisternae (Fig. 7A, double arrows). By contrast the junctional regions of well visualized terminal cisternae were either not labeled (Fig. 6, a, b, and Fig. 7A, TC and thick and double arrows) or very sparsely labeled (Fig. 6, b and d, stars) for the 94-kDa glycoprotein. Because of the dilation of the T-tubules it was quite clear that labeling for the 94-kDa glycoprotein was not detected over the luminal and nonjunctional portion of T-tubules (Figs. 6 and 7, T).

Examination of longitudinal sections labeled for the ryanodine receptor showed clusters of colloidal gold particles to be confined to the triad regions (Fig. 7B, single arrowheads and double arrows) where they were nonuniformly distributed. Within the triad region labeling over the junctional region of the triad and the lumen of well visualized terminal cisternae sectioned perpendicular to the triad junction was rarely observed (Fig. 7B, TC and thick arrows). Rather specific labeling was distributed in small clusters mostly located in close vicin-

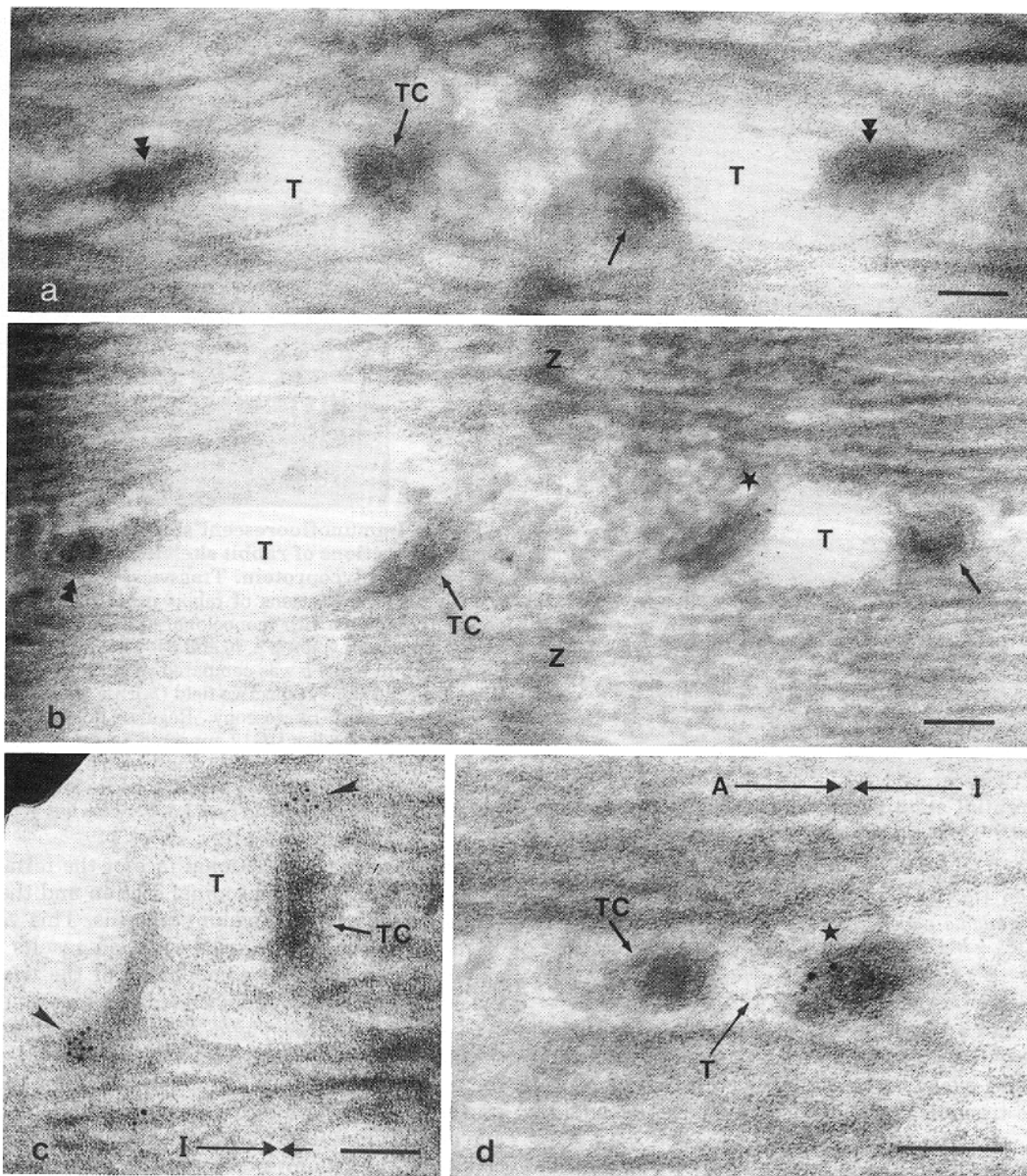


FIG. 6. Subcellular distribution of the 94-kDa glycoprotein in rabbit skeletal muscle. Electron micrographs of various triad regions of cryofixed, freeze-dried, and Lowicryl K4M-embedded rabbit skeletal muscle tissue labeled for the 94-kDa glycoprotein by the immunocolloidal gold labeling procedure. The various fields are presented to illustrate that specific labeling is present in close vicinity of some (panel *c*, arrowheads), but not all terminal cisternae (TC; arrows and double arrowheads) and only occasionally is sparse labeling of the junctional region of the triad observed (panels *b* and *d*, stars). T, T-tubules; Z, Z-line; A, A-bands; I, I-band. Magnification: panel *a*, $\times 115,000$; panel *b*, $\times 113,000$; panel *c*, $\times 130,000$; panel *d*, $\times 178,000$. Scale bar = 0.096 μm .

ity to well visualized terminal cisternae (Fig. 7B, arrowheads). Since the T-tubules are dilated because of the presence of the cryoprotectant polyvinylpyrrolidone (Jorgensen *et al.*, 1988b), it is quite obvious that labeling for the ryanodine receptors was not detected over the luminal and nonjunctional portion of T-tubules (Fig. 7B, TC and arrows).

DISCUSSION

Several protein components of the skeletal muscle triad junction have been identified and shown to play essential roles in excitation-contraction coupling. However, the precise means by which activation of the dihydropyridine receptor by membrane depolarization leads to Ca^{2+} release through the ryanodine receptor/ Ca^{2+} release channel is not known. The most widely accepted hypothesis is that there is a direct interaction between the two receptors which allows a conformational change in the dihydropyridine receptor to lead di-

rectly to a conformational change in the calcium release channel (Takeshima *et al.*, 1989). The best data to support this hypothesis come from high resolution electron micrographs which show that the foot structures/calcium release channel are aligned with oval particles in the T-system which may represent the dihydropyridine receptor (Block *et al.*, 1988). However, to our knowledge, there is no published evidence that the receptors are biochemically associated. We have attempted to copurify the receptors using various detergents and ionic conditions but as yet have been unable to show any type of association.² Given the abundance of data on these receptors and ample time to test the above hypothesis, it seems likely that others have been unsuccessful at showing an association between the receptors. In fact, studies have looked for associations of the ryanodine receptor (Chad-

² C. M. Knudson and K. P. Campbell, unpublished data.

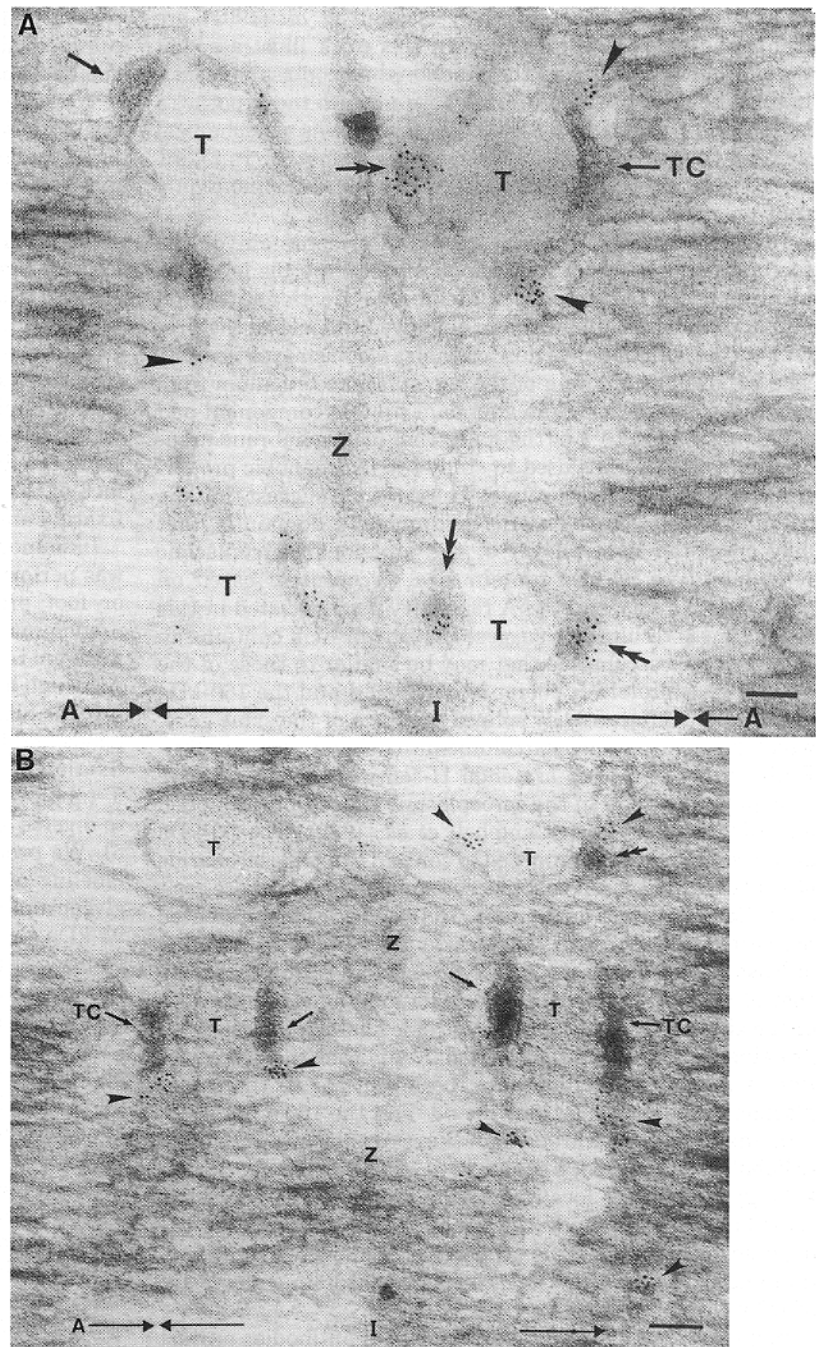


FIG. 7. Subcellular distribution of the 94-kDa glycoprotein and the ryanodine receptor in rabbit skeletal muscle. A longitudinal ultrathin section of cryofixed, freeze-dried, and Lowicryl K4M-embedded rabbit psoas muscle was labeled with monoclonal antibody IIG12 against the 94-kDa glycoprotein (*panel A*) and with affinity-purified antibodies to rabbit ryanodine receptor (*panel B*) by the indirect immunocolloidal gold (5 nm) labeling technique as described under "Experimental Procedures." In both cases, colloidal gold particles are confined to the triad region of the myofiber. Within the triad region labeling is only occasionally observed to be densely distributed over the terminal cisternae (*TC* and *arrows*). By contrast most of the small clusters of colloidal gold particles are located in regions in very close vicinity to well visualized regions of terminal cisternae. *T*, T-tubules; *Z*, Z-line; *A*, A-bands; *I*, I-band. Magnification: *panel a*, $\times 95,000$; *panel b*, $\times 110,000$. Scale bar = $0.1 \mu\text{m}$.

wick *et al.*, 1988) and the dihydropyridine receptor (Brandt *et al.*, 1990; Kim *et al.*, 1990) with other junctional proteins and have not reported a direct interaction between the two proteins. However, despite the paucity of supportive biochemical data, the possibility that the two receptors maintain a highly labile interaction that is sensitive to membrane fractionation, detergent extraction, and/or ionic disruption cannot be excluded. Alternatively, additional proteins may be involved in the transmission of this signal. Thus, a number of recent studies have focused on the identification of additional protein components that are localized to the triad junction and may be important in calcium regulation. Proteins of 71 (Chadwick *et al.*, 1988), 106 (Zaidi *et al.*, 1989a, 1989b), and 95 kDa (Kim *et al.*, 1990) have been identified and proposed to be localized to the triad junction and important in calcium homeostasis. But despite these results, the mechanism of excitation-contraction coupling and the molecular

interactions that occur at the triad junction remain controversial.

Brandt *et al.* (1990) described a 95-kDa protein that they propose be named "triadin" based on its localization and their proposal that it directly interacts with both the dihydropyridine receptor and the ryanodine receptor/ Ca^{2+} release channel (Caswell *et al.*, 1991). Aside from their molecular mass, the 95-kDa protein and the 94-kDa glycoprotein described here have several other properties in common. Both proteins appear to be junctional specific proteins that are found in the terminal cisternae of the sarcoplasmic reticulum. Similarly, both proteins are absent or reduced in T-system and non-junctional sarcoplasmic reticulum. Caswell *et al.* (1991) showed that the 95-kDa protein has a similar immunostaining pattern on SDS-PAGE when run in the absence of reducing agents. This highly characteristic property makes it extremely likely that the 94-kDa glycoprotein described here and the

95-kDa protein characterized by Caswell *et al.* (1991) are identical. The results presented in this work illustrate that the protein is localized to the junctional sarcoplasmic reticulum. Although these results are consistent with the hypothesis by Caswell *et al.* that the 95-kDa protein acts as a mechanical link between the dihydropyridine receptor and the ryanodine receptor, in our view this proposed function remains highly controversial (Knudson *et al.*, 1993).

In this study, an abundant 94-kDa glycoprotein of the sarcoplasmic reticulum from rabbit skeletal muscle has been identified and characterized. Membrane fractionation and immunological techniques were employed to localize the protein to the junctional region of the sarcoplasmic reticulum. The 94-kDa glycoprotein of the sarcoplasmic reticulum from rabbit skeletal muscle is an abundant protein component and composes almost 10% of the junctional face membrane preparation. [¹²⁵I]TID was used to study the hydrophobic properties of the 94-kDa glycoprotein. These results suggest that the 94-kDa glycoprotein contains hydrophobic domain(s) that may cross the lipid bilayer of the junctional sarcoplasmic reticulum. The 94-kDa protein is a glycoprotein based on endo H sensitivity and ConA reactivity demonstrated in this study. These results also suggest that the protein contains an N-linked oligosaccharide that may be similar to those of the 53-kDa glycoprotein (Leberer *et al.*, 1989b) and the 160-kDa glycoprotein, alternatively spliced products of the same gene, which has been termed sarcalumenin (Leberer *et al.*, 1989a). Both of these proteins are endo H-sensitive, ConA-positive glycoproteins found in the sarcoplasmic reticulum (Campbell and MacLennan, 1981; Campbell *et al.*, 1983). The 53-kDa glycoprotein has been proposed to contain two oligosaccharide chains each composed of 2 glucosamine and 9 mannose residues (Campbell and MacLennan, 1981). The results presented here are consistent with the 94-kDa glycoprotein containing at least a single oligosaccharide with a composition similar to that of the 53-kDa glycoprotein. The functional significance of the similarity in oligosaccharide side chains of these three proteins is not known. One possibility is that the glycosylation is important for targeting these proteins to the sarcoplasmic reticulum. However, the 53- and the 160-kDa glycoproteins are found throughout the sarcoplasmic reticulum (Michalak *et al.*, 1980; Leberer *et al.*, 1990), whereas the 94-kDa glycoprotein is localized to the junctional sarcoplasmic reticulum. Regardless of the functional significance of the oligosaccharide chain, this property may allow for identification and purification of the 94-kDa glycoprotein using lectin affinity chromatography.

Immunocytochemistry with monoclonal antibodies against the 94-kDa glycoprotein was performed at both the light and electron microscopic level of resolutions. Staining of serial sections shows that the 94-kDa glycoprotein is detected in both slow and fast fibers. The immunostaining pattern of the 94-kDa glycoprotein in slow and fast fibers is similar to that of the dihydropyridine receptor (Jorgensen *et al.*, 1989) and to the ryanodine receptor (data not shown). This is presumably because of the greater abundance of T-system and junctional sarcoplasmic reticulum found in type II fibers in comparison with type I fibers (Eisenberg, 1983). Although the presence of isoforms of the 94-kDa glycoprotein cannot be excluded, the data presented here can most easily be explained by a single isoform. No fiber type-specific isoforms for either the dihydropyridine receptor or the ryanodine receptor in rabbit skeletal muscle have been identified using either protein biochemistry (Imagawa *et al.*, 1987; Lai *et al.*, 1988) or molecular biological techniques (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990). However, these results do not exclude the

presence of fiber type-specific isoforms of the 94-kDa glycoprotein which comigrate on SDS-PAGE gels and are indistinguishable using our monoclonal antibodies.

We have demonstrated previously by immunoferritin labeling of cryoultramicrotomy sections that calsequestrin is confined to the lumen of the terminal cisternae in rabbit skeletal muscle (Jorgensen *et al.*, 1983). Using the same approach, Kawamoto *et al.* (1986) demonstrated that the junctional sarcoplasmic reticulum component biochemically characterized as the foot protein is localized to the triad region in skeletal muscle. More recently biochemical and ultrastructural characterization of the ryanodine receptor has demonstrated that this protein corresponds to the individual foot structures observed in freeze-fracture images of junctional sarcoplasmic reticulum. Thus we assume that the subcellular distribution of the ryanodine receptor illustrated here represents the distribution of a protein confined to the junctional face of the terminal cisternae, when the tissue is exposed to fixation and embedding as described in this study.

Immunocolloidal gold labeling of the 94-kDa glycoprotein was performed and is compared with the ryanodine receptor or foot protein. The 94-kDa glycoprotein was distributed predominantly over the terminal cisternae, and the labeling was generally indistinguishable from the ryanodine receptor. Although both proteins were distributed over triads, the labeling was highly variable with clusters of labeling around some triads and little or no labeling of others. We propose that the fixation and embedding of the tissue lead to dilatation of the T-tubule and reorientation of the triad junctions, which lead to diverse exposure of the triad junction after sectioning (Fig. 6). We propose that this exposure accounts for the variable labeling of triads with antibodies against both the 94-kDa glycoprotein and the ryanodine receptor. The absence of both 94-kDa glycoprotein and ryanodine receptor labeling over the transverse tubules and the lack of these proteins in isolated transverse tubules suggest that they are more closely associated with the terminal cisternae of the sarcoplasmic reticulum than with the T-tubule membrane. Although the 94-kDa glycoprotein labeling of individual triads was variable and often on the perimeter of the triad, the nearly identical staining pattern of the ryanodine receptor suggests that the proteins colocalize at the triad junction. Thus, we feel that location and high abundance of the 94-kDa glycoprotein in the junctional sarcoplasmic reticulum indicate that it performs an important functional or structural role in calcium homeostasis in skeletal muscle.

Acknowledgments—We thank Joseph B. Snook and David Zoloto for membrane preparations and Steven D. Kahl, Suzanne W. Sernett, Amy C. Y. Shen, Wayne Arnold, and Jonathan Rudd for expert technical assistance.

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