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THE SARCOPLASMIC RETICULUM Ca^{2+}-ATPase, SERCA1A, CONTAINS ENDOPLASMIC RETICULUM TARGETING INFORMATION

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The fast-twitch skeletal muscle Ca^{2+}-ATPase isoenzyme, SERCA1a, is localized in chick skeletal myotubes to both the sarcoplasmic reticulum (SR) and to the nuclear envelope, an extension of the endoplasmic reticulum (ER). The ER labeling remained after cycloheximide treatment, indicating that it did not represent newly synthesized SERCA1a in transit to the SR. Expression of the cDNA encoding SERCA1a in cultured non-muscle cells led to the localization of the enzyme in the ER, as indicated by organelle morphology and the co-localization of SERCA1a with the endogenous ER luminal protein, BiP. Immunopurification analysis showed that SERCA1a was not bound to BiP, nor was any degradation apparent. Thus, the SR Ca^{2+}-ATPase appears to contain ER targeting information.

During myogenesis the SR is postulated to arise by budding from the ER (6), but the relationship between these two membrane systems in fully differentiated muscle is unclear. SR and ER are distinctly different in their morphology and protein composition, suggesting that mechanisms exist for the selective sorting of ER and SR components. The major protein constituent of the SR is Ca^{2+}-ATPase, accounting for approximately two-thirds of the total protein of that organelle, which functions to concentrate calcium in the SR for subsequent release to initiate muscle contraction. The SR Ca^{2+}-ATPase is synthesized on the rough ER (10) and during myogenesis moves into the nascent SR, possibly by lateral diffusion through membrane continuities between the two organelles (6). Immunolocalization studies have shown that Ca^{2+}-ATPase is present not only in the SR, but also in the nuclear envelope (14,15), an extension of

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Abbreviations: SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; DEAE dextran, diethyl aminoethyl dextran; DMSO, dimethyl sulfoxide; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; PBS, phosphate-buffered saline, pH 7.4; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MEM, Eagle's minimal essential medium; DMEM, Dulbecco's modified Eagle's medium.

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the ER. The mechanism by which the subcellular distribution of Ca^{2+}-ATPase is established is not known.

The localization of proteins in intracellular membranes is postulated to be an active process, i.e., proteins are sorted via specific targeting sequences which presumably function through interaction with receptors (22). Thus, ER and SR proteins are predicted to contain structural information which governs their subcellular distribution. Targeting sequences have been identified in both soluble (22) and transmembrane (12) ER proteins. A lysine-rich motif at the cytoplasmically-exposed C-terminus of some transmembrane proteins was described by Jackson *et al.* (12) which conferred ER localization (although a more complex retention signal at the C-terminus has been postulated [8]).

Five SR/ER Ca^{2+}-ATPases which are the product of distinct genes and alternative splicing have been identified (3,4,5,21) and termed "SERCA" (sarco/endoplasmic reticulum calcium ATPase; ref. 5). While one of the five, SERCA3, contains a C-terminal lysine-rich domain which may function as an ER retention signal (5), the mechanism by which this family of enzymes is sorted is unknown. The Ca^{2+}-ATPase isoform (SERCA1a) found in adult fast-twitch skeletal muscle SR does not contain any of the sequences previously identified as ER targeting signals. We report here that, in addition to being found in the SR, SERCA1a is stably localized to the nuclear envelope/ER of skeletal myotubes, and is targeted to the ER when expressed in non-muscle cells. These results indicate the fast muscle SR isoform of Ca^{2+}-ATPase contains ER targeting information.

Materials and Methods

Materials. Fertilized white leghorn chicken eggs were obtained from Texas A&M University (Bryan, Texas). Cell culture media powders, and horse, chicken and fetal calf sera were obtained from GIBCO Laboratories (Grand Island, NY). Iron-supplemented calf serum was from Hyclone Laboratories, Inc. (Logan, UT). Fluorescent-conjugated immunoglobulins were purchased from Cappel/Organon Teknica (West Chester, PA) and isotopes were from Amersham (Arlington Heights, IL). The cloning of the cDNA encoding avian SERCA1a (FCa cDNA), and its insertion into the eukaryotic expression vector pSVDF (23) to yield pSVFCa was reported previously (15).

Cell Culture and Transfections. Skeletal myoblasts were isolated from the breast muscle of day 11 chick embryos (1), plated onto collagen-coated glass coverslips and allowed to differentiate into myotubes. Culture medium, which was replaced every other day, consisted of MEM containing 10% horse serum and 2% chick embryo extract. Chick fibroblasts were isolated from the trypsinized skin of day 11 embryos and maintained in MEM supplemented with 10% horse serum and 4% chicken serum. Mouse Ltk^- cells and African green monkey kidney epithelial cells, CV-1, were grown in DMEM containing either 10% fetal calf serum or 10% iron-supplemented calf serum with identical results. Chick fibroblasts, Ltk^- and CV-1 cells were transfected using DEAE dextran/DMSO shock (9) with 2 μg DNA per 35mm culture dish containing approximately 5 x 10^5 cells. Twenty four hours after transfection, fresh medium containing 10mM sodium butyrate was added (18) and the cells were maintained for another 48 h. All cells were maintained in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Immunofluorescent Labeling of Cells. Transfected cells and myotubes on glass coverslips were fixed and permeabilized by immersion in 100% methanol for 10 min at -20°C, rinsed with 0.1% bovine serum albumin in Hank's balanced salt solution, then incubated for 10 min at room temperature in 1% bovine serum albumin in Hank's balanced salt solution. Cells were incubated for 1 h at room temperature with a monoclonal antibody (5 μg/ml in 20mM HEPES-buffered MEM [pH 7.2], 10% horse serum) directed against avian SERCA1a (CaF1 IgG; ref. 14), washed with 0.1% bovine serum albumin, then labeled with fluorescein-conjugated goat anti-mouse IgG for 1 h. For the double-labeling of SERCA1a and BiP, transfected cells were sequentially incubated in (1) CaF1, (2) rhodamine-conjugated goat anti-mouse, (3) a 1:40 dilution of hybridoma supernatant containing anti-BiP antibodies (ref. 2), and (4) fluorescein-conjugated goat anti-rat IgG (no cross-reactivity to mouse IgG). Coverslips were rinsed and mounted in 90% glycerol, 10% PBS, 1 mg/ml p-phenylene diamine to minimize photobleaching (13), then photographed with appropriate filters using a Nikon Optiphot epifluorescence microscope.

Immunopurification. Ltk$^-$ cells on 60mm dishes were transfected as described above with either pSVFCa (15) or with vector alone. Following incubation in butyrate-containing medium for 48 hours, cells were rinsed with PBS and maintained at 37°C for 2 h in methionine-free DMEM (no serum) supplemented with 80 μCi [^{35}S]methionine per ml. The labeling medium was replaced with serum-containing medium and the cells incubated for 1 h at 37°C. This labeling/chase period was employed to maximize incorporation of ^{35}S into BiP. A parallel dish of unlabeled cells contained a coverslip which was immunostained as described above to verify the success of the transient transfection. The following steps were performed at 0-4°C. Cells were rinsed twice with PBS and lysed with 1 ml 1% triton X-100, 10mg/ml bovine serum albumin in PBS. The lysates were cleared by centrifugation at 100,000 x g for 30 min. To preserve the possible association of the binding protein, BiP, to SERCA1a, lysates were ATP-depleted by the addition of D-glucose to 400μM and 10 U hexokinase (Sigma Chemical Co., St. Louis, MO.) for 2 h (20). Thirty μl of a 50% suspension of goat anti-mouse IgG agarose (Sigma) was also added as an "irrelevant antibody" to reduce background binding in subsequent steps. The lysates were spun briefly to pellet the beads and the supernatants transferred to fresh tubes. Fifty μl of a 50% slurry of CaF1 coupled to CNBr-activated Sepharose 4B (Sigma) or 50 μl of anti-BiP beads were added and incubated for 4 h. Anti-BiP beads were prepared by incubating 50 μl of anti-BiP hybridoma supernatant with 50 μl of a 50% suspension of Protein A-Agarose beads (Sigma) at room temperature for 3 h, then washing with PBS and resuspension in 25 μl PBS. The beads containing immunocomplexes were washed three times each in lysis buffer and PBS, then separated by SDS-PAGE on 9% gels (19). Gels were soaked in Fluoro-Hance (Research Products International Corp., Mt. Prospect, IL), dried and fluorographed at -70°C.

Results and Discussion

The presence of SERCA1a in the nuclear envelope/ER of skeletal muscle could represent either newly-synthesized enzyme in transit to the SR, or Ca^{2+}-ATPase that is a stable resident of this organelle. To distinguish between these alternatives, chick embryo myotubes were maintained for 2 h in the presence or absence of the protein synthesis inhibitor, cycloheximide. The presence of SERCA1a in the nuclear envelope was unaffected by cycloheximide, as can be seen by comparing Figures 1a and 1b Had the nuclear envelope labeling represented SERCA1a that was being transported out of the ER to the SR, the protein synthesis inhibitor would have been expected to decrease or eliminate this staining. Since the halftime for the exit of proteins from the ER is between 15 minutes and two hours (22), the two hour incubation

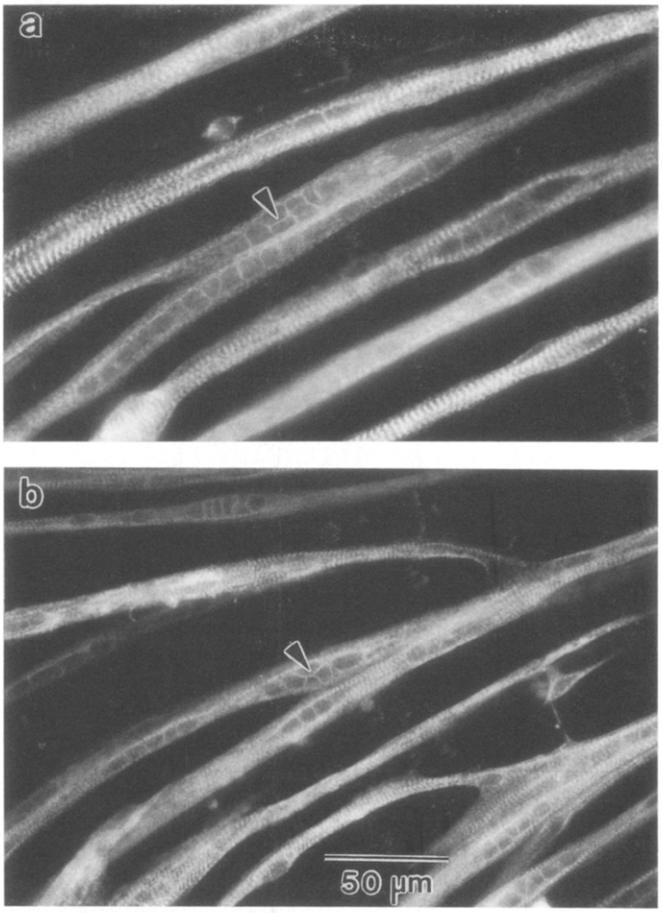

FIG. 1. Immunofluorescent localization of SERCA1a in chick myotubes incubated in the presence or absence of cycloheximide. Skeletal myotubes were incubated for two hours at 37°C in normal growth medium or medium containing 50 μg/ml cycloheximide. The cells were then fixed and immunolabeled with the anti-SERCA1a antibody, CaF1, as described in Materials and Methods. SERCA1a labeling can be seen in both sets of myotubes in the SR, characterized by a striated appearance, and in the nuclear envelope (arrowheads). (a) control, (b) + cycloheximide; bar = 50 μm.

period used here should have been sufficient to effect at least a diminution in nuclear envelope labeling. We have not ruled out a very slow exit of newly-synthesized SERCA1a from the ER. That SERCA1a remains in the ER indicates that this enzyme is either stably localized there or represents a population of Ca^{2+}-ATPase molecules that are part of an exchangeable ER/SR pool, either of which implies the presence of ER targeting information in this enzyme.

This postulate was tested by expressing the cDNA encoding SERCA1a in a variety of cultured non-muscle cell lines. Immunofluorescent localization of SERCA1a in transfected mouse Ltk^- cells, monkey CV-1 cells, and chick embryo fibroblasts revealed a similar pattern (Fig. 2). In each case, SERCA1a was localized to a diffuse intracellular membrane system and to the nuclear envelope, the morphology expected of the ER. At higher magnifications, a

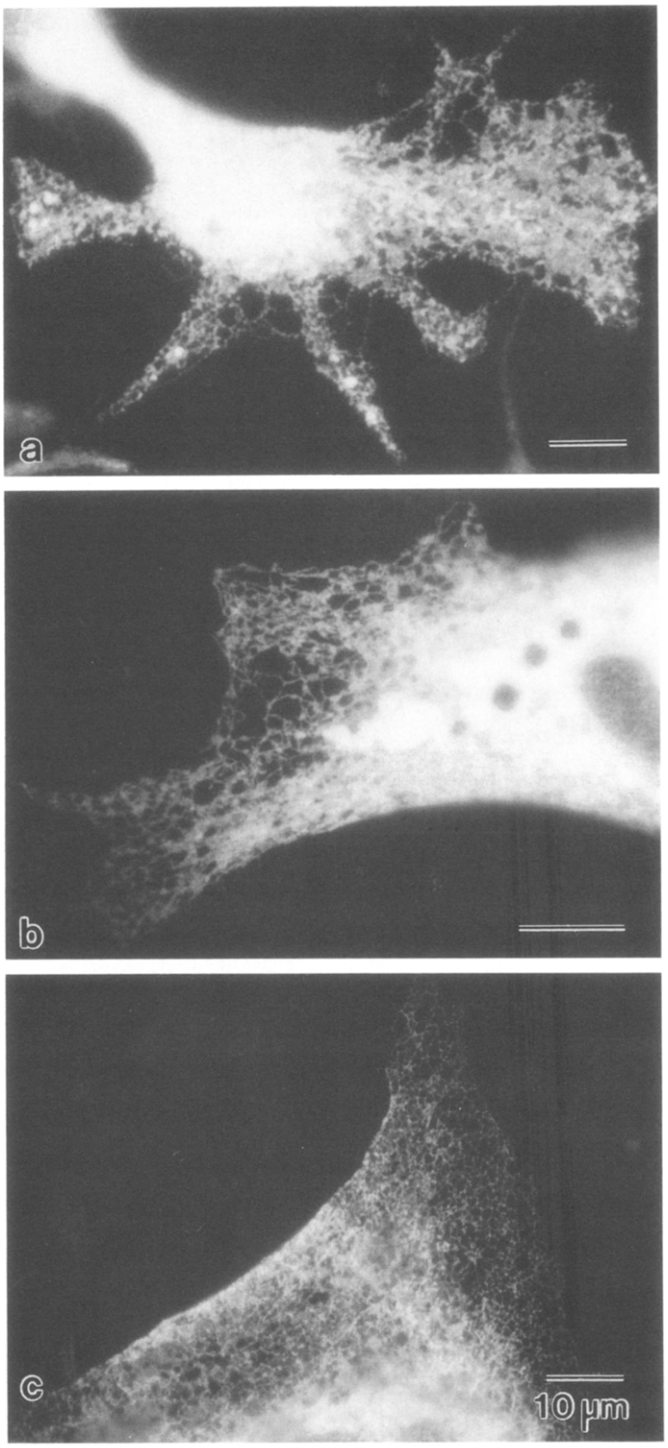

FIG. 2. Immunofluorescent localization of SERCA1a in transfected non-muscle cells. Cells were plated on coverslips and transfected with pSVFCa, then fixed and immunolabeled with CaF1. (a) Ltk$^-$, (b) chick embryo fibroblasts, (c) CV-1; bar = 10 μm.

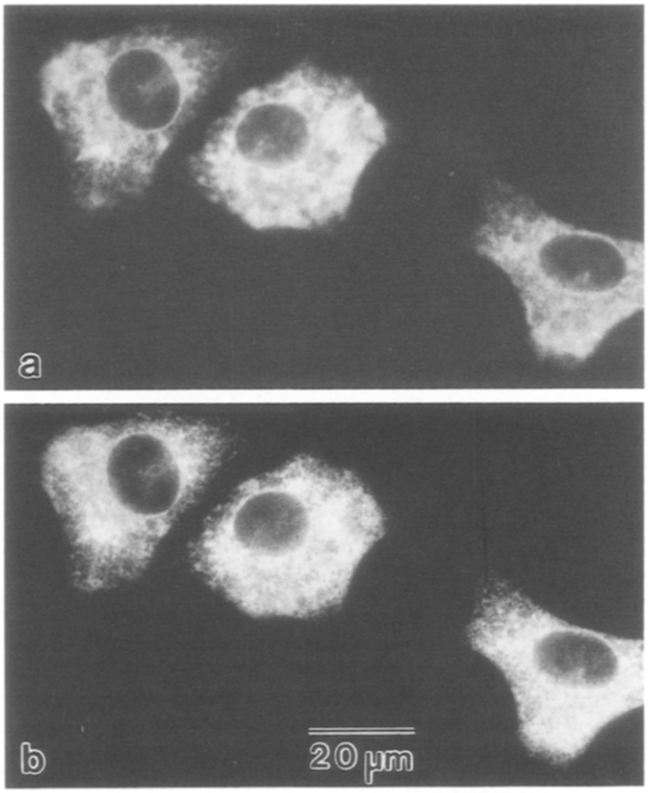

FIG. 3. Double-label immunofluorescent localization of SERCA1a and BiP in Ltk⁻ cells transfected with pSVFCa. Methanol-fixed cells were sequentially immunolabeled with CaF1 and anti-BiP. (a) SERCA1a, (b) BiP; bar = 20 μm.

characteristic net-like reticulum was commonly observed, especially near the periphery of transfected cells (Fig. 2). The identification of the membrane to which SERCA1a was targeted as ER was verified by double-label immunofluorescence using antibodies against SERCA1a and the luminal ER protein, BiP (Fig. 3).

The co-localization of SERCA1a with BiP in transfected Ltk⁻ cells (Fig. 3) invoked the possibility that SERCA1a is retained in the ER because it is bound to BiP, which is known to associate with misfolded proteins (2,11,16,17,20). In this eventuality, BiP molecules should co-immunopurify with SERCA1a. Ltk⁻ cells were transfected with SERCA1a cDNA, metabolically radiolabeled, and SERCA1a was immunopurified (Fig. 4). A single band corresponding to SERCA1a was observed (lane B) with no co-purification of BiP, a protein of approximately 78 kDa (lane C). This experiment was performed under ATP-depleting conditions since ATP induces the release of BiP from the proteins to which it is bound (20). The absence of BiP in the SERCA1a immunopurification cannot be attributed to insufficient labeling of BiP, as can be seen from Figure 4 (lane C) which shows the result of immunopurification of a parallel culture with an anti-BiP antibody. We do not consider it likely that SERCA1a would selectively

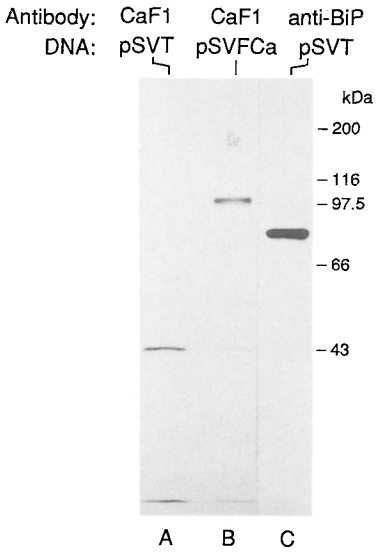

FIG. 4. Immunopurification of SERCA1a from transfected mouse Ltk⁻ cells. Cells were transfected and labeled with [^{35}S]methionine as described in Materials and Methods. ATP-depleted lysates were prepared from cells transfected with either vector alone (pSVT) or with vector containing the SERCA1a cDNA (pSVFCa), then incubated with immunobeads prepared from either CaF1 IgG or with anti-BiP. Eluates from washed immunobeads were separated by SDS-PAGE and fluorographed. Immunopurifications from (A) control cells with CaF1 beads, (B) from SERCA1a-expressing cells with CaF1 beads, and (C) control cells with anti-BiP beads. BiP does not co-purify with SERCA1a purified from pSVFCa-transfected cells. The labeled band at 43 kDa in lanes A and B represents non-specific association of actin and is observed in variable amounts.

associate with a pool of unlabeled BiP. Importantly, the results in Figure 4 also showed that the immunoreactive SERCA1a protein is full-length (105 kDa), as opposed to a collection of breakdown products that might result from the degradation of the enzyme if it were non-specifically held in the ER (16). Since tryptic digestion of SERCA1a does not abolish CaF1 binding (not shown), it is unlikely that proteolysis of SERCA1a would go undetected. Thus, SERCA1a is not retained non-specifically in the ER by BiP in these transfected fibroblasts.

While several luminal SR proteins have been reported as having ER targeting sequences (7), no sorting sequences have been identified on SR transmembrane proteins. Jackson *et al.*, (12) have proposed that a short linear sequence (KKXX or KXKXX) at the cytoplasmic C-terminus of a number of transmembrane ER proteins is responsible for their subcellular distribution (although Gabathuler and Kvist (8) suggested a more complex signal at the C-terminus). One of the family of SR/ER Ca^{2+}-ATPase's, SERCA3, which is expressed in both muscle and non-muscle tissue contains a C-terminal sequence (KKDLK) resembling this consensus (5), but the subcellular distribution of this Ca^{2+}-ATPase isoform is not known. SERCA1a does not have a recognizable ER targeting domain at its C-terminus.

SR proteins are synthesized in the rough ER, which in early development is continuous with the SR (6). One explanation for the localization of SERCA1a in the ER is that structural continuities between the ER and SR remain in differentiated muscle, or that there is vesicle traffic between the ER and SR, and that Ca^{2+}-ATPase distributes equally between these two membrane systems. In this case, one sorting signal might be sufficient to explain the presence of this enzyme in the ER and SR. Alternatively, it is possible that SR proteins contain distinct ER and SR targeting signals. It is not clear whether transmembrane ER proteins are restricted from leaving this organelle, of if they retrieved from a "salvage compartment," as is the case for luminal ER proteins (22). No escape of SERCA1a from the ER was detectable, in spite of high levels of expression; Jackson *et al.* (12) noted a similar phenomenon in their studies of the adenovirus E3/19K protein, a membrane-spanning protein localized in the ER of infected cells. This suggests that the sorting machinery was not saturated with SERCA1a, and may indicate that this protein was retained in the ER by a retrieval mechanism. Experiments are underway which are designed to identify the regions of SERCA1a involved in targeting it to both the ER and SR, and to define the means by which this distribution occurs.

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