Association of Sorcin With the Cardiac Ryanodine Receptor*

(Received for publication, May 17, 1995, and in revised form, August 30, 1995)

Marian B. Meyers§, Virginia M. Pickel†, Shey-Shing Sheu, Virendra K. Sharma, Kathleen W. Scotto**, and Glenn I. Fishman‡‡

From the †Department of Medicine, Section of Molecular Cardiology, Albert Einstein College of Medicine, Bronx, New York 10461, the ‡Department of Neurology and Neuroscience, Cornell University Medical College, New York, New York 10021, the †Department of Pharmacology, University of Rochester Medical Center, Rochester, New York 14642, and the **Molecular Pharmacology and Therapeutics Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Sorcin is a 22-kDa calcium-binding protein initially identified in multidrug-resistant cells; however, its patterns of expression and function in normal tissues are unknown. Here we demonstrate that sorcin is widely distributed in rodent tissues, including the heart, where it was localized by immunoelectron microscopy to the sarcoplasmic reticulum. A >500-kDa protein band immunoprecipitated from cardiac myocytes by sorcin antiserum was indistinguishable in size on gels from the 565-kDa ryanodine receptor/calcium release channel recognized by ryanodine receptor-specific antibody. Association of sorcin with a ryanodine receptor complex was confirmed by complementary co-immunoprecipitations of sorcin with the receptor antibody. Forced expression of sorcin in ryanodine receptor-negative Chinese hamster lung fibroblasts resulted in accumulation of the predicted 22-kDa protein as well as the unexpected appearance of ryanodine receptor protein. In contrast to the parental host fibroblasts, sorcin transfectants displayed a rapid and transient rise in intracellular calcium in response to caffeine, suggesting organization of the accumulated ryanodine receptor protein into functional calcium release channels. These data demonstrate an interaction between sorcin and the ryanodine receptor and suggest a role for sorcin in modulation of calcium release channel activity, perhaps by stabilizing the channel protein.

Sorcin was initially identified as a 22-kDa protein in cultured cells selected for resistance to natural product cancer drugs, such as vincristine, adriamycin, and actinomycin D, i.e. multidrug-resistant cells (1–5). One of the major mechanisms of resistance in these cells is mediated by overexpression of the membrane-bound drug transporter, P-glycoprotein (6). Molecular cloning studies demonstrated that the sorcin and P-glycoprotein genes are tightly linked and that both may be amplified during the acquisition of multidrug resistance. However, while P-glycoprotein overexpression correlates with resistance development, increased sorcin expression is not obligatory, and its abundance does not correlate with the degree of resistance (4–9). Complementary DNA for sorcin has been isolated from hamster (2) and human (10) multidrug-resistant cells, which amplify the sorcin gene. The highly conserved sequence, with 95% homology between hamster and human sorcin, predicts a 22-kDa protein with four putative Ca2+-binding domains, two with strong homology to calmodulin "EF hand" motifs (2). In a classification of Ca2+-binding proteins based on sequence, sorcin was placed among members of the closely related calpain and sarcoplasmic Ca2+-binding protein subfamilies (11). Direct 45Ca2+-binding to sorcin has been demonstrated by in vitro assays (3), and Ca2± affinity in the μM range has been determined by fluorescence spectroscopy (12). Sorcin also contains putative recognition sites for protein kinase A, and phosphorylation of sorcin in drug-resistant cell-free extracts or of purified sorcin in the presence of the catalytic subunit of protein kinase A has been observed (4, 5, 13). Although initially characterized as a soluble protein, our recent studies have shown that sorcin undergoes Ca2+-mediated translocation from soluble to cellular membrane sites (12). Despite these biochemical data suggesting a role for sorcin in Ca2± handling, a function for sorcin in multidrug resistance or in P-glycoprotein activity remains speculative (2–5).

In the present study, we began to characterize the expression of sorcin in normal tissues and gain some insight into its function. We found that sorcin is widely expressed in mammalian tissues, including the heart, where it was localized to cardiac sarcoplasmic reticulum (SR).1 Co-immunoprecipitation studies revealed an association between sorcin and the ryanodine receptor (RyR), the calcium release channel located in muscle SR at the junction of transverse tubules and SR terminal cisternae (14). Sorcin transfectants in DC-3F Chinese hamster lung fibroblasts were generated to study a possible role for sorcin in intracellular calcium transport. These cells were characterized immunocytochemically for sorcin and ryanodine receptor expression as well as functionally, by digital Ca2+ imaging for their response to caffeine, a potentiator of Ca2± release from the SR through RyR/Ca2± release channels (14, 15).

MATERIALS AND METHODS

Antibodies—A mouse monoclonal antibody raised against a peptide from the sorcin N terminus (amino acids 21–56) (2) (Berkeley Antibody Company, Richmond, CA) and rabbit polyclonal antiserum raised against a peptide from the sorcin C terminus (amino acids 178–198) (2) (Poono Rabbit Farm and Laboratory, Inc., Canadensis, PA) were developed for these studies. Both antibodies recognized a 22-kDa protein as the predominant immunoreactive species in immunoblot analysis of

1 The abbreviations used are: RyR, ryanodine receptor; SR, sarcoplasmic reticulum.
multidrug-resistant cells, bacterially expressed recombinant protein (12), and in vitro translation products. In some cases minor higher molecular weight bands were also observed. Detection of all immuno-reactive bands was abrogated when antibodies were preadsorbed with appropriate antigenic peptide for 15 min at 4°C. Guinea pig antiserum recognizing cardiac and brain RyR (GP561) was a generous gift from Dr. Kenner F. Stuehr, Howard Hughes Medical Institute (University of Iowa) (16) and a mouse monoclonal antibody raised against canine cardiac RyR (C3–33) was a generous gift from Dr. Gerhard Meissner, University of North Carolina (17).

Cardiac Lines—The spontaneously transformed Chinese hamster lung cell line, DC-3F, vincristine-resistant subline, DC-3F/VCrd-5L, and actinomycin D-resistant line, DC-3F/AD, all together with the DC-3F/VCRd-5L line, were characterized in previous studies (1). The cells were maintained in 1:1 Eagle's minimum essential medium and Ham's F12 supplemented with 5% fetal bovine serum, and, for the multidrug-resistant cells, 50 μg/mL of vincristine (generously supplied by Eli Lilly and Co., Indianapolis, IN) or 10 μg/mL actinomycin D (Sigma). DC-3F/VCrd-5L cells express 10–15 times more sorcin than DC-3F (as a result of amplification of the sorcin gene), unlike DC-3F/AD X cells, which do not overexpress sorcin and do not contain amplified genes for sorcin (18). The two resistant lines express equivalent, high levels of P-glycoprotein (19).

Preparation of Tissue and Cells—Tissue samples from adult BALB/c mice were minced and sonicated in 50 mM Hepes buffer (pH 7.4) containing 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 10% phenylmethylsulfon fluoride to obtain a 10% homogenate. Cardiac myocytes were enzymatically dissociated from neonatal Sprague-Dawley rat heart as described previously (20) and plated in 60-mm tissue culture dishes for short term culture. In some experiments cardiac myocytes were separated from fibroblasts (20). Metabolic labeling with 100 μCi/mL [35S]methionine (DuPont NEN) in methionine-free medium was accomplished by overnight incubation of monolayer cells at 37°C. Labelled or unlabelled cardiac myocytes or cultured Chinese hamster cells and sublines were lysed in 20 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl, 2 mM EDTA, and 1 mM phenylmethylsulfon fluoride by needle extraction. Where noted, antibody preadsorption experiments were conducted with 3.3 mg/mL bovine serum albumin and 0.05% Tween 20 (Sigma) or in primary antibody preincubated with 10 μg/mL of the corresponding antigenic peptide for 15 min at 4°C. The papers were washed with diluting buffer and finally incubated with peroxidase-conjugated goat anti-mouse IgG and/or rhodamine-conjugated goat anti-rabbit IgG antibody (21). All procedures involving use of animal tissues were undertaken in accordance with institutional guidelines.

Immunohistochemical Analysis—Aliquots of tissue or cell homogenates were subjected to electrophoresis and transferred to nitrocellulose according to published procedures (3, 21, 22). For Western blot analysis with sorcin peptide antisemur, nitrocellulose papers were incubated in 5% dry milk in 0.1 mM Tris buffer (pH 7.4) for 1 h and then in primary antibody diluted in 0.1% Triton buffer (pH 7.4), containing 0.15 M NaCl and 1% bovine serum albumin (Sigma) or in the corresponding antigenic peptide for 15 min at 4°C. The papers were washed with diluting buffer and finally incubated with peroxidase-conjugated Protein A (Bio-Rad Laboratories, Hercules, CA) before analysis by chemiluminescence (ECL Western blotting System, Amersham Corp.). For immunoprecipitations, aliquots of cell homogenates containing 100–200 μg of protein were incubated for 1 h at room temperature with 1:500 dilutions of antibodies to sorcin or to RyR (C3–33) (17) in buffer containing 10 mM Tris (pH 7.4), 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% sodium dodecyl sulfate. Antibody-antigen complexes were precipitated with immobilized Protein A (for antisemur to sorcin C terminus peptide) or G (for N terminus peptide antibody or RyR antibody) (Sigma). Antigens were solubilized by heating the washed complexes in Laemmlli buffer (21) at 100°C for 2 min (for sorcin detection) or by treating the complexes with 2% sodium dodecyl sulfate in 6.25 mM Tris·HCl and 10% glycerol for 10 min at room temperature (for RyR detection) before examination by gel electrophoresis (21) and autoradiography (for labeled samples) by exposure of dried gels to Kodak X-AR film for 30 h or Western blot analysis with sorcin peptide antibody as described above.

Northern Blot Analysis—Total RNA samples were isolated from rat and mouse heart and spleen and the use of TRIzol reagent (Life Technologies, Inc.) according to the manufacturer's procedures, and poly(A)~+~ RNA was prepared with Dyna beads mRNA Direct kit (Dynal A.S., Oslo, Norway). RNA samples were electrophoresed on 0.8% agaroseformaldehyde gels and capillary-blotted onto Nytran membranes (Schleicher and Schuell). Membranes were prehybridized at 42°C in 5 x SSC, 50 mM sodium phosphate, pH 7.4, 1 x Denhardt's solution, 2% sodium dodecyl sulfate with 100 μg/mL denatured salmon sperm DNA and then hybridized in that buffer with random prime-labeled sorcin c6 probe (2, 18) for 16 h at 42°C. The c6 probe contains the 1.0-kilobase sorcin coding region isolated from hamster (2). Blots were washed with 0.2 x SSC, 0.2% SDS at 60°C and exposed to Kodak X-AR film (Kodak, Rochester, NY) for 20 h.

Electron Microscopic Immunocytochemistry—Adult male Sprague-Dawley rats (200–300 g) were deeply anesthetized with sodium pentabarbitol (50 mg/kg intraperitoneally). The thoracic cavity was opened, and the excised heart ventricle was removed and placed in a solution containing 3.4% acrolein and 2% paraformaldehyde in 0.1 mM phosphate buffer at pH 7.4. Sections (30–50 μm in thickness) were cut with the use of a Lancer Vibratome and collected in phosphate buffer. Tissue sections were rinsed in 0.3 M sodium borohydride solution in the same buffer for 30 min to neutralize aldehydes prior to immunocytochemical labeling (24). Tissue sections were incubated for 24 h at room temperature with antibody raised against the sorcin N terminus or C terminus peptide or preadsorbed control antibody at dilutions ranging from 1:100 to 1:1000 in 0.1 mM Tris-buffered saline containing 1% bovine serum albumin and 0.035% Triton X-100. Preadsorbed control antisemur was prepared by incubating 1 ml of the working dilution of antibodies with excess sorcin peptidk. After primary antibody incubations, sections were washed, placed for 30 min each in biotinylated goat anti-mouse or anti-rabbit IgG (1:400) and peroxidase-avidin complex (ABC, Elite Kit, Vector Laboratories, CA), which were reacted with 3.3 mg/mL of 0.01% hydrogen peroxide for 6 min. All incubations and washes between each step were carried out in 0.1 mM Tris-buffered saline. Immunolabeled sections were then processed for electron microscopy using conventional methods (24). Ultrathin sections from outer and inner portions of the tissue sections were examined with a Phillips CM-10 electron microscope. Selective peroxidase immunoreactivity was detected by detection of a granular precipitate that was seen only near the accessible surface of tissue sections incubated with the nonadsorbed antibody.

Sorcin Expression Vector, Transfection, and Immunocytochemistry—Stable transfectants that overexpressed sorcin were generated in DC-3F host cells. Transfections were carried out by the calcium phosphate/DNA precipitation technique (26) with pflag sorcin expression plasmid, in which transcription was directed by the human CMV promoter and enhancer (a generous gift from Dr. Piet Borst, Director of the Netherlands Cancer Institute) (27). In brief, cells were transfected with 15 μg of pflagCMV/DSR and 5 μg of p308 plasmid (American Type Culture Collection, Rockville, MD, number 37613) containing the neo gene (28). Cells were selected in 100 μg/mL G418 (Sigma) for approximately 2 weeks until distinct colonies were visible. Clonal populations of G418-resistant cells were obtained by serial dilution. Control DC-3F cells transfected with the p308 neo gene alone were generated in parallel. Integration of the sorcin expression gene was confirmed by Southern blot analysis (29) of genomic DNA from individual transfectants. The transfectants were screened for resistance to 50 μg/mL G418 and were to niracin D, and adriamycin (drugs to which cells become multidrug-resistant). The clones were not resistant to those drugs; doses for 50% cell kill were identical in transfectants and DC-3F host cells. Increase in P-glycoprotein expression in sorcin transfectants was not observed (data not shown). DC-3F, DC-3F/VCrd-5L, transfected DC-3F cells and other controls were grown on two-chamber glass slides (Lab-Tek Chamber Slide System, VWR Scientific, Rochester, NY) or glass coverslips. The cells were rinsed and fixed as described above for heart tissue. Cells were incubated for 12–18 h in 0.1 mM Tris-buffered saline containing 0.1% bovine serum albumin and either a 1:1000 dilution of the rabbit antisemur against sorcin C terminus peptide or a 1:500 dilution of GP561, a polyclonal antisemur raised in guinea pig against purified rabbit brain ryanodine receptor. The cells were then processed with the ABC method described above, and the bound peroxidase was visualized.
by light microscopy.

Digital Ca\textsuperscript{2+} Imaging—Cells were plated on cover glasses (25-mm circle, VWR Scientific) in 35-mm tissue culture dishes. The cells were maintained and experiments were performed in a Hepes-buffered (pH 7.4) Krebs-Henseleit solution containing 145 mM NaCl, 5 mM KCl, 2 mM CaCl\textsubscript{2}, 2 mM MgCl\textsubscript{2}, 10 mM glucose, 10 mM Hepes, and 0.1% bovine serum albumin with or without 10 mM caffeine (Sigma) and loaded with 5 \mu M fura-2/AM (Molecular Probes, Inc., Eugene, OR) for 30–40 min at 37 °C to initiate the experiments. The cells were then transferred to Hepes-buffered solution without fura-2/AM and incubated for 1 h, and the cover glasses were placed in a tissue chamber mounted on the stage of a Nikon microscope as described previously (30). Sequential excitation at 340 and 380 nm was used, and the emitted fluorescent images were acquired at 500 nm by a silicon-intensified target camera (Dage MTI 65, Michigan City, IN). After determination of base-line Ca\textsuperscript{2+} levels, cells were perfused with medium containing 10 mM caffeine until termination of the experiment. Fluorescent ratio images were calculated off-line on a pixel-by-pixel basis by dividing the 340-nm image by the 380-nm image after background subtraction. Intracellular Ca\textsuperscript{2+} levels, correlated with the instrument-derived false color spectrum, were determined by in vitro calibration (31). Quantitation of the effect of caffeine on intracellular Ca\textsuperscript{2+} levels in the cells was based on values obtained for an average of 10 determinations with at least 50 cells/microscope field.

RESULTS

Sorcin Distribution among Normal Tissues—Western blot analysis of mouse tissue homogenates with the sorcin C terminal peptide antiserum revealed that the 22-kDa protein was present in a wide variety of tissues, including heart, spleen, lung, skeletal muscle, liver, and brain (Fig. 1). The slightly faster migrating band in kidney samples was consistently observed and may represent a degradation product. Antibody specificity was demonstrated by immunoblot analysis of DC-3F/VCRd-5L and heart proteins with preadsorbed antiserum (Fig. 2). The predominant 22 kDa species as well as a minor 35 kDa band observed in some tissue and cultured cell samples, were not detected by preadsorbed antiserum. Quantitative Western blotting showed that the abundance of sorcin in lysates of heart is 50–70-fold less than that found in the DC-3F/
Sorcin mRNA in Heart and Spleen—Two major sorcin transcripts of ~1.0 and 2.5 kilobases were detected in normal mouse heart and spleen poly(A)
+ samples, using a hamster cDNA probe (Fig. 3). These sizes were identical to sorcin transcripts expressed in DC-3F/VCRd-5L cells (18), where they have been shown to represent the use of alternative polyadenylation sites (2). The transcripts were present at markedly lower levels in the normal tissue samples, in parallel with the protein quantitation comparison, and required examination of purified mRNA samples for adequate detection. The relative abundance of sorcin mRNA in normal tissues, i.e. heart and spleen, paralleled the amount of encoded protein detected immunologically (Fig. 1).

Neonatal Rat Heart Cells Are Sorcin-positive by Immunofluorescent Labeling—Preparations enriched for cardiac myocytes were double-labeled with antibody to sarcomeric myosin heavy chain (Fig. 4, left panel) and to the antibody raised against the sorcin C terminus (Fig. 4, right panel). Myocytes were identified by their characteristic filamentous labeling pattern (20, 23). These cells were strongly reactive with the sorcin antisemum; immunolabeling was mainly extranuclear and was distributed throughout the cytoplasm, although some punctate nuclear labeling was observed. Antibody raised against the N terminus sorcin peptide produced an identical labeling pattern (data not shown). Preferential expression of sorcin in myocytes was observed; the occasional non-myocyte was not positive for myosin heavy chain and had reduced or nondetectable sorcin fluorescence.

Cardiac SR Is Selectively Labeled with Sorcin Antibody—The intracellular localization of sorcin within cardiac cells was investigated with immunoelectron microscopy. Intense immunoperoxidase labeling of SR tubules in rat ventricular cardiomyocytes was observed with either the C or N terminus peptide antibodies. N terminus peptide antibody labeling is shown in Fig. 5; use of C terminus peptide antiserum produced the same pattern. Punctate labeling of the lateral sacculles of the reticulum was also detected when myocyte lysates were immunoprecipitated with antibody to sorcin N terminus peptide (lane 5), and antiserum to sorcin C terminus peptide (lane 6); and cardiac fibroblasts were immunoprecipitated with RyR antibody (C3–33) (lane 7). Aliquots of homogenates containing 200 μg of protein (at equivalent specific radioactivities) were analyzed by immunoprecipitation on 5% acrylamide gels. The ~500-kDa band present in lanes 1 and 2 was also detected when myocyte lysates were treated with diithiothreitol (Dithiobis succinimidylpropionate) cross-linking agent before immunoprecipitation.
lum was seen near the transverse tubules (Fig. 5A). Mitochondrial membranes were not immunolabeled except at points directly in contact with or in close proximity to the SR near the transverse tubules (Fig. 5B). Plasma membranes also had no detectable sorcin except near points of contact with SR. Control sections immunolabeled with adsorbed antibody and specifically labeled sections collected at depths within the tissue having limited access to primary antibody showed no immunolabeling (Fig. 5C).

Cross-immunoprecipitation with Antibodies to RyR and Sorcin Indicate an Association between the Two Proteins—Antibodies to peptides representing N and C termini of sorcin immunoprecipitated a >500-kDa protein from rat or mouse cardiac myocytes metabolically labeled with [35S]methionine (Fig. 6, lanes 1 and 2), which co-migrated with immunoprecipitated RyR (Fig. 6, lane 3; arrow at left indicates RyR) and with RyR detected in isolated myocyte SR by Western blot with RyR antibodies (not shown). Neither the sorcin nor RyR antibody immunoprecipitated high molecular weight proteins from cardiac fibroblasts (Fig. 6, lane 7) or DC-3F/VCRd-5L cells (Fig. 6, lanes 4–6). However, both RyR antibody (Fig. 6, lane 4) and sorcin antiserum (Fig. 6, lanes 5 and 6) immunoprecipitated a ∼110-kDa protein from the drug-resistant cells, similar in size to a 106-kDa SR protein shown to bind ryanodine and have Ca2+ release channel properties when incorporated into planar lipid bilayers (14, 32).

To address the question of whether the >500-kDa band represented aggregated material or a true association between sorcin and the RyR, additional immunoprecipitation experiments were carried out with the use of RyR-specific antibody. Immunoprecipitates of proteins from rat cardiac myocytes were solubilized in Laemmli buffer containing 2-mercaptoethanol and examined by Western blot analysis with sorcin antiserum, resulting in display of a prominent 22-kDa protein (Fig. 7, lane 1). Interestingly, no sorcin was detected when the myocytes were initially lysed in the presence of a reducing agent (Fig. 7, lane 2), suggesting a disulfide-linkage between sorcin and the RyR complex. RyR antibody did not recognize sorcin, nor did sorcin antibodies recognize RyR, by Western blot analysis.

Sorcin Transfectants Express both Sorcin and the RyR—To identify a functional role for sorcin, cell lines that stably overexpressed the protein were generated, and 10 clones of DC-3F cells co-transfected with pFRCMVSOR and p308 and five clones transfected with p308 alone were analyzed for sorcin expression by Western blot. Nine sorcin transfectant clones overexpressed sorcin, and one of those, DC-3F/sor.3, was selected for further analysis by immunocytochemistry. Surprisingly, in addition to the expected accumulation of sorcin in the transfected cell line, RyR immunoreactivity was now detected (Fig. 8, panels E (sorcin) and F (RyR)). In contrast, the DC-3F parental line (Fig. 8, panels C and D) was negative for both sorcin and RyR. Sham transfectants (DC-3F/neo), as well as the non-sorcin-overproducing drug-resistant DC-3F/AD X cells, were also negative for these proteins (not shown). To further
establish a relationship between sorcin and the RyR, DC-3F/VCRd-5L drug-resistant cells, which constitutively overexpress sorcin as a result of gene amplification, were assessed. In these cells, like the DC-3F/sor.3 transfectants, both sorcin and RyR protein were detected (Fig. 8, panels A (sorcin) and B (RyR)), suggesting that the increase in sorcin was associated with the coordinate accumulation of the RyR protein.

RyR-positive Sorcin Transfectants Exhibit Caffeine-sensitive Ca\(^{2+}\) Release from Intracellular Stores—To examine whether increased expression of sorcin and RyR protein was associated with functional Ca\(^{2+}\) channel release activity, the effects of caffeine on intracellular Ca\(^{2+}\) in the transfectant clone (DC-3F/sor.3) and the various control lines were carried out with the use of digital fluorescent Ca\(^{2+}\) imaging with fura-2/AM indicator. In DC-3F/sor.3 cells, 10 mM caffeine stimulated a transient increase in intracellular Ca\(^{2+}\) from about 100 nM (A) to about 250 nM (B), followed by a gradual decay toward base line in 4–8 min (C', D'). More than 90% of sorcin-transfected or drug-resistant cells in each field were caffeine-responsive. The rainbow spectrum on the right correlates with Ca\(^{2+}\) concentration as calculated from in vitro calibration methods described under “Materials and Methods.” Addition of EGTA to extracellular solutions during caffeine perfusion did not affect these results.

**Fig. 9.** Effect of 10 mM caffeine on intracellular Ca\(^{2+}\) by digital fluorescent Ca\(^{2+}\) imaging with fura-2/AM indicator. False color images show levels of intracellular Ca\(^{2+}\) in sham-transfected DC-3F cells (DC-3F/neo) of about 100 nM in the absence (A) or presence (B) of 10 mM caffeine. Caffeine stimulates an increase in intracellular Ca\(^{2+}\) in DC-3F/sor.3 sorcin-transfected cells from about 100 nM (C) to 240 nM (D). Sorcin-overproducing DC-3F/VCRd-5L cells also respond to 10 mM caffeine with a rise in intracellular Ca\(^{2+}\) from about 100 nM (A'), to about 250 nM (B'), followed by a gradual decay toward base line in 4–8 min (C', D'). More than 90% of sorcin-transfected or drug-resistant cells in each field were caffeine-responsive. The rainbow spectrum on the right correlates with Ca\(^{2+}\) concentration as calculated from in vitro calibration methods described under “Materials and Methods.” Addition of EGTA to extracellular solutions during caffeine perfusion did not affect these results.

**DISCUSSION**

Sorcin was initially identified as a protein differentially expressed during the acquisition of multidrug resistance (1–5). Although the mechanism underlying sorcin’s enhanced expression in these cells has been determined, its pattern of expression and function in normal tissues have not previously been characterized. With the use of immunological reagents, we now demonstrate that 22-kDa sorcin is widely expressed in mam...
malian tissues and is highly conserved among mammalian species. Antibodies raised against hamster sorcin peptide sequences recognized the 22-kDa protein in a number of other species, including mouse, rat (this paper), and human (4, 5). Higher molecular weight immunoreactive bands are observed in some tissue and cultured cell samples. Their identity has not been determined; however, they may represent different forms of sorcin or sorcin dimers. A related 28-kD protein, grancalcin, has been shown to exist in dimeric form, possibly through covalent linkage stable to thiol reagents (33).

The primary sequence of sorcin gives some clues as to its potential function. The two EF hand Ca$^{2+}$-binding domains are homologous with those in a number of Ca$^{2+}$-binding proteins, including calpain and calmodulin (2), and sorcin has been shown to bind Ca$^{2+}$ by in vitro assays (3, 12). Immunoelectron microscopy directly localized sorcin to the SR, at or near transverse tubules, suggesting that sorcin might participate in SR-mediated intracellular Ca$^{2+}$ regulation.

Biochemical studies presented here suggest that sorcin interacts directly with the cardiac RyR. Sorcin antisera immunoprecipitated a >500-kDa species from cardiac myocytes that co-migrated on gels with the protein immunoprecipitated by antibody raised against the 565-kDa RyR receptor. This finding suggests that sorcin is part of a complex, and an association between sorcin and the cardiac RyR was confirmed by co-immunoprecipitation of 22-kDa sorcin from cardiac myocytes with RyR-specific antibody. Immunoblot analysis of isolated SR protein with antibody to RyR and to sorcin revealed the presence of the 565-kDa RyR and of 22-kDa sorcin in a location protected from proteolysis by proteinase K (not shown), supporting the possibility of an intramembrane interaction between sorcin and the RyR complex.

Sorcin transfectants, generated to study a function for the Ca$^{2+}$-binding protein, were unexpectedly found to be RyR-positive. DC-3F/VCRd-5L cells, which overproduce sorcin by an entirely different mechanism, also displayed RyR immunoreactivity. The species accounting for the immunoreactivity in sorcin transfectants and in DC-3F/VCRd-5L cells suggested that sorcin influences the abundance of RyR protein. Given the demonstrated biochemical association between the two proteins, it is conceivable that sorcin directly stabilizes the RyR protein and retards its degradation, a mechanism that seems more plausible than a transcriptional effect on RyR gene expression. Clearly, sorcin alone is insufficient to promote the accumulation of the RyR in all cell types, since the former protein is much more widely expressed than the latter. However, in the appropriate cellular environment, such as sarcomeric muscle, the levels of sorcin may influence the abundance of co-expressed RyR. Thus, it will be of interest to compare the profiles of sorcin and RyR expression during myogenic differentiation, both in vivo and in cell culture model systems, such as the C2C12 mouse myoblast cell line (34), where our preliminary studies suggest a marked accumulation of sorcin during the process of differentiation into myotubes. It should be pointed out, with regard to a possible role for sorcin in multidrug-resistant cells, that forced expression of sorcin in Chinese hamster lung cells did not, on its own, confer the multidrug resistance phenotype.

Both DC-3F/VCRd-5L and DC-3F/sor.3 cells, with increased levels of sorcin and RyR protein, demonstrated a characteristic property of the RyR/Ca$^{2+}$ release channel, i.e. caffeine-induced intracellular Ca$^{2+}$ release. The temporal profile of Ca$^{2+}$ movement exhibited by the sorcin-transfected fibroblasts in response to caffeine paralleled that observed in similarly treated cardiac myocytes. These results suggest that nonexcitable cells may serve as a useful model to study some aspects of intracellular Ca$^{2+}$ movement normally associated with excitable tissues, analogous to the recent use of such cells for the study of contractile protein function (35). The ultimate goal of such analyses is to produce new information about cardiac or muscle cell function and diagnose dysfunction.

Although these data suggest an important interaction between sorcin and the RyR, they do not indicate whether interaction between sorcin and RyR is direct or through a third or intermediary protein. The data also do not address whether sorcin modulates any gating parameters of the Ca$^{2+}$ release channel. Detailed functional studies using expression systems in which sorcin and/or RyR abundance can be manipulated and Ca$^{2+}$ movement determined will be required to investigate the latter possibility.

Acknowledgments—We gratefully acknowledge the gift of the pRCl-oMVSOR construct from Drs. Piet Borst and Frank Baas (The Netherlands Cancer Institute), the GP561 antisera against ryanodine receptor from Dr. Kevin Campbell (an Investigator at the Howard Hughes Medical Institute), and the C33-ryanodine receptor antibody from Dr. Gerhard Meissner (University of North Carolina). We thank Ms. Aileen Heras, Jue Chan, Kathy Clark, and Chan Hee Song for valuable technical assistance.

REFERENCES

1. Meyers, M. B., Spengler, B. A., Chang, T. D., Meiera, P. W., and Biedler, J. L. (1985) in Stimulus-Response Coupling: The Role of Intracellular Calcium-Binding Proteins, pp. 159–171, CRC Press, Inc., Boca Raton, FL.

2. Endicott, J. A., and Ling, V. (1989) in Novel Calcium-Binding Proteins: Fundamentals and Clinical Implications (Heizman, C. W., ed) pp. 385–399, Springer-Verlag, Heidelberg.

3. Meyers, M. B. (1990) in Stimulus Response Coupling: The Role of Intracellular Calcium-Binding Proteins (Smith, V. L., and Dedman, J. R., eds) pp. 159–171, CRC Press, Inc., Boca Raton, FL.

4. Endicott, J. A., and Ling, V. (1989) Annu. Rev. Biochem. 58, 137–171.

5. Meyers, M. B. (1993) in Novel Calcium-Binding Proteins: Fundamentals and Clinical Implications (Heizman, C. W., ed) pp. 385–399, Springer-Verlag, Heidelberg.

6. Endicott, J. A., and Ling, V. (1989) Annu. Rev. Biochem. 58, 137–171.

7. Jongsma, A. P. M., Spengler, B. A., Van der Bliek, A. M., Meyers, M. B., Biedler, J. L., Hes, E., and Borst, P. (1986) EMBO J. 5, 3201–3208.

8. Meyers, M. B. (1987) Biochem. Pharmacol. 36, 2373–2380.

9. Meyers, M. B. (1990) in Stimulus Response Coupling: The Role of Intracellular Calcium-Binding Proteins (Smith, V. L., and Dedman, J. R., eds) pp. 159–171, CRC Press, Inc., Boca Raton, FL.

10. Meyers, M. B. (1993) in Novel Calcium-Binding Proteins: Fundamentals and Clinical Implications (Heizman, C. W., ed) pp. 385–399, Springer-Verlag, Heidelberg.

11. Endicott, J. A., and Ling, V. (1989) Annu. Rev. Biochem. 58, 137–171.

12. Jongsma, A. P. M., Spengler, B. A., Van der Bliek, A. M., Borst, P., and Biedler, J. L. (1987) Cancer Res. 47, 2875–2878.
Association of Sorcin with Ryanodine Receptor

11. Moncrief, N. D., Krebsinger, R. H., and Goodman, M. (1990) J. Mol. Evol. 30, 522–562
12. Meyers, M. B., Zamparelli, C., Verzili, D., Dick, A. P., Blanck, T. J. J., and Chiancone, E. (1995) FEBS Lett. 357, 230–234
13. Meyers, M. B. (1989) Cancer Commun. 1, 233–241
14. Coronado, R., Morissette, J., Sukhareva, M., and Vaughan, D. M. (1994) Am. J. Physiol. 266, C1485–C1504
15. Su, J. Y., and Hasselbach, W. (1984) Pfluegers Arch. 400, 14–21
16. Sharp, A. H., McPherson, P. S., Dawson, T. M., Aoki, C., Campbell, K. P., and Snyder, S. H. (1993) J. Neurosci. 13, 3051–3063
17. Lai, F. A., Liu, Q. Y., Xu, L., El-Hashem, A., Kramarcy, N. R., Sealock, R., and Meissner, G. (1992) Am. J. Physiol. 263, C365–C372
18. de Bruijn, M. H. L., Van der Bliek, A. M., Biedler, J. L., and Borst, P. (1986) Mol. Cell. Biol. 6, 4717–4722
19. Peterson, R. H. F., Meyers, M. B., Spengler, B. A., and Biedler, J. L. (1983) Cancer Res. 43, 222–228
20. De Leon, J. R., Buttrick, P. M., and Fishman, G. I. (1994) J. Mol. Cardiol. 26, 379–389
21. Laemmli, U. K. (1970) Nature 227, 680–685
22. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
23. Crow, M. T., and Stockdale, F. E. (1984) Exp. Biol. Med. 9, 165–174
24. Leranth, C., and Pickel, V. M. (1989) in Neuroanatomical Tract-tracing Methods (Heimer, L., and Zaborsky, L., eds) pp. 129–172, Plenum Publishing Co., New York
25. Hsu, S., Raine, L., and Fanger, H. (1981) J. Histoch. Cytochem. 29, 577–580
26. Graham, F. L., and van der Eb, A. J. (1973) Virology 52, 456–467
27. Van der Bliek, A. M., Koilman, P. M., Schneider, C., and Borst, P. (1988) Gene (Amst.) 71, 401–411
28. Southern, P. J., and Berg, P. (1982) J. Mol. Biol. Genet. 1, 327–341
29. Southern, E. M. (1975) J. Mol. Biol. 98, 503–517
30. Williford, D. J., Sharma, V. K., Korth, M., and Sheu, S. S. (1990) Circ. Res. 66, 241–248
31. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
32. Salama, G., Nigam, M., Shome, K., Finkel, M. S., Lagaur, C., and Zaidi, N. F. (1992) Cell Calcium 13, 635–647
33. Teahan, C. G., Totty, N. F., and Segal, A. W. (1992) Biochem. J. 286, 549–554
34. Yaffe, D., and Saxel, O. (1977) Nature 270, 725–727
35. Vikstrom, K. L., Rowner, A. S., Saax, C. G., Bravo-Zehnder, M., Straceski, A. J., and Leinwand, L. A. (1993) Cell Motil. Cytoskeleton 26, 192–204