HSP70 Binds to the Fast-twitch Skeletal Muscle Sarco(endo)plasmic Reticulum Ca\(^{2+}\)-ATPase (SERCA1a) and Prevents Thermal Inactivation*

This study examined whether HSP70 could bind to and protect against thermal inactivation of SERCA1a, the SERCA isoform expressed in adult fast-twitch skeletal muscle. Sarco(endo)plasmic reticulum vesicles prepared from rat gastrocnemius muscle were incubated with purified HSP70 at both 37 and 41 °C for either 30, 60, or 120 min. Maximal SERCA1a activity (μmol/g protein/min) in the absence of HSP70 was reduced progressively with time, with greater reductions occurring at 41 °C compared with 37 °C. HSP70 protected against thermal inactivation of SERCA1a activity at 37 °C but not at 41 °C and only at 30 and 60 min but not at 120 min. HSP70 also protected against reductions in binding capacity for fluorescein isothiocyanate, a fluorescent probe that binds to Lys\(^{+}\) in the nucleotide binding domain of SERCA, at 30 and 60 min but not at 120 min, an effect that was independent of temperature. HEK-293 cells were co-transfected with cDNAs encoding rabbit SERCA1a and human HSP70 to prevent protein aggregation and assist with refolding seen in other thermal conditions both in vitro and in HEK-293 cells. Modeling showed that the fluorescein isothiocyanate-binding site of intact SERCA1a in the E2 form lies in its close proximity to a potential interaction site between SERCA1a and HSP70. These results indicate that HSP70 can bind to SERCA1a and, depending on the severity of heat stress, protect SERCA1a function by stabilizing the nucleotide binding domain.

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
vitro by blocking the aggregation of SERCA1a at 25 °C (30). However, it is unknown whether HSP70 can interact physically with SERCA1a and prevent inactivation of SERCA1a activity that normally occurs with oxidative stress. To explore this possibility, we prepared SR membrane fractions from rat fast-twitch skeletal muscle and mixed them with a purified recombinant HSP70 just prior to prolonged incubation at temperatures known to cause sulfhydryl (SH) oxidation and thermal instability of SERCA1a in vitro (13). We found that HSP70 binds to SERCA1a, prevents inactivation of maximal SERCA1a activity, and preserves FITC binding capacity of SERCA1a during heat stress in vitro. Experiments using HEK-293 cells that co-expressed SERCA1a and HSP70 also confirmed that the two proteins can interact physically in intact mammalian cells. However, functional protection of SERCA1a and, to a lesser extent, preservation of FITC binding capacity by HSP70 were lost with progressively greater severity of heat stress. We propose that HSP70 can bind to SERCA1a and, depending on the severity of heat stress, protect SERCA1a function by stabilizing the nucleotide binding (N) domain.

EXPERIMENTAL PROCEDURES

Materials—Protein G-Sepharose and a primary immunoprecipitation kit for measurement of co-immunoprecipitation was from Pierce. A chemiluminescence kit for immunoblotting was from Amersham Biosciences. The protease inhibitor mixture (Complete, Mini) was from Roche Applied Science. Recombinant rat HSP70 protein (SP-7585), mouse anti-HSP70 monoclonal antibody SPA-810, and rabbit anti-HSP70 polyclonal antibody SPA-812 were from Stressgen Biotechnologies. Fluorescein isothiocyanate (FITC) and the anti-fluorescein/Oregon Green monoclonal antibodies 4-4-20 were from Molecular Probes. Anti-rabbit FITC- and anti-mouse Cy5 (cyanine 5.29)-conjugated secondary antibodies were from Jackson ImmunoResearch. Monoclonal antibodies A25 and A52 against SERCA1 have been described previously (31).

Culture and Transfection of HEK-293 Cells—The culture of HEK-293 cells and their transfection with cDNAs have been described in earlier publications (32–34). cDNA encoding human HSP70-EYFP (35) and rabbit SERCA1 (20, 32) were used in these experiments. Plasmid DNAs were purified by using Qiagen columns (Mississauga, Ontario, Canada) and were subsequently transfected into HEK-293 cells using the Ca2+- phosphate precipitation method (32–34). Cells were harvested 48 h after transfection with three washes in phosphate-buffered saline (PBS) at 4 °C, and samples were suspended in 250 mM sucrose, 5 mM HEPES, pH 7.6, 1 mM PMFS, and 20 µg/ml aprotinin.

Immunofluorescence in HEK-293 Cells—Immunofluorescence experiments in HEK-293 cells were grown in 18 × 18-mm glass coverslips and transfected with 10 µg of plasmid DNA using Ca2+-phosphate. In co-localization experiments, SERCA1a plasmid DNA was co-transfected in a 1:1 (w/w) ratio with HSP70-EYFP plasmids. Forty eight hours post-transfection, cells were fixed with 2% paraformaldehyde for 15 min at 4 °C and washed in PBS. Samples were then permeabilized by using antibody buffer (0.5% Triton X-0.2% Nonidet P-40 in PBS) for 30 min at 4 °C and were blocked for 30 min at 4 °C using antibody buffer containing 5% bovine serum albumin. Cells were then incubated with the antibody buffer containing the appropriate anti-rabbit FITC- and anti-mouse Cy5 (cyanine 5.29)-conjugated secondary antibodies (Jackson ImmunoResearch; West Grove, PA) were applied to the samples for 1 h at 4 °C. Coverslips were then washed three times for 15 min with PBS, mounted on glass slides using glycerol/PBS solution, and sealed using nail polish.

Images with each excitation wavelength (488 and 633 nm) were collected separately (FITC and EYFP fluorescence, −495 to 550 nm; Cy5, −645–720 nm), using a Leica DM IRBE inverted microscope equipped with a Leica TCS SP laser scanning confocal system (Leica Microsystems; Canada; Richmond Hill, Ontario, Canada). Images were assembled using Adobe Photoshop 7.0 (Ottawa, Ontario, Canada). To quantify immunofluorescence results, a minimum of 200 cells in five random fields of view from at least three experiments was counted under the ×40 microscope.

Preparation of Isolated SR Membrane Fractions—SR membrane fractions were isolated from white portions of gastrocnemius muscles from 3- to 4-month-old female Sprague-Dawley rats weighing 238 ± 3.5 g (mean ± S.E.), suspended in a solution containing 5 mM HEPES, pH 7.5, 250 mM sucrose, 0.2% Na2ATP, and 0.5 mM PMFS, and frozen at −80 °C, as described earlier (36, 37). Total protein concentration was measured by the method of Lowry, as modified by Schacterle and Pollock (38). Experimental protocols were approved by the Animal Care Committee of the University of Waterloo.

SERCA Activity Measurements—To determine whether HSP70 could protect against thermal inactivation of SERCA1a activity, SR samples (5 µg of total protein) were diluted in 1 ml of the assay buffer used for measurement of SERCA activity (described below) and incubated at both 37 and 41 °C either with or without 1 µg of total purified rat HSP70 protein. At 30, 60, and 120 min of incubation, measurements of SERCA activity were made at 37 °C by using a spectrophotometric assay as described previously (39). Briefly, the assay buffer contained 100 mM RCl, 20 mM HEPES, 10 mM MgCl2, 5 mM NaCl, 10 mM P-endolpyruvate, and 1 mM EDTA, pH 7.0. Immediately before the reaction was started by the addition of CaCl2, 5 mM ATP, 18 units/ml of both lactate dehydrogenase and pyruvate kinase, 0.3 mM NADH, and 1 µM Ca2+ ionophore A-23187 (Sigma, C-7522) were added to the diluted sample. Basal activity was measured in the absence of Ca2+ and recorded prior to the addition of CaCl2. Experiments were also performed at 37 °C with or without samples that were incubated for either 30, 60, or 120 min in SERCA activity assay buffer plus 1 mM CaCl2. The 1 mM Ca/EGTA buffer would have a free Ca2+ concentration of about 10 µM. The data were analyzed by nonlinear regression with computer software (Graph Pad Software), and the Kcat and Hill coefficient (nH) values were calculated by using an equation for a general cooperative model for substrate activation. The values for maximal SERCA1a activity were also measured at pH 5.6 to 5.2. They were taken directly from the experimental data and normalized for total protein concentration. Maximal SERCA1a activity values are reported as percentages of control (CTL) values, which were measured in SR samples that were kept on ice and set to 100%. Samples from all conditions were analyzed during the course of a single day.

Co-immunoprecipitation and Western Blot Analysis—To determine whether HSP70 could bind to SERCA1a, co-immunoprecipitation of SERCA1a and HSP70 was carried out following the method described in the Seize-X primary immunoprecipitation kit (Pierce). SR samples were diluted in SERCA activity assay buffer (no ATP) to a final concentration of 1 mg/ml in a total of 85 µl, mixed with purified HSP70 (3 µg), and incubated for 30, 60, and 120 min at either 37 or 41 °C. The samples were then mixed with an equal volume of 40 mM HEPES-NaOH, pH 7.5, 300 mM NaCl, 2 mM EDTA, 1% SDS, 1% PMSF, 1% Tween 20, vortexed for 30 s, and centrifuged in a Beckman GS-15R centrifuge for 30 min at 16,000 × g. The supernatants were mixed with an agarose gel/anti-HSP70 monoclonal antibody affinity support complex and rotated overnight at 4 °C. After washing the affinity support complex with 25 mM Tris, pH 7.2, 150 mM NaCl, the samples were eluted with a glycine elution buffer (pH 2.8) supplied with the kit. The eluted samples were loaded on 7% polyacrylamide gels, and proteins were separated using standard SDS-PAGE protocols (40) and transferred to nitrocellulose membranes. After blocking with a skim milk suspension, the membranes were treated with anti-SERCA1a monoclonal antibody A25, washed in Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 (Tris-buffered saline/0.1% Tween), and treated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology). Membranes were washed in Tris-buffered saline, 0.1% Tween, and the signals were detected with an enhanced chemiluminescence kit (Amer sham Biosciences) using a bio-imaging system and the GeneSnap software (Syngene) obtained from Fisher. Immunoprecipitations of proteins from microsomal fractions that were isolated from HEK-293 cells were performed as described previously (41, 42). Western blot analysis was also used to determine whether proteolysis of SERCA1a occurred under the heat stress conditions that were employed in this study.

FITC Binding Capacity—FITC binding capacity of SERCA1a was measured to assess the structural integrity of the N domain under conditions of heat stress. SR samples (5 µg of total protein) were diluted in SERCA activity assay buffer at 250 µg/ml and incubated under all conditions at 37 °C for 60 min in the dark. FITC-labeled samples were dissolved in 125 mM Tris-HCl, pH 6.8, 5% SDS, 10% (w/v) glycerol, 0.01% bromphenol, 5% (w/v) mercaptoethanol, to stop the reaction, applied to the same
RESULTS

Ca$^{2+}$-dependent SERCA1a Activity—To determine the effects of HSP70 on SERCA1a function in response to heat stress, we mixed isolated SR membrane fractions prepared from rat fast-twitch skeletal muscle with recombinant rat HSP70 protein, and we determined Ca$^{2+}$ dependence of SERCA1a activity immediately following 30-, 60-, and 120-min incubations (in 1 mM EGTA, see “Experimental Procedures”) at both 37 and 41 °C. At saturating Ca$^{2+}$ levels (pCa 5.6 to 5.2) the time-dependent inactivation of maximal SERCA1a activity was greater (p < 0.05) at 41 °C compared with 37 °C (Fig. 1A). Relative to CTL, in the absence of HSP70, following 30, 60, and 120 min of incubation, maximal SERCA1a activity was reduced at 41 °C (p < 0.05) by 30, 38, and 57% compared with reductions at 37 °C (p < 0.05) of 11, 19, and 31%, respectively. When SR membrane fractions were premixed with purified HSP70, maximal SERCA1a activity was protected at 37 °C following both 30- and 60-min incubations and, perhaps, even activated at 30 min. For all other incubation conditions, the percent inactivation of maximal SERCA1a activity was not different (p > 0.05) between samples that were exposed to elevated temperature in the presence or absence of purified HSP70.

In order to determine whether HSP70 function itself was impaired during prolonged periods at elevated temperatures thereby explaining the lack of protection of maximal SERCA1a activity at 120 min at 37 °C or >30 min at 41 °C, we performed experiments where purified HSP70 was incubated for 120 min at 41 °C, then mixed with SR membranes that were then incubated for 60 min at 37 °C in 1 mM EGTA, with maximal SERCA1a activity being determined between pCa 5.6 and 5.2 (Fig. 1B). SR samples that were mixed with HSP70 that had been preincubated for 120 min at 41 °C were fully protected against thermal inactivation of maximal SERCA1a activity compared with samples that were incubated for 60 min at 37 °C in the absence of HSP70, where maximal SERCA1a activity was reduced by 19% compared with CTL. These results indicate that direct thermal effects on HSP70 function cannot explain the lack of protection of SERCA1a function by HSP70 that was observed under some conditions of elevated temperature used in this study.

Because the Ca$^{2+}$-bound state of SERCA is more stable than the Ca$^{2+}$-free state (11, 44), it was of interest to assess thermal inactivation of SERCA1a in the presence of Ca$^{2+}$, both with and without HSP70. Maximal SERCA1a activity was partially stabilized at 37 °C in the presence of 1 mM Ca$^{2+}$/EGTA, which was added prior to the start of incubation. Compared with CTL, in the absence of HSP70, maximal SERCA1a activity was unaltered (p < 0.05) at 30 min but was reduced (p < 0.05) by 16 and 20% at 60 and 120 min, respectively. Similar to our findings with 1 mM EGTA, HSP70 prevented inactivation of maximal SERCA1a activity at 60 but not at 120 min of incubation (data not shown).

The effects of HSP70 on the SERCA1a enzyme kinetic properties in response to heat stress are illustrated in Table I. A main effect (p < 0.05) of time of incubation was found for both the $K_{Ca}$ (the negative logarithm of the Ca$^{2+}$ concentration that gives half-maximal SERCA enzyme activity expressed in pCa units) (120 = 60 < 30 min = CTL) and the Hill coefficient (nH) (120 < 60 < 30 min = CTL) (Fig. 2). These effects were not specific to temperature level and were independent of HSP70 treatment. Compared with CTL and 30 min, $K_{Ca}$ was shifted...
TABLE I  
Effects of HSP70 on SERCA1a enzyme kinetic properties  
in response to heat stress

| Temperature | Incubation time | $K_{Ca}^a$ | $n_H^b$ |
|-------------|-----------------|-----------|---------|
| 37 °C       | No HSP70        | 6.47 ± 0.06 | 2.05 ± 0.11 |
|             | HSP70           | 6.39 ± 0.07 | 1.89 ± 0.17 |
|             | No HSP70        | 6.36 ± 0.13 | 1.82 ± 0.21 |
|             | HSP70           | 6.21 ± 0.09 | 1.73 ± 0.08 |
|             | No HSP70        | 6.26 ± 0.05 | 1.54 ± 0.07 |
|             | HSP70           | 6.32 ± 0.08 | 1.57 ± 0.12 |
| 41 °C       | No HSP70        | 6.36 ± 0.08 | 1.84 ± 0.09 |
|             | HSP70           | 6.33 ± 0.08 | 1.84 ± 0.05 |
|             | No HSP70        | 6.17 ± 0.09 | 1.55 ± 0.20 |
|             | HSP70           | 6.19 ± 0.08 | 1.78 ± 0.04 |
|             | No HSP70        | 6.18 ± 0.03 | 1.49 ± 0.19 |
|             | HSP70           | 6.43 ± 0.03 | 1.45 ± 0.27 |

$K_{Ca}^a$ ($pCa$) is the negative logarithm of the Ca$^{2+}$ concentration required to attain 50% of the maximal SERCA activity. There was a significant ($p < 0.05$) time-dependent main effect (120 < 60 < 30 min = CTL) across conditions (see Fig. 2).

The Hill coefficient was determined based on the SERCA1a activity between 10 and 90% of maximal value. There was a significant ($p < 0.05$) time-dependent main effect (120 < 60 = 30 min = CTL) across conditions (see Fig. 2).

TABLE II  
Effects of HSP70 on SERCA1a FITC binding capacity  
in response to heat stress

| Temperature | Incubation time, min |
|-------------|----------------------|
| 37 °C       | 30 | 60 | 120 |
| No HSP70    | 91.4 ± 6.27 | 92.2 ± 3.65 | 74.0 ± 3.89 |
| HSP70       | 99.7 ± 6.58 | 102 ± 5.08 | 67.2 ± 2.73 |
| 41 °C       | No HSP70    | 89.8 ± 2.28 | 83.0 ± 5.08 | 79.6 ± 8.61 |
| HSP70       | 104 ± 6.61 | 94.3 ± 6.15 | 79.4 ± 8.96 |

FITC Binding Capacity—FITC covalently labels Lys$^{515}$ (45), which is located within the N domain close to the ATP-binding site (46). Inactivation of SERCA1a activity with oxidative stress has been associated with structural modification to the N domain as indicated by reductions in FITC binding capacity (17, 18, 36, 47, 48). Moreover, it has been shown that thermal inactivation of SERCA1a activity is because of denaturation of the N domain (11). Therefore, it was of interest to determine the effects of HSP70 on FITC binding capacity of SERCA1a in response to heat stress. Immediately following thermal incubations of SR samples that were treated either with or without recombinant rat HSP70 protein, SERCA1a was labeled with FITC, and the FITC binding capacity of SERCA1a was assessed by Western blotting using the anti-fluorescein/Oregon Green monoclonal antibody 4-4-20. Table II and Fig. 3. Differences in FITC binding capacity between conditions were independent of temperature. When the temperature data were combined and evaluated in relation to time, we observed that FITC binding capacity was reduced relative to CTL ($p < 0.05$) in the absence of HSP70 by 11, 14, and 26% at 30, 60, and 120 min, respectively. However, in the presence of HSP70, FITC binding capacity was not different from CTL ($p > 0.05$) at 30 and 60 min but was 27% lower relative to CTL ($p < 0.05$) at 120 min, which was not different ($p > 0.05$) from the percent reduction observed in the absence of HSP70 at 120 min.

Western Blot Analysis—Because SERCA inactivation would occur as a result of proteolysis, it was of interest to determine whether proteolysis of SERCA1a occurred under the heat stress conditions employed in this study. Western blotting was carried out on SR samples immediately following incubation in the absence of HSP70 at either 37 or 41 °C for 30, 60, and 120 min (Fig. 4). We did not find any evidence to suggest that proteolysis of SERCA1a occurred in our samples with heat stress because no lower molecular weight bands were detected using the monoclonal antibody A25.

Co-localization of SERCA1 and HSP70—We examined the subcellular localization of SERCA1 and HSP70 in HEK-293 cells by transfecting cDNAs encoding rabbit SERCA1 or human SERCA1-EYFP and carrying out confocal analyses 48 h later using anti-SERCA1 (A52; Fig 5A, left panel) and anti-HSP antibodies (SPA-812; Fig 5A, middle panel). In addition, we visualized EYFP fluorescence without any antibodies (Fig 5A, right panel). In these experiments, immunofluorescence patterns for SERCA1 indicated that its presence was restricted to the endoplasmic reticulum (34). HSP70 showed a strong level of cytoplasmic staining with clear aggregates throughout the cytoplasm of the cell. Similar staining patterns were observed by using anti-HSP antibodies or with EYFP fluorescence. Cellular stress experiments were performed on HEK-293 cells that...
were co-transfected with both SERCA1 and HSP70 (Fig 5B). For these experiments, transfected cells were subjected to 40 °C for 1 h and then fixed and analyzed. We observed nearly complete co-localization of SERCA1 with HSP70 under these conditions.

During these experiments, we also determined that SERCA1a expression appeared to be significantly affected under heat stress, an effect ameliorated by the presence of HSP70 (Table III). In an attempt at quantifying our findings, we counted the total number of cells positive for HSP70 or SERCA1a fluorescence in five random fields of view in each culture. In control experiments, we observed 63 ± 4 HSP70-positive cells and 44 ± 2 SERCA1a-positive cells. However, following heat stress in cultures transfected with SERCA1a only, we observed less than 10 SERCA1a-positive cells. This contrasts with the 66 ± 5 HSP-positive cells in HSP-only transfected cells. In cultures co-transfected with both HSP70 and SERCA1a and subjected to heat stress, we visualized 58 ± 4 HSP70-positive and 36 ± 2 SERCA1a-positive cells. These findings would indicate that heat stress results in the reduced expression of SERCA1a fluorescence, either through loss of the total number of SERCA1a-expressing cells or partial unfolding of SERCA1a and loss of immunoreactivity. The co-expression of
FIG. 6. Co-immunoprecipitation of SERCA1a and HSP70 in vitro. SR membrane fractions that were prepared from rat fast-twitch muscle were mixed with purified rat HSP70 prior to in vitro incubations (in 1 mM EGTA) at both 37 and 41 °C. At 30, 60, and 120 min of incubation, samples were processed for immunoprecipitation using the SPA-810 antibody against HSP70 as described under “Experimental Procedures.” The presence of SERCA1a associated with HSP70 in the samples was then detected by Western blotting and detection procedures using the anti-SERCA1a monoclonal antibody A25. Right lane, SR membrane fractions (10 μg of total protein) that were prepared from rat fast-twitch muscle was used as a positive control for SERCA1a.

HSP70 together with SERCA1a results in the rescue of SERCA1a immunoreactivity following such stress.

Co-immunoprecipitation of SERCA1a with HSP70—A direct protective effect of HSP70 on SERCA1a function during heat stress would likely result through a physical association between the two proteins. However, evidence of direct binding between SERCA1a and HSP70 has not been reported to date. Thus, it was of interest to use co-immunoprecipitation to test for physical association between SERCA1a and purified HSP70 following conditions of heat stress in vitro, not only where functional protection of maximal SERCA1a activity was observed but also where HSP70 failed to protect against thermal inactivation of maximal SERCA1a activity. Co-immunoprecipitation of SERCA1a with HSP70 was carried out using the SPA-810 antibody against HSP70. Fig. 6 shows that physical association occurred between SERCA1a and HSP70 in samples that were incubated for 30, 60, and 120 min at both 37 and 41 °C. CTL SR samples were used as a positive control in the immunoblots. HSP70 binding to SERCA1a was specific and likely depended on SERCA1a SH oxidation because no binding between HSP70 and SERCA1a occurred when samples were incubated with 100 mM DTT (data not shown).

It was also of interest to determine whether SERCA1a and HSP70 could interact physically in HEK-293 cells. For these studies, co-immunoprecipitations were performed by using cells transfected with both HSP70 and SERCA1a. Samples were first immunoprecipitated by using the HSP70 antibody, and lysates were then subjected to immunoblotting by using the A52 antibody against SERCA1a and the HSP70 antibody (Fig. 7). In the SERCA1a immunoblots (Fig. 7, left panel), we did not observe any immunoreactivity in the cultures transfected with HSP70 only, whereas a very clear SERCA1a band was present in the cultures co-transfected with both HSP70 and SERCA1a. SERCA1a-transfected cells were used as a positive control in the immunoblots. In the HSP70 immunoblots, clear bands were observed, as expected, in HSP70-only transfected cultures and HSP70 and SERCA1a co-transfected cultures. Purified HSP70 was included in the immunoblots as a positive control. Together, these findings verify our in vitro co-immunoprecipitation data and demonstrate that HSP70 and SERCA1a interact in vivo.

Modeling of the Interaction between HSP70 and SERCA1a—On the basis that HSP70 is largely localized in the cytoplasm (21), it seemed likely that HSP70 would bind to a cytoplasmic domain on SERCA1a. Based on our FITC binding data, a likely candidate would be the N domain that contains the FITC-binding site (Lys515). The minimum energy of the most stable binding form was predicted at −138.2 kcal/mol and placed 7HSC on SERCA1a near Lys515, but the binding site of 7HSC was facing away from SERCA1a (Fig. 8A). For the second best predicted binding form, with −135.6 kcal/mol of binding energy, the interaction site for the HSP70-SERCA1a complex was through a cytoplasmic domain on SERCA1a but did not involve Lys515 (Fig. 8B). A third model shows that it is possible for HSP70 and SERCA1a to form a complex through an interaction between the binding domain of HSP70 and the N domain of SERCA1a very close to Lys515 (Fig. 8C). This model, which is consistent with our FITC binding data, scored −79.4 kcal/mol of predicted binding energy.

DISCUSSION

To assess whether HSP70 could bind to SERCA1a and thereby provide protection against thermal inactivation of SERCA1a, SR membrane fractions that were prepared from rat fast-twitch muscle were incubated at temperatures known to cause SH oxidation and thermal instability of SERCA1a in vitro (13) in the presence and absence of purified HSP70. We showed that HSP70 can form a complex with SERCA1a in vitro, and notably, we also verified that these two proteins interact physically in vivo, in response to heat stress. Molecular modeling predicted that HSP70 could bind to the N domain of SERCA1a at a site involving Lys515, a model that is strengthened by our results showing that heat-induced reductions in FITC binding capacity of SERCA1a are not observed in the presence of HSP70, at least with incubations for up to 60 min. In addition, we show that HSP70 binding provides functional protection to SERCA1a; however, the ability of HSP70 to prevent thermal inactivation of SERCA1a is limited and depends on the severity of heat stress.

Our general findings for thermal inactivation of SERCA1a in the absence of HSP70 confirm our most recent findings from in vitro studies which showed the following: 1) that maximal SERCA1a activity is reduced progressively with increasing temperature and time of incubation; and 2) that SERCA1a enzyme kinetics are less sensitive to thermal inactivation compared with maximal SERCA1a activity, although reductions in both the apparent Ca2⁺ affinity and the Hill coefficient do occur with increasing severity of heat stress (13). Our results are also consistent with previous studies investigating heat stress in SR membrane fractions in vitro (11, 12). As with our study, these investigators reported only a small stabilizing effect of Ca2⁺ on maximal SERCA1a activity in response to heat stress.

The main finding of this study was that HSP70 could bind to
FITC-binding site of SERCA1a (Lys515) is displayed in the Protein Data Bank code 7HSC; between the binding domain of HSP70 (the substrate binding domain of HSP70 complex. The FITC-binding site of SERCA1a (Lys515) is displayed in the yellow balls model on Protein Data Bank code 1W0L and the binding site of HSP70 is displayed in the red balls model on the Protein Data Bank code 7HSC. A and B show the most stable and the second most stable configurations, respectively. One of the predicted configurations indicated that the binding site of HSP70 interacts with SERCA1a at sites close to Lys515 (C).

**Fig. 8. Potential binding configuration for the SERCA1a-HSP70 complex.** Pictures show possible candidates of binding configurations derived from a computational prediction of interaction between the binding domain of HSP70 (the substrate binding domain of Protein Data Bank code 7HSC; red ribbon model) and the E2 form of SERCA1a (Protein Data Bank code 1W0L; green ribbon model). The FITC-binding site of SERCA1a (Lys515) is displayed in the yellow balls model on Protein Data Bank code 1W0L and the binding site of HSP70 is displayed in the red balls model on the Protein Data Bank code 7HSC. A and B show the most stable and the second most stable configurations, respectively. One of the predicted configurations indicated that the binding site of HSP70 interacts with SERCA1a at sites close to Lys515 (C).

SERCA1a and thereby stabilize SERCA1a against thermal inactivation. Inactivation of SERCA1a was likely not through complete denaturation because SERCA1a denatures at much higher temperatures (~49 °C) than those employed in this study (11). We also showed that proteolysis of SERCA1a was not responsible for its inactivation with heat stress. However, because ROS are produced by heat stress (49–51), and because DTT greatly attenuates heat-induced inactivation of SERCA1a activity (13), it is likely that thermal inactivation of SERCA1a results from SH oxidation.

As support for the view that thiols are involved in the denaturation of SERCA1a, we found that if DTT was present during thermal incubations, no interaction between HSP70 and SERCA1a was detected (data not shown). In this study, HSP70 and SR membrane fractions were incubated in the absence of ATP. Given that ATP drives the release of protein substrates from the substrate binding domain of HSP70 (52) and because HSP70-peptide complexes are poorly dissociated by reducing agents (53), it is unlikely that any physical interaction between HSP70 and SERCA1a occurred in the presence of DTT. These results suggest that thiols in SERCA1a were maintained in a reduced state during heat stress in the presence of DTT which precluded HSP70 binding to SERCA1a. By contrast, in the absence of DTT, SH oxidation and exposure of hydrophobic residues on SERCA1a would progress with prolonged heat stress (15). This could be a trigger for HSP70 binding to SERCA1a. This would be consistent with the general mechanism of binding of HSP70, which binds specifically to hydrophobic sites in denatured or partially denatured proteins (52). There is also evidence that HSP70 forms mixed disulfides with proteins following ROS exposure (54).

HSP70 has the capacity to repair unfolded proteins; however, this requires the presence of ATP (22, 29). In our assay system for Ca2+-ATPase, we added ATP directly to the incubation mix and then started the Ca2+-ATPase reaction by the addition of Ca2+. Recognizing that SERCA1a with oxidized thiols and bound HSP70 would be inactive, we assume that protection by HSP70 occurs in the form of repair of partially unfolded SERCA1a rather than through binding which prevents its unfolding. Alternatively, if oxidation is involved in thermal inactivation of SERCA1a, protection by HSP70 could be due to nonspecific scavenging of ROS. However, addition of a non-chaperone protein (bovine serum albumin) to the SR samples prior to heat stress provided no protection against thermal inactivation of SERCA1a (data not shown).

Although a number of cytoplasmic regions of SERCA1a are sensitive to oxidants and are modified by exposure to ROS (16), numerous studies support the view that ROS can inactivate SERCAs by inducing structural alterations to the nucleotide-binding site (17, 36, 47, 48, 55). By using a FITC binding assay, we tested the hypothesis that modification of a region of the N domain containing the nucleotide-binding site is responsible for thermal inactivation of SERCA1a. In support of our hypothesis, we found, in the absence of HSP70, that FITC binding capacity of SERCA1a was reduced progressively over time, an effect that was independent of temperature. To provide further support for our hypothesis, we also showed that HSP70 could preserve FITC binding capacity of SERCA1a under the same conditions that it prevented thermal inactivation of SERCA1a.

If we compare the time course and temperature dependence of thermal inactivation and changes in FITC binding capacity of SERCA1a in the absence of HSP70 in relation to results obtained in the presence of HSP70, we find a dissociation between the ability of HSP70 to provide functional protection and its ability to preserve FITC binding capacity of SERCA1a under some conditions. Specifically, HSP70 could preserve FITC binding capacity with 30- and 60-min incubations at both 37 and 41 °C whereas, under the same conditions, functional protection was only observed at 37 °C. These results suggest that another region on SERCA1a, which may not be able to form an interaction with HSP70, is modified at 41 °C and is responsible for inactivation of SERCA1a under those conditions. One postulate is that the transmembrane or Ca2+-binding region of SERCA1a is modified, which is consistent with our results showing a reduction in the apparent Ca2+ affinity and the Hill slope of SERCA1a under these conditions, even if HSP70 is present and is bound to SERCA1a. It is clear from studies of the structures of SERCA1a that there is long range transmission of conformational changes between cytosolic and transmembrane domains (56).

Indirectly, all of our results point to a potential interaction site between the substrate binding domain of HSP70 and the N domain of SERCA1a. Based on the known structures of HSP70 and SERCA1a, we predicted the binding configurations of the two proteins by performing global scan calculations of protein to protein docking on literally thousands of possible binding candidates. One model (Fig. 8C), which had a high binding energy, predicted that the interaction between the substrate binding domain of HSP70 and the N domain of SERCA1a occurred at a site close to Lys515 on SERCA1a. However, we cannot assume that HSP70 does not form other binding configurations with SERCA1a such as the one shown in Fig. 8B. Moreover, it must be emphasized that HSP70 would not be expected to bind to intact SERCA1a but only if it is partially unfolded; therefore, it is not easy to predict the exact interaction sites of these two proteins. Despite this limitation, our model is at least consistent with the published structures of the two proteins and with our FITC binding data, further suggesting that HSP70 binds to the N domain of SERCA1a in response to heat stress. It is unclear why HSP70 binding was unable to preserve FITC binding capacity of SERCA1a at 120 min at both 37 and 41 °C, but this would indicate that the N domain was irreversibly denatured under those conditions.

Ca2+ overload is a hallmark of cellular injury produced by oxidative stress and has long been implicated in cell death (57,
Preservation of SERCA function during oxidative stress would be expected to help maintain Ca\textsuperscript{2+} homeostasis and improve cellular responses to stress. Thus, our results suggest that SERCA is a key substrate that is protected by HSP70 against oxidative stress, which may at least partially explain the cytoprotective effects of HSP70. Consistent with this view, a previous study showed that induction of HSP70 in rat myocardium by heat shock was associated with protection of SR Ca\textsuperscript{2+} cycling and myocardial physiological performance against ischemia-reperfusion (59). This would suggest that HSP70 can also interact with the cardiac isoform of SERCA, namely SERCA2a, although evidence of a direct association has yet to be reported. Most interestingly, there is also evidence that HSP70 can form a functional interaction with the Na\textsuperscript{+}/K\textsuperscript{−}-ATPase (60), which, structurally, is remarkably similar to SERCA (61).

In summary, we have found that HSP70 binds to SERCA1a and protects SERCA1a function during heat stress. Functional protection of SERCA1a in vitro was associated with preserved FITC binding capacity of SERCA1a indicating that HSP70 binding stabilizes the N domain of SERCA1a. We also confirmed that HSP70 can bind to SERCA1a in vivo in response to heat stress. These results imply that HSP70 could function to maintain cellular Ca\textsuperscript{2+} homeostasis during oxidative stress which might explain the well known cytoprotective effects of HSP70.
HSP70 Binds to the Fast-twitch Skeletal Muscle Sarco(endo)plasmic Reticulum Ca$^{2+}$-ATPase (SERCA1a) and Prevents Thermal Inactivation

A. Russell Tupling, Anthony O. Gramolini, Todd A. Duhamel, Hiroya Kondo, Michio Asahi, Shauna C. Tsuchiya, Michael J. Borrelli, James R. Lepock, Kinya Otsu, Masatsugu Hori, David H. MacLennan and Howard J. Green

J. Biol. Chem. 2004, 279:52382-52389. doi: 10.1074/jbc.M409336200 originally published online September 14, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M409336200

Alerts:

- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 60 references, 15 of which can be accessed free at http://www.jbc.org/content/279/50/52382.full.html#ref-list-1