Identification of Novel Ryanodine Receptor 1 (RyR1) Protein Interaction with Calcium Homeostasis Endoplasmic Reticulum Protein (CHERP)*

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The ryanodine receptor type 1 (RyR1) is a homotetrameric Ca2+ release channel located in the sarcoplasmic reticulum of skeletal muscle where it plays a role in the initiation of skeletal muscle contraction. A soluble, 6×-histidine affinity-tagged cytosolic fragment of RyR1 (amino acids 1–4243) was expressed in HEK-293 cells, and metal affinity chromatography under native conditions was used to purify the peptide together with interacting proteins. When analyzed by gel-free liquid chromatography mass spectrometry (LC-MS), 703 proteins were identified under all conditions. This group of proteins was filtered to identify putative RyR interacting proteins by removing those proteins found in only 1 RyR purification and proteins for which average spectral counts were enriched by less than 4-fold over control values. This resulted in 49 potential RyR1 interacting proteins, and 4 were selected for additional interaction studies: calcium homeostasis endoplasmic reticulum protein (CHERP), endoplasmic reticulum–Golgi intermediate compartment 53-kDa protein (LMAN1), T-complex protein, and phosphorylase kinase. Western blotting showed that only CHERP co-purified with affinity-tagged RyR1 and was eluted with imidazole. Immunofluorescence showed that endogenous CHERP co-localizes with endogenous RyR1 in the sarcoplasmic reticulum of rat soleus muscle. A combination of overexpression of RyR1 in HEK-293 cells with siRNA-mediated suppression of CHERP showed that CHERP affects Ca2+ release from the ER via RyR1. Thus, we propose that CHERP is an RyR1 interacting protein that may be involved in the regulation of excitation–contraction coupling.

In skeletal muscle excitation-contraction (EC)4 coupling is prominent among the processes controlled by Ca2+ (1). The sequence of EC coupling events begins with the depolarization of sarcolemmal and transverse tubular membranes that triggers a conformational change in the dihydropyridine receptor (DHPR). Protein-protein interactions between the DHPR and the ryanodine receptor type 1 (RyR1) lead to the activation of the Ca2+ release channel function of RyR1, the release of Ca2+ from the SR via RyR1, and the generation of skeletal muscle contraction. Subsequently, relaxation is initiated by re-uptake of intracellular Ca2+ through the action of the sarco/endoplasmic reticulum Ca2+-ATPase type 1 (SERCA1a). The correct functioning of the contraction/relaxation cycle relies on a precise balance between Ca2+ release and re-uptake. Disruptions in this balance, such as those that result from mutations in the proteins involved in the Ca2+ uptake and release processes, lead to muscle diseases, including Brody disease, malignant hyperthermia, central core disease, and cardiomyopathies (2–5).

RyR1 has numerous interacting protein partners; that is, both integral membrane proteins such as DHPR, junction, and triadin (6) and soluble proteins such as FK506-binding proteins, calmodulin, protein kinases, and phosphatases (6–9). These protein interactors are proposed to comprise an RyR1 Ca2+ release channel complex and to regulate RyR1 function at the molecular level. Previous studies elucidating RyR1 structure and topology showed that RyR1 can be subdivided into a soluble, N-terminal, cytosolic domain (amino acids 1–4243) and an insoluble, C-terminal, pore-forming sector (amino acids 4244–5038) containing at least 6 transmembrane sequences and a cytosolic tail (10).

In this study we attached codons for a 6×-histidine affinity tag to the 3’ end of rabbit cDNA encoding amino acids 1–4243 of RyR1. The soluble, C-terminal-tagged cytosolic domain (cytosolic RyR1) was then expressed in HEK-293 cells for the purpose of isolating highly purified RyR1 and interacting protein partners under non-denaturing conditions. Liquid chromatography mass spectrometry (LC-MS) was employed as a first screen in identifying these interacting proteins. Here, we identified calcium homeostasis endoplasmic reticulum protein (CHERP), as a protein that interacts with RyR1 and regulates its Ca2+ release channel function.

plasmic reticulum protein; Ni-NTA, nickel-nitriotriacetic acid; ERGIC-S3, endoplasmic reticulum Golgi intermediate complex 53; TCP, T-complex protein; DHPR, dihydropyridine receptor; PHK-α, phosphorylase kinase α-subunit; SR, sarcoplasmic reticulum; TM, transmembrane.
EXPERIMENTAL PROCEDURES

**Vector Construction**—The cDNA encoding the cytosolic N-terminal fragment of RyR1, described previously (10), was inserted into the Qiagen pQE expression vector system containing the dual tandem affinity tags encoding 6×-histidine and streptavidin-binding peptide to form the C-terminal His6 tag. The His6-tagged cytosolic RyR1 cDNA was then excised with XbaI and HindIII and treated to form a blunt end and ligated in-frame into the pcDNA3.1 vector via its EcoRV site.

**Expression of Cytosolic RyR1 in HEK-293 Cells and Preparations of the Protein Extract**—The His6-tagged RyR1 protein was expressed in HEK-293 cells by transfection with 10 µg of the cDNA construct per 100-mm plate using the standard calcium phosphate transfection protocol (11). Cells were harvested 48 h after transfection in an ice-cold solution of PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) containing 5 mM EDTA. The cells were then pelleted by centrifugation at 4100 rpm for 5 min at 4 °C and resuspended and washed in ice-cold PBS alone. After removal of the supernatants, the cells were resuspended in 2 ml of lysis buffer (5% sucrose, 150 mM NaCl, 50 mM Tris-Hepes, pH 7.0, 1 mM PMSF, 1× EDTA-free protease inhibitor mixture tablets (Roche Applied Science), 0.01% Triton X-100, and 5 mM β-mercaptoethanol. The cells were lysed using 25 strokes in a loose-fitting Dounce homogenizer. To remove cell debris, the lysate was then centrifuged at 8000 rpm for 20 min at 4 °C. The supernatant was retained for analysis.

**Purification of RyR1 Complexes Using Ni-NTA Resin**—Two µl of 50% Ni-NTA resin slurry per plate of transfected HEK-293 cells was added to the protein extract, and the solution was rotated for 2 h at 4 °C to allow the tagged proteins to bind the Ni2+ via the His6 tag. Proteins bound to the Ni-NTA resin were then separated from the flow-through by centrifugation (4100 rpm, 5 min, 4 °C) and washed 3 times in 1 ml of ice-cold Ni-NTA binding buffer (lysis buffer with 25 mM imidazole added) by rotation (5 min; 4 °C) followed by centrifugation (4100 rpm; 5 min; 4 °C). Bound proteins were eluted twice for 1 h with 100 µl of ice-cold elution buffer (binding/lysis buffer with 500 mM imidazole).

**Protein Sample Preparation and Proteomic Analysis**—Aliquots containing 100 µg of total protein from the elution fraction were prepared for trypsin digestion by the addition of ammonium bicarbonate, pH 8.5, to 50 mM and CaCl2 to a final concentration of 1 mM in a total volume of 180 µl. Samples were incubated overnight at 37 °C with 0.02 mg/ml proteomics grade trypsin (Roche Applied Science) as described previously (12, 13). Before application to the LC-MS columns, the digested peptides were solid-phase extracted using OMIX C-18 pipette tips (Varian Inc.) according to the manufacturer’s protocol.

Comprehensive gel-free shotgun sequencing of tryptic digests was performed essentially as described previously (13, 14). Briefly, for peptide separation, individual samples were first loaded onto microcapillary fused silica columns with an internal diameter of 75 μm packed with 7 cm of reversed phase C18 resin (Magic C18). The columns were aligned with an LTQ linear ion trap mass spectrometer, and the peptides were eluted using a 2-h water/acetonitrile gradient and ionized via electrospray ionization. The tandem mass spectra generated were matched to peptide sequences in the human IPI protein sequence data base using the X!Tandem algorithm. Only proteins identified with at least two unique peptides were accepted for further experimental consideration.

**Immunoblot Analysis**—Immunoblotting was performed on protein fractions collected throughout the purification process. Thirty µg of protein from the cellular lystate, flow-through, final wash, and elution fractions from RyR1 Ni-NTA purifications were subjected to standard Western blotting techniques (15). The blots were incubated first with the following commercial antibodies to target specific proteins: mouse monoclonal against RyR1 (Affinity Bioreagents), rabbit polyclonal against CHERP (Abcam), rabbit polyclonal against endoplasmic reticulum Golgi intermediate complex 53 (ERGIC-53) (Sigma), goat polyclonal against T-complex protein (TCP) (Abcam), mouse monoclonal against DHPR (Affinity Bioreagents), and rabbit polyclonal against phosphorylase kinase α-subunit (PHK-α) and β-subunit (PHK-β) (Abcam). Blots were then incubated with the corresponding horseradish peroxidase-conjugated secondary IgG. Finally, the blots were incubated with the Supersignal ultra chemiluminescent substrate (Pierce) for 5 min and then exposed to a BioMax film (Eastman Kodak Co.).

**Rat Skeletal Muscle Preparation and Confocal Immunofluorescence Microscopy**—Skeletal muscle soleus fibers were isolated from the hind limbs of adult female Sprague-Dawley rats after euthanization via CO2 asphyxiation. Immediately after isolation, the tissue was incubated in excess ice-cold PBS for 30 min before fixation in 2% paraformaldehyde for 30 min at 4 °C. Two 1-ml washes were then performed with freshly prepared permeabilization buffer (0.2% Tween 20, 0.5% Triton X-100 in 1× PBS) at 4 °C for 15 min each. Immunofluorescence was performed on the skeletal muscle tissue as described by Sharma et al. (16).

**Ca2+ Transient Analysis**—A microfluorimetry system was used to measure the effect of caffeine on Ca2+ release in HEK-293 cells. Cells were transfected at ~50% confluence with full-length RyR1 cDNA via the calcium phosphate method (11). The medium was changed after 24 h, and 2 h later siRNA was transfected using Lipofectamine reagent (Invitrogen). Ca2+ photometry was performed on intact cells 48 h after siRNA transfection. The cells were loaded with 2 µM Fura-2 AM (Invitrogen) for 1 h at pH 7.4 and room temperature in a physiological medium. The glass coverslips were placed in a holder on the stage of an inverted Olympus IX87 microscope. The samples were excited alternately at 340 and 380 nm with a dual monochromator. The emitted fluorescence was fed into a charge-coupled device camera. Acquired digital images were analyzed with the Quorum Analysis software.

For the 340/380-nm ratio acquisitions, cytosolic Ca2+ concentrations were read over a period of 60 s with fluorescence measurements taken every 3 s, with 5–15 cells measured per run. At 30 s, the appropriate concentration of caffeine was added to induce Ca2+ release. No attempt was made to wash out caffeine during the procedure.

**Statistical Analysis**—Data are reported as the mean ± S.D. Statistical differences were evaluated by analysis of variance. A p value <0.05 was considered statistically significant.
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FIGURE 1. Expression analysis of the H11001 affinity-tagged cytosolic RyR1 rabbit cDNA. HEK-293 cells were transiently transfected with cytosolic RyR1 cDNA using the calcium phosphate protocol. A, confocal microscopy images with antibody raised against RyR1 show that the cytosolic RyR1 construct (amino acids 1–423) remains in the cytoplasm. B, phase contrast imaging of the same cells shows transfection of HEK-293 cells. C, Coomassie-stained protein gel analysis of wash and elution fractions obtained during the purification of cytosolic RyR1 on Ni-NTA columns shows a purified and concentrated RyR1 band (red arrow) along with potential interacting proteins (black arrows). D, immunoblot analysis of purification products from HEK-293 cells probed with the 34C antibody is shown.

RESULTS

Expression and Purification of Cytosolic RyR1 Proteins in HEK-293 Cells—To test for expression of the cytosolic RyR1 construct, confocal microscopy imaging was performed on cytosolic-RyR1-transfected HEK-293 cells (Fig. 1A) using the 34C antibody against RyR1. Phase contrast imaging of the same cells (Fig. 1B) showed good transfection efficiency. WT HEK-293 control cells showed no signal (data not shown); thus, it can be deduced that the proteins visualized in Fig. 1 were generated from the cDNA construct and contain the His6 affinity tag.

To ensure that an overexpressed protein of the size of cytosolic RyR1 could be purified efficiently, we examined eluants from Ni-NTA columns in Coomassie-stained gels (Fig. 1C). Purified cytosolic RyR1, indicated by a red arrow, was accompanied by multiple additional protein bands (black arrows), representative of co-purified potential RyR1 interacting proteins. To ensure that the protein detected by the red arrow in Fig. 1C was RyR1, immunoblot analysis was performed (Fig. 1D). The immunoblots show that a significant amount of the expressed recombinant protein was purified and concentrated in the elution fraction.

Identification of RyR1 Interacting Partners via LC-MS—A total of 9 Ni-NTA purifications were performed from HEK-293 cells, and the elution fractions were analyzed by MS. Of these, three were RyR1 purifications, three were negative control bead purifications, and three were His6-tagged DPYSL3 protein control purifications. This control was selected because DPYSL3 is a soluble cytosolic protein with few Gene Ontology terms associated with Ca2+ signaling and EC coupling (17, 18). In every experimental run, only proteins that were supported by at least 2 unique peptides were accepted for further consideration. A total of 703 proteins were identified from all 9 purifications including controls and RyR tags. These proteins are represented in a heat map (Fig. 2, left) prioritized by ascending spectral count in the RyR1 MS runs.

Of the 703 proteins detected, 323 were identified in at least 1 of the 3 RyR1 purifications, and the remaining 380 proteins were not considered further. To identify RyR1 interacting proteins with higher confidence, the subsets of identified proteins were filtered using two criteria. When proteins were found only in RyR and not in controls, they were included only if they were present in at least two of the three RyR1 runs but not in any of the six control runs. For proteins that were found in both control and RyR purifications, spectral counts were averaged for each identified protein over the three RyR1 runs and for the six control runs to generate an enrichment value over controls. Only those proteins that were enriched by a factor of four were included. Forty-nine proteins passed these criteria, and they are represented in Fig. 2 (right) and listed in Table 1.

To further filter this list, each protein was subjected to a literature search to assess its potential as an RyR1 interacting protein. Proteins classified as originating in the ribosome, the mitochondria, the spliceosome, or the histone fraction or as being associated with metabolism or heat shock were eliminated. This resulted in only five candidates passing these criteria. They were CHERP, TCP-α, PHK, ERGIC-53, and MYCBP2. MYCBP2 is an ubiquitin ligase (19, 20). It was removed from consideration, as ubiquitin-related pathways are not likely to be involved in cellular Ca2+ homeostasis. CHERP, TCP-α, PHKB, and ERGIC-53 were selected for subsequent interaction validation studies.

CHERP, an integral sarco/endoplasmic reticulum (SR/ER) membrane protein. It is a 916 (human)- and 936 (mouse)-amino acid protein. The human sequence is predicted to contain 1 luminal to cytosolic, transmembrane (TM) sequence near its N terminus (amino acids 233–252; predicted with “very high certainty” on the basis of a high hydrophobicity score of 1.25 using the Kyte-Doolittle scale (21)). It also has a second potential TM sequence (amino acids 641–659; predicted with “putative” probability based on a 0.78 hydrophobicity score) (22). This domain structure would suggest a luminal N terminus of 230 amino acids and either a long cytosolic domain of 664 amino acids (if 1 TM sequence) or 388 amino acids (if 2 TM sequences). A cytosolic coiled-coil domain at amino acids 290–312 is also predicted.

Because CHERP has been described as playing a role in cytosolic Ca2+ cycling in various cell types (21, 23), it was selected for further study. TCP-α is a molecular chaperone and a member of the chaperonin-containing TCP1 complex (24). Because it is unknown how a protein channel the size of RyR1 is organized and folded in vivo, TCP-α was deemed a potential candidate. Phosphorylase kinase is a serine/threonine-specific protein kinase composed of several subunits, including calmodulin (the δ subunit) (25). The phosphorylation of various RyR1 residues, in particular Ser-2843, activates RyR1 (26). Thus, the rationale for including PHK as a potential RyR1 interacting protein was its potential for phosphorylating RyR1. ERGIC-53 is a type 1 transmembrane glycoprotein receptor protein localized...
to the ER-Golgi intermediate compartment that is involved in protein trafficking from the ER to the Golgi. Its affinity for substrates is dependent on cytosolic Ca\(^{2+}\) levels (27).

**Immunoblot Analysis of Ni-NTA Eluates to Verify RyR1 Pull-down of Candidate Proteins**—Western blotting was performed on 25 \(\mu\)g of protein from the flow-through, first wash, final wash, and eluant fractions of the Ni-NTA purification step. Using antibodies against the four candidate proteins, we verified the presence of RyR1 interacting candidates in the purified samples together with RyR1 (Fig. 3A). ERGIC-53, CHERP, TCP, and PHK-\(\alpha\) were all detected by immunoblotting after purification of affinity-tagged RyR1 on Ni-NTA resin. PHK-\(\beta\) staining was found to be unreliable and nonspecific and not pursued (results not shown). However, when the same experiments were performed in untransfected HEK-293 cells, faint bands for ERGIC-53, TCP, and PHK-\(\alpha\) elutions indicated that those proteins were bound to the Ni-NTA resin in the absence of RyR1. Accordingly, they were eliminated as potential RyR1 interacting candidates. CHERP, however, was not found in any control blots.

We were not able to perform successful co-immunoprecipitation assays from mouse or rabbit skeletal muscle either in a forward or reverse direction with any of the available RyR antibodies under numerous experimental conditions. These results are consistent with the extremely limited data concerning RyR immunoprecipitations in the literature.

To ensure the effective suppression of CHERP levels, Western blotting was used to compare protein samples from HEK-293 cells expressing either RyR1 alone, RyR1 together with CHERP siRNA, or RyR1 together with a scrambled CHERP siRNA. Fig. 3B shows that RyR1 was expressed at comparable levels in all three treated cells and that endogenous CHERP was expressed at comparable levels in the cells expressing RyR1 or RyR1 plus scrambled siRNA. However, in those cells treated with CHERP siRNA, the expression of endogenous CHERP was reduced dramatically.

To ensure that CHERP is expressed in WT skeletal muscle, we analyzed WT mouse skeletal muscle using Western blotting against protein samples from two different mice. Fig. 3C...
shows that CHERP is present in mouse skeletal muscle in the same samples that express RyR1 and skeletal myosin heavy chain.

**Immunofluorescence Co-localization of CHERP and RyR1 in Rat Skeletal Muscle**—In skeletal muscle, RyR1 is retained in the junction terminal cisternae of the SR membrane (10). Through immunofluorescence staining, RyR1 can be resolved as double rows of punctate immunosignals, with each spot representing a single triad structure. The Z-line lies between the RyR1 doublet (28). In skeletal muscle co-stained with CHERP and RyR1 antibodies, co-localization and partial overlap pattern was evident (Fig. 4A). Co-localization and partial overlap was observed at both of the RyR1 double rows and was most consistent on the side facing the Z-line throughout the longitudinal section. This gave the appearance of two RyR1 rows, split by the Z-line, each with a CHERP row proximal to the Z-line.

Fig. 4B shows the three-dimensional reconstitution of a Z-stack of two-dimensional images from mouse skeletal muscle tissue co-stained with RyR1 (green) and CHERP (red) antibodies. The Imaris analysis software allowed us to calculate the percentage of co-localization and overlap observed in a three-dimensional image. The co-localization software employed a computational algorithm to determine the presence of each signal in one voxel (a three-dimensional pixel), thus determining the percentage of co-localized voxels containing both green and red signals. Through this analysis, 22% of the voxels within the region of interest were found to colocalize. To ensure that colocalization results were not affected by the nonspecific binding of fluorescent secondary antibodies, we incubated rat soleus muscle with Alexa 488 and 633 secondary antibodies followed by confocal imaging. Under these conditions, co-staining could not be resolved, so we could not reconstruct three-dimensional images from a Z-series of images (data not shown).

Our three-dimensional pattern of co-localization of RyR1 and CHERP (Fig. 4B) indicates that CHERP and RyR1 sit adja-

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**TABLE 1**

Forty-nine filtered proteins from the RyR1 purifications

| Protein name | Spectral count-RyR1 LC-MS |
|--------------|--------------------------|
|              | Run 1 | Run 2 | Run 3 |
| RYR1, isoform 1 of ryanodine receptor 1 | 98    | 113   | 59    |
| VIM, vimentin | 81    | 108   | 0     |
| LMAN1, protein ERGIC-53 precursor | 53    | 52    | 0     |
| CAD protein | 42    | 42    | 11    |
| RPS15, 40 S ribosomal protein S15 | 40    | 43    | 0     |
| SFRS10, isoform 1 of splicing factor, arginine/serine-rich 10 | 28    | 32    | 0     |
| MYCBP2, MYC-binding protein 2 | 21    | 10    | 0     |
| PRPF8, pre-mRNA-processing-splicing factor 8 | 20    | 16    | 0     |
| ASCC3L1, isoform 1 of U5 small nuclear ribonucleoprotein helicase | 17    | 14    | 3     |
| DHX30, isoform 1 of putative ATP-dependent RNA helicase DHX30 | 15    | 10    | 0     |
| GYS1, GYS1 protein | 14    | 4     | 0     |
| KRT14, keratin, type I cytoskeletal 14 | 10    | 19    | 0     |
| RPS9, 40 S ribosomal protein S9 | 8     | 2     | 0     |
| CCT2, T-complex protein 1 subunit β | 8     | 12    | 34    |
| SF3B1, splicing factor 3B subunit 1 | 8     | 6     | 15    |
| CCT8, 59-kDa protein | 7     | 4     | 44    |
| EFTUD2, 116-kDa U5 small nuclear ribonucleoprotein component | 7     | 9     | 2     |
| GEMIN4, component of gems 4 | 6     | 7     | 0     |
| CCT5, T-complex protein 1 subunit e | 6     | 4     | 41    |
| LUC7L2, isoform 1 of putative RNA-binding protein Luc7-like 2 | 6     | 12    | 0     |
| MRPS22, mitochondrial 28 S ribosomal protein S22 | 5     | 2     | 0     |
| RPS4X, 40 S ribosomal protein S4, X isoform | 5     | 6     | 0     |
| CCT6A, T-complex protein 1 subunit ξ | 5     | 6     | 20    |
| SF3B2, splicing factor 3B subunit 2 | 5     | 4     | 0     |
| RPS24, isoform of 40 S ribosomal protein S24 | 4     | 3     | 0     |
| PTCD3, isoform 1 of pentatricopeptide repeat-containing protein 3 (mito) | 4     | 3     | 0     |
| RPS16, 40 S ribosomal protein S16 | 4     | 3     | 0     |
| MRPS31, 28 S ribosomal protein S31, mitochondrial precursor | 4     | 2     | 0     |
| GTPBP4, nucleolar GTP-binding protein 1 | 3     | 6     | 6     |
| PRPF6, pre-mRNA-processing factor 6 | 3     | 2     | 0     |
| EDC4, isoform 1 of enhancer of mRNA-decaping protein 4 | 3     | 2     | 0     |
| SMN2, isoform SMN of survival motor neuron protein | 3     | 1     | 0     |
| MRPS10, mitochondrial 28 S ribosomal protein S10 | 3     | 1     | 0     |
| TCP1, T-complex protein 1 subunit α | 3     | 8     | 17    |
| RPS18L,LOC100130553, 40 S ribosomal protein S18 | 3     | 2     | 0     |
| RPS6, 40 S ribosomal protein S6 | 2     | 4     | 0     |
| ATP5B, ATP synthase subunit β, mitochondrial precursor | 2     | 3     | 0     |
| RPL11, isoform 1 of 60 S ribosomal protein L11 | 2     | 3     | 0     |
| MRPS25, mitochondrial 28 S ribosomal protein S25 | 2     | 3     | 0     |
| WDR26, isoform 1 of WD repeat-containing protein 26 | 2     | 2     | 0     |
| RSBN1, round spermatid basic protein 1 | 2     | 2     | 0     |
| YTHDC1, isoform 1 of YTH domain-containing protein 1 | 2     | 2     | 0     |
| MRPS9, mitochondrial ribosomal protein S9 | 2     | 2     | 0     |
| HERC1, guanine nucleotide exchange factor p532 | 2     | 0     | 1     |
| CHERP, calcium homeostasis endoplasmic reticulum protein | 2     | 0     | 11    |
| RPS13, 40 S ribosomal protein S13 | 1     | 2     | 0     |
| RPS26, OTTHUMP00000018641 | 1     | 2     | 0     |
| PHKB, isoform 4 of phosphorylation b kinase regulatory subunit β | 1     | 2     | 0     |
| FKBPA1, FK506-binding protein 1A | 1     | 0     | 14    |
cent to each other in the ER/SR membrane. CHERP is predicted to be an integral ER membrane protein with at least 1 TM sequence and a large cytosolic domain (21). With this in mind we generated a schematic model of interaction between these two proteins (Fig. 4C).

To further validate our three-dimensional fluorescence data, we generated a three-dimensional reconstruction of mouse skeletal muscle tissue RyR1 (green) co-stained with DHPR (red), a protein known to interact with RyR1 (Fig. 4D). The co-localization algorithm determined that 28% of the voxels were co-localized and that the staining pattern was stacked. This is exactly what is predicted, as the DHPR is located in the transverse tubular membrane, whereas RyR1 is embedded in the SR membrane, as indicated in Fig. 4C.

**Ca**²⁺ Transient Analysis of HEK-293 Cells Expressing Full-length RyR1 Alone and with siRNA Suppression of CHERP—To study the effect of CHERP suppression on **Ca**²⁺ transients in HEK-293 cells expressing full-length RyR1, we acquired 340/380-nm fluorescence ratios from cells treated with caffeine, an RyR1 activator (Fig. 5A).

The amplitudes of the **Ca**²⁺ transients seen in Fig. 5B represent the increase in the fluorescence ratio over baseline values upon stimulation with 5 mM caffeine. The fluorescence ratio is directly proportional to the level of intracellular **Ca**²⁺. Each condition was repeated 3 or more times with emissions from clusters of 5–12 cells measured in each repeat. These amplitudes are quantified in Fig. 5C. Fluorescence amplitude was calculated by dividing each point throughout the curve by the

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**FIGURE 3.** Immunoblot analysis of His₆-tagged RyR1 overexpression, CHERP siRNA knockdown, and endogenous expression of RyR1 and CHERP in mouse skeletal muscle. A, fractions analyzed were the cell lysate, the flow-through, or unbound fraction, which is the lysate after incubation with Ni-NTA resin, the final wash, and the fraction eluted with 500 mM imidazole. DHPR, known to interact with RyR1, was used as positive control. B, HEK-293 cells were transfected with full-length RYR1 cDNA 24 h after plating and 2 h later with either blank Lipofectamine (+RyR1) or siRNAs, knocking down either CHERP (+RyR1 + CHERP) or scramble siRNA (+RyR1 + SCRAM). Twenty µg of protein from each sample were run on SDS-PAGE and analyzed by Western blotting. C, WT mouse skeletal muscle was harvested, homogenized, and centrifuged at 8000 rpm to obtain a soluble cell lysate. Twenty µg of protein were run on SDS-PAGE and analyzed by Western blotting to determine endogenous levels of RyR1 and CHERP in WT skeletal muscle.

**FIGURE 4.** Subcellular co-localization of CHERP and RyR1 in rat skeletal muscle. A, shown is immunofluorescent analysis of the subcellular distribution of endogenous CHERP and RyR1 in rat soleus skeletal muscle. CHERP was detected by labeling with Alexa 488 (green), and RyR1 was labeled with Alexa 633 (red). Regions of overlap are represented by yellow in the third column. B, three-dimensional reconstruction of a two-dimensional Z-stack of images illustrates the subcellular distribution of endogenous CHERP and RyR1 in rat soleus skeletal muscle (left panel). Analysis of images for three-dimensional colocalization shows that 22% of the voxels within the region of interest seen above were co-localized. Right panel, co-localized voxels only. C, shown is a schematic representation of co-localization between RyR1 and CHERP. D, shown is a three-dimensional reconstruction of a two-dimensional Z-stack of images illustrating the subcellular distribution of endogenous RyR1 and DHPR in mouse soleus skeletal muscle (left panel). Analysis of images for three-dimensional colocalization showed that 28% of the voxels within the region of interest seen above were co-localized. Right panel, co-localized voxels only.
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FIGURE 5. Effect on RyR1-mediated Ca\(^{2+}\) release in HEK-293 cells after knocking down CHERP levels by introduction of siRNAs. HEK-293 cells were transfected with full-length RYR1 cDNA (+ RYR1) or siRNAs knocking down either CHERP (+ RYR1 - CHERP), or scramble siRNA (+ RYR1 - Scramble), which does not knock down any protein (see “Experimental Procedures”). Ca\(^{2+}\) photometry was carried out using a dual monochromator to provide activating light at 340 or 380 nm combined with reading of emission at 510 nm. Measurements were made every 3 s over a period of 60 s after the addition of caffeine to 5 mM caffeine – 30 s after readings commenced. All values were normalized by dividing by the baseline value. A, 340/380 ratio for Ca\(^{2+}\) transients of HEK-293 cells expressing RyR1 show that lowered CHERP levels induce a marked decrease in the amplitude of the Ca\(^{2+}\) transient when the cells were stimulated with caffeine. B, the percentage increase of the 340/380 nm ratio over base-line values after the addition of 5 mM caffeine was calculated; after siRNA knockdown of CHERP, Ca\(^{2+}\) release was significantly reduced (*, p = 0.05). C, dose-response curves were obtained by Ca\(^{2+}\) photometry of HEK-293 cells transfected with wild-type RYR1 cDNA or with wild-type RYR1 cDNA followed by transfection with CHERP or scrambled siRNA. The amplitude of each peak caffeine response was normalized to the amplitude of the peak caffeine response. Changes in fluorescence ratio are presented as ΔR = (R - R_{max})/R_{max}, where R refers to the 340/380 nm fluorescence ratio at each caffeine concentration, and R_{max} and R_{min} refer to the fluorescence ratio under resting conditions and at the highest response to caffeine. R is plotted as a function of caffeine concentration. Upon stimulation with 12 mM caffeine, the upper limit of RyR1 caffeine response, there was a significant reduction in fluorescence response in cells suppressing CHERP (*, p = 0.05).

DISCUSSION

In this study we screened for RyR1 interacting proteins that might affect RyR1 function by overexpressing and purifying the soluble, cytosolic domain of RyR1 in HEK-293 and determining by mass spectrometry, immunoblotting, and immunofluorescence microscopy whether interacting proteins might co-purify and colocalize with RyR1. Our studies show that CHERP co-purifies with RyR1 and that it co-localizes with RyR1 in endogenous rat skeletal muscle. Subsequently, we showed that a decrease in the expression of endogenous CHERP in HEK-293 cells expressing full-length, functionally active RyR1 reduced Ca\(^{2+}\) efflux from the ER via RyR1, suggesting that protein-protein interactions between CHERP and RyR1 contribute to the activity of RyR1 Ca\(^{2+}\) release channels and, by analogy, may be involved in the regulation of EC coupling in muscle cells.

Expression and Purification of Cytosolic RyR1 Proteins and Co-purification of Interacting Proteins from HEK-293 Cells—To identify novel RyR1 protein interactions, metal affinity chromatography was applied to extracts of HEK-293 cells expressing a soluble, truncated form of RyR1. This method was chosen for its efficiency, potential for high-throughput application, and capacity for purifying protein complexes (from in vivo systems. HEK-293 cells provided a large cell mass, an easily transfected cell line, and generation of a high overall yield of soluble bait and interacting proteins. Concerning the RyR1 purification itself, the recovery and concentration of bait protein from the cell lysate was performed successfully; in the RyR1 purifications and subsequent MS runs, RyR1 spectral counts greatly exceeded those of any other protein.

minimum fluorescence, thus generating a base-line value of ~1.0. The amplitude thus represents the percentage change in the fluorescence ratio.

The percentage increase in the fluorescence ratio in HEK-293 expressing full-length RyR1 in the presence of endogenous CHERP (36.1 ± 4.8%; p value < 0.05) was significantly higher than the percentage increase in fluorescence observed when endogenous CHERP expression was suppressed by siRNA (8.1 ± 1.0%). In all runs, after the caffeine-induced Ca\(^{2+}\) release event, a decrease was observed in the 340/380-nm ratio, although the base line remained elevated, as we did not attempt to wash out added caffeine during this period. This represents subsequent Ca\(^{2+}\) reuptake by the ER after the Ca\(^{2+}\) release event and establishes a Ca\(^{2+}\) transient. In every run the uptake or decrease in 340/380-nm ratio was in the range of 25–40% of the initial increase in 340/380-nm ratio. This response to caffeine-induced Ca\(^{2+}\) release is consistent with reports in the literature.

When we measured caffeine dose dependence curves for HEK-293 cells expressing RyR1 and RyR1 with suppressed CHERP, we observed a decrease in caffeine maximal efficacy on Ca\(^{2+}\) release from RyR1 in those cells with low expression of CHERP (Fig. 5C). Cells were treated across a range of caffeine concentrations up to 12 mM, the upper limit of an RyR1 response to caffeine-induced Ca\(^{2+}\) release (29). At 12 mM there was a significant difference in the percentage of maximal fluorescence ratio observed in cells with low expression of CHERP (p < 0.05, analysis of variance).
Our stringent data-filtering criteria removed some known RyR1 interactors from our final candidate list. For example, DHPR, which physically interacts with RyR1 in skeletal muscle (30), was excluded because it appeared in only one RyR1 mass spectrometry run. FKBPIA, another known interacting protein (7), was retained because it was identified in 2 runs, and FKBPIA spectral counts were, on average, ∼4-fold enriched over controls.

A subset of 49 potential RyR1 interacting proteins was identified after the filtering of the MS data. Individual analysis of these proteins revealed that many were involved in biological processes that are not directly related to RyR1 and Ca$^{2+}$ regulation, so they were eliminated as potential interacting proteins. The identification of these proteins on the basis of their known functions probably represents nonspecific binding of contaminant proteins to bait-binding resin, which is a common limitation of affinity chromatography (31). Washing the Ni-NTA resin throughout the purification process did not remove all contaminants, allowing these proteins to pass even stringent criteria for MS identification. Many contaminant proteins are commonly found in proteomics experiments. They are present either by accident or through unavoidable contamination of protein samples. These proteins are listed online in the contaminant repository of adventitious proteins (cRAP; The Global Proteome Machine Organization).

**CHERP as a Potential RyR1 Interacting Protein**—Of the 49 RyR1 co-purified proteins that are candidates for interaction with RyR1, a select number were identified in the literature as being involved in Ca$^{2+}$ homeostasis and muscle processes, allowing us to filter the MS data list down to a handful of proteins for further analysis. These proteins, PHK, TCP-α, ERGIC-53, and CHERP, were subjected to immunoblot analysis to determine whether they could be identified in the eluant fractions of RyR1 purifications. Although all of the proteins were identified in these fractions, only CHERP was not found in control experiments where Ni-NTA resin was incubated in WT HEK-293 cellular lysate. This suggests that these candidate proteins bound to the Ni-NTA resin non-specifically and were contaminant proteins.

CHERP was first identified through its interaction with a monoclonal antibody raised against inositol 1,4,5-triphosphate receptor-rich dense-tubular-system membranes of platelets (23). This antibody inhibited Ca$^{2+}$ release by inositol 1,4,5-triphosphate receptor from internal membrane vesicles derived from platelets, cerebellum, smooth muscle, sea urchin eggs, and human erythroleukemia cells (23). CHERP is found in many cell types, including HEK-293 cells (this study) and mammalian skeletal muscle. It has been found to play a role in the regulation of intracellular Ca$^{2+}$ mobilization. Our study has identified CHERP as a RyR1 interacting and regulatory protein, which may be involved in the regulatory mechanism of EC coupling.

More in-depth, functional work has been performed with CHERP in Jurkat cells. Transfection of Jurkat cells with a lac-regulated mammalian expression vector containing CHERP antisense cDNA caused a knockdown of CHERP and impaired the rise of cytoplasmic Ca$^{2+}$ levels caused by phytohemagglutinin and thrombin (21). A 50% fall of CHERP decreased the phytohemagglutinin-induced rise of the cytoplasmic free Ca$^{2+}$ concentration, but Ca$^{2+}$ influx was unaffected. Furthermore, cell proliferation was slowed. Although these findings provide further evidence that CHERP is an important component of ER Ca$^{2+}$ mobilization and that its loss impairs Ca$^{2+}$-dependent biochemical pathways and progression through the cell cycle, no mechanism of these effects was ever offered. However it was shown that Ca$^{2+}$ influx from extracellular spaces was not affected.

Jurkat cells express RyR1 according to an earlier study elucidating the expression of RyR isoforms in immune cells (32). It was found that 4-chloro-m-cresol, a specific activator of RyR1, induced Ca$^{2+}$ release, thereby confirming functional expression of RyR in Jurkat cell lines. Because Ca$^{2+}$ influx was unaffected and because Jurkat cells express RyR1, it is plausible that CHERP suppression was responsible for lower cytosolic Ca$^{2+}$ levels because of reduced RyR1 activity.

In the present study CHERP suppression affected cytosolic Ca$^{2+}$ levels in a manner similar to that observed in the O’Rourke et al. study (21). Here, siRNA knockdown of CHERP resulted in a reduction of total Ca$^{2+}$ release from the ER by 56%. Based on the 4-fold enrichment of CHERP spectra in MS-analyzed RyR1 purifications, in our CHERP-RyR1 co-localization results, which show subcellular colocalization of CHERP and RyR1 in skeletal muscle SR (22% co-localized voxels, when analyzed by three-dimensional software), we suggest that CHERP interacts directly with RyR1 to promote Ca$^{2+}$ release in skeletal muscle cells.

Cytosolic Ca$^{2+}$ levels are determined by numerous factors, including Ca$^{2+}$ uptake by sarco/endoplasmic reticulum Ca$^{2+}$-ATPase and extracellular Ca$^{2+}$ release by the plasma membrane Ca$^{2+}$ ATPase and the sodium calcium exchanger and the NCX as well as Ca$^{2+}$ uptake and release by the mitochondria. The properties of these channels, however, leave RyR as the only component of Ca$^{2+}$ flux capable of generating the magnitude of Ca$^{2+}$ release observed in our Ca$^{2+}$ transient results. Thus, regulation of RyR1 function by CHERP of the magnitude that we observed would not be trivial in the context of Ca$^{2+}$ handling in muscle cells.

**Potential Physiological Relevance of a CHERP-RyR1 Interaction on EC-coupling Regulation**—The amino acid sequence of CHERP described by O’Rourke et al. (21) suggested an integral ER membrane protein that appears to contain several relevant domains that might interact with RyR1. Recent analyses of the topology of CHERP suggest that it contains either 1 or 2 TM regions (233–252 and 641–661) (22). As a result, 1 or 2 TM sequences would lead to either a 388- or 664-amino acid cytosolic domain. Because we used a large cytosolic domain of RyR for interaction analysis, we predict that CHERP-RyR1 interactions occur in the cytosol via cytosolic domains. It is also likely that CHERP interacts with RyR1 at or near the RyR1 membrane sector and not at the most distal cytosolic portion, believed to interact with the DHPR.

In future studies, it will be important to assess the function of CHERP through muscle cell lines for both the RyR1 (skeletal) and RyR2 (cardiac) isoforms. It will also be important to localize and characterize CHERP-RyR1 binding sites and to define the regulatory mechanism whereby CHERP exerts its effects on...
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RyR1 Ca\textsuperscript{2+} release channel function. At this stage we cannot conclude whether CHERP knockdown results in direct inhibition of Ca\textsuperscript{2+} release channel function or, alternatively, whether our results reflect depletion of the ER/SR Ca\textsuperscript{2+} store induced by loss of CHERP. Given the relative abundance of CHERP in skeletal muscle, as indicated in our morphological studies, and its intimate proximity to RyR1, it is probable that CHERP plays a significant role in ER/SR function.

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