A 37-Amino Acid Sequence in the Skeletal Muscle Ryanodine Receptor Interacts with the Cytoplasmic Loop between Domains II and III in the Skeletal Muscle Dihydropyridine Receptor

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Interactions between the Ca\(^{2+}\) release channel of skeletal muscle sarcoplasmic reticulum (ryanodine receptor or RyR1) and the loop linking domains II and III (II-III loop) of the skeletal muscle L-type Ca\(^{2+}\) channel (dihydropyridine receptor or DHPR) are critical for excitation-contraction coupling in skeletal muscle. The DHPR II-III loop was fused to glutathione-S-transferase or His-peptide and used as a protein affinity column for the skeletal muscle sarcoplasmic reticulum (the ryanodine receptor or RyR1). The II-III loop was bound specifically to the DHPR II-III loop column, but the corresponding fragment from the cardiac ryanodine receptor (RyR2) did not. The use of chimeras indicated that amino acids, Arg1076–Asp1112, in RyR1. The RyR1 922–1112 fragment did not bind to the cardiac DHPR II-III loop but did bind to the skeletal muscle Na\(^+\) channel II-III loop. The skeletal DHPR II-III loop double mutant K677E/K682E lost most of its capacity to interact with RyR1, suggesting that two positively charged residues are important in the interaction between RyR and DHPR.

E-C coupling linking electrical stimulation of skeletal muscle and the release of Ca\(^{2+}\) from the sarcoplasmic reticulum to muscle contraction almost certainly involves a direct interaction between the Ca\(^{2+}\) release channel of skeletal muscle sarcoplasmic reticulum (the ryanodine receptor or RyR1) and the L-type Ca\(^{2+}\) channel of skeletal muscle (the dihydropyridine receptor or DHPR) located in the transverse tubule (1–4).

Extracellular Ca\(^{2+}\) is not necessary for E-C coupling in skeletal muscle but is required in cardiac muscle (5). Skeletal muscle-type E-C coupling was rescued in RyR1 knockout mice by injection with RyRI cDNA (6) and in myotubes cultured from dysgenic mouse skeletal muscle, in which functional DHPR and E-C coupling are lacking, by injection with cDNA encoding skeletal DHPR (7, 8). Tanabe et al. (8) used chimeras between skeletal and cardiac DHPRs to define a region linking DHPR domains II and III as the determinant of skeletal versus cardiac type E-C coupling. The DHPR II-III loop activates RyR1 in single channel recordings (9, 10). Ca\(^{2+}\) release from triads has also been elicited by the addition of the DHPR II-III loop peptide (11).

In this study, we purified the DHPR II-III loop following its expression in Escherichia coli. RyR1 fragments were prepared from a cell-free in vitro translation system. The purified DHPR II-III loop was bound to a gel matrix to permit affinity chromatography, and \(^{35}\)S-labeled RyR1 fragments were then passed through the column to demonstrate direct interaction between specific, expressed fragments of the two proteins. This strategy allowed us to identify a 37-amino acid sequence of RyR1 that interacts specifically with the DHPR II-III loop.

MATERIALS AND METHODS

Chemicals and Reagents—NiNTA resin was from Qiagen and glutathione-Sepharose 4B from Pharmacia Biotech Inc. Translational grade [\(^{35}\)S]Met was obtained from Amersham Life Science. The coupled in vitro transcription and translation kit (TNT Quick) was from Promega. Rabbit skeletal and cardiac muscle cDNA was purified from λ phage CDNA libraries (15, 16) using mediprep columns from Qiagen. The rSkM1 rat skeletal muscle Na\(^+\) channel cDNA (17) was a generous gift from Dr. P. Backx (University of Toronto). The full-length ryanodine receptor cDNA clone, pBS SRR10, was described previously (18).

Preparation of Fusion Proteins—The loops linking domains II and III were amplified and cloned using the polymerase chain reaction (PCR): nucleotides 1990–2381 of the cDNA fragment encoding the rabbit skeletal muscle Na\(^+\) channel as template; nucleotides 2574–2772 of the cardiac DHPR II-III loop with the cardiac muscle cDNA library as template; nucleotides 2389–3059 of the rat skeletal muscle Na\(^+\) channel with rSkM1, the rat skeletal muscle Na\(^+\) channel cDNA (17) as template. Oligonucleotide primers flanking the cDNA sequence for the respective loops were designed with exterior BamHI and EcoRI restriction endonuclease sites for in-frame cloning into the pTrcHisC vector (Invitrogen) or the pGEX 3X vector (Pharmacia). All cloned fragments were then verified by DNA sequence analysis. The oligonucleotide attgagttttgacccacacacacacctagttcaacatca and its complementary oligonucleotide were used to add 10 His residues to the C-terminal end of glutathione S-transferase (GST) by self-annealing of the two oligonucleotides, endonuclease restriction digestion at the EcoRI sites flanking the His\(_{10}\) sequence, and ligation into the EcoRI site of pGEX3X. E. coli strain DH5α (Life Technologies, Inc.) was used for expression. Proteins were purified following standard procedures (19) in the pres-
ence of 20 mM imidazole, pH 7.0, and protease inhibitors. His-peptide (Invitrogen) fusion proteins were purified with NINTA resin, and GST fusion proteins were purified with glutathione-Sepharose 4B. After washing, the His-peptide fusion proteins were eluted with 0.5 M imidazole, pH 7.0, in phosphate-buffered saline (PBS). GST fusion proteins were eluted with 10 mM reduced glutathione and dialyzed against PBS overnight. Eluted proteins were analyzed by SDS-PAGE and Coomassie Blue staining, and yield was determined by the Bradford assay (Bio-Rad).

Preparation of Ryanodine Receptor Fragments—Fragments for in vitro transcription and translation were prepared from pBS SRR10 (18). pBS SRR10 was digested with EcoRI (New England Biolabs) and XhoI (New England Biolabs), blunt ended with Klenow, and self-ligated to make pBS RYR1 F1. Pe1 re-

Fig. 1. Affinity of RyR1 fragments for the skeletal DHPR II-III loop. In vitro translated fragments of RyR1 were passed through 0.5 mg/ml DHPR II-III His-peptide fusion protein columns as described under "Materials and Methods." A, schematic of RyR1 fragments cloned in-frame with a Kozak consensus start sequence (20) and an initiator ATG codon for in vitro translation. B, autoradiogram of in vitro translated RyR1 fragments (F1–F10) representing 5% of input and 20% of fragments eluted from GST-His10 (lanes G) and skeletal DHPR (lanes D) affinity columns. C, percentage of specific binding of in vitro translated RyR1 fragments to the skeletal DHPR affinity columns, quantitated by densitometry and expressed as the means ± S.E. from at least four separate experiments. Specific binding was defined as total binding to DHPR II-III His-peptide fusion protein columns, less nonspecific binding to GST-His10.

RESULTS

Identification of an RyR1 Sequence Interacting with the DHPR II-III Loop—We immobilized the DHPR II-III loop on a Ni2+ column as a His-peptide fusion protein and passed 35S-labeled in vitro translated fragments of RyR1 through it (Fig. 1). We found that 20 ± 2.7% of in vitro translated fragment 922–1220 was retained on the DHPR II-III loop affinity column compared with less than 5% retention for any of the other fragments of RyR1 binding to the Ni2+ column (Fig. 1, B and C). Reduction of the RyR1 fragment to 190 amino acids, spanning residues 922–1112, increased the proportion of the in vitro translated fragment retained on DHPR II-III loop columns to 32 ± 1.6% (Fig. 2). To test isoform specificity, we passed 35S-labeled in vitro translated fragments of RyR2 over the affinity column. We did not detect any specific binding of
sequence within the skeletal muscle DHPR activation region is KAKAEEERKRRKMSR (Fig. 3C). The corresponding amino acid sequence in the skeletal muscle Na\textsuperscript{+} channel is RGKILSPKEIK11 in vitro and Na\textsuperscript{+} channel II-III loop so that the mutated skeletal muscle DHPR sequence, KAAEERKRRKMSR, would more closely resemble the cardiac sequence in this region. This double mutation resulted in a 66 ± 7.1% decrease in binding of the C3 chimera, from 12 ± 0.4% binding to 4 ± 0.7% binding.

**DISCUSSION**

In this study, we localized the binding site for the skeletal DHPR II-III loop to 190 amino acids between 922 and 1122 in *in vitro* transcribed and translated products of RyR1. Decreasing the size of the fragment resulted in loss of *in vitro* translation, and GST fusion proteins of fragments shorter than 190 amino acids were unstable, suggesting that this sequence might form a stable structural domain. RyR1, RyR2, and RyR3 contain four repeat sequences (15, 16, 21). The 922–1122 fragment of RyR1 contains part of repeat 1 and all of repeat 2. We made use of these repeat domain boundaries to create three RyR1-RyR2 chimeras (Fig. 2A). The 37-amino acid sequence between Arg\textsuperscript{1076} and Asp\textsuperscript{1112} of RyR1 is important for binding the DHPR II-III loop.

**DHPR Specificity in the RyR1-DHPR Interaction—**The chimeric construct (C3), which binds to the skeletal muscle DHPR II-III loop His-peptide fusion protein, did not bind above background to either GST columns or to a GST fusion protein constructed from the II-III loop of the cardiac DHPR receptor (cdDHPR in Fig. 3, A and B). A GST fusion protein affinity column with the II-III loop of the skeletal muscle DHPR II-III (skDHPR in Fig. 3, A and B) contained 12 ± 0.4% of the input of the in vitro-transcribed C3 chimera (Fig. 3, A and B). Surprisingly, 14 ± 0.4% of the input of in vitro-translated C3 chimera bound to a GST fusion protein affinity column containing the loop between domains II and III of the homologous skeletal muscle voltage-gated Na\textsuperscript{+} channel (skNa in Fig. 3, A and B).

The region of the skeletal muscle DHPR II-III loop important for activation of RyR1 has been localized to the amino acid sequence between positions 671 and 690 (11). A 14-amino acid sequence in this region is RGKILSPKEIK11. The corresponding sequence in the cardiac sequence is RGKILSPKEIK11. The 37-amino acid sequence between positions 671 and 690 (11) would more closely resemble the cardiac sequence in this region. This double mutation resulted in a 66 ± 7.1% decrease in binding of the C3 chimera, from 12 ± 0.4% binding to 4 ± 0.7% binding.

**FIG. 2. Affinity of chimeric RyR1-RyR2 constructs for the skeletal DHPR II-III loop.** A, chimeric RyR1-RyR2 constructs prepared as described under “Materials and Methods.” The first line represents a region in RyR1 containing two repeat sequences and defines their respective boundaries. Amino acid numbering refers to RyR1 residues and cardiac Ca\textsuperscript{2+} channel II-III loops. The context of the skeletal muscle Na\textsuperscript{+} channel II-III loop is illustrated in Fig. 2A. The first line represents a region in RyR1 containing two repeat sequences and defines their respective boundaries. Amino acid numbering refers to RyR1 residues.

**A** chimeric constructs

| Construct | Repeat 1 | Repeat 2 |
|-----------|----------|----------|
| RyR1 (922–1112) |  |  |
| RyR2 (933–1126) |  |  |
| RyR2 (922–1107)/RyR1 (1076–1112) |  |  |
| RyR2 (922–1075)/RyR1 (1076–1112) |  |  |
| RyR2 (922–953)/RyR1 (954–1112) |  |  |

**B** autoradiogram

| Column | Input | R1 | R2 | C1 | C2 | C3 |
|--------|-------|----|----|----|----|----|
| GST | skDHPR | skDHPR | skDHPR | skDHPR | skDHPR | skDHPR |
| skDHPR KE | skDHPR | skDHPR | skDHPR | skDHPR | skDHPR | skDHPR |
| ccdDHPR | skDHPR | skDHPR | skDHPR | skDHPR | skDHPR | skDHPR |
| skNa | skDHPR | skDHPR | skDHPR | skDHPR | skDHPR | skDHPR |

**C** percentage binding

| % Bound | R1 | C1 | C2 | C3 |
|---------|----|----|----|----|
| 0 | 5 | 10 | 15 | 20 |

**FIG. 3. Affinity of chimeric RyR1-RyR2 constructs for skeletal and cardiac Ca\textsuperscript{2+} and Na\textsuperscript{+} channel II-III loop-GST fusion proteins.** A, autoradiogram of *in vitro* translated RyR2(922–953)-RyR1(954–1112) chimeric fragment (C3) representing 5% of input and 20% of fragments eluted from different 0.1 mg/ml GST fusion protein affinity columns: GST (GST); skeletal DHPR II-III loop (skDHPR); skeletal DHPR II-III loop double mutant K677E/K682E (skDHPR KE); cardiac DHPR II-III loop (ccdDHPR); skeletal Na\textsuperscript{+} channel II-III loop (skNa). B, percentage of specific binding of *in vitro* translated chimeric fragment (C3) to the II-III loop affinity columns, quantitated by densitometry and expressed as the means ± S.E. from at least four separate experiments. Specific binding was defined as total binding to GST fusion protein columns, less nonspecific binding to GST. C, sequence alignment of segments of skeletal and cardiac muscle Ca\textsuperscript{2+} and Na\textsuperscript{+} channel II-III loops.
Ry1-DHPR interaction but may form part of a structural domain that includes the DHPR binding site. Only 10 residues between Arg1076 and Asp1112 differ in Ry1 and RyR2, and several of these residues are likely to form part of the interaction site between the two proteins.

The newly identified Ry1-DHPR interaction site is distinct from the D2 region (amino acids 1303–1406), which was shown to be important for E-C coupling in skeletal muscle (22) by reconstituting E-C coupling in myotubes from RyR1 knockout mice. Takekura et al. (23) have reported that a functional ryanodine receptor must be provided for the formation of normal junctions between the transverse tubule and the sarcoplasmic reticulum in RyR1 knock-out mice. Thus, deletions in Ry1 may lead to the loss of E-C coupling because the interaction site between DHPR and RyR1 is missing or because Ry1 structure is so altered that proper junctions can no longer form, even though interaction sites are intact. The second possibility could explain the apparent discrepancy between the findings of Yamazawa et al. (22) and our own results.

In vivo studies have shown that Ry1, but not RyR2, can restore skeletal muscle-type E-C coupling (24). Peptides from II-III loops of the skeletal muscle DHPR that activate Ca\(^{2+}\) currents in RyR1 reconstituted into lipid bilayers cannot activate RyR2 (9). In our studies, RyR2 sequences did not bind to the DHPR II-III loop (Fig. 2). In contrast to previous in vitro studies in which peptides from either the skeletal or cardiac II-III loops were equally capable of activating RyR1 Ca\(^{2+}\) release channel function (9, 10), we have found that binding of the RyR1 954–1112 fragment is specific to the skeletal DHPR II-III loop (Fig. 3). Our results are also in agreement with in vivo studies showing that skeletal-type E-C coupling in dystrophic mice lacking DHPR could only be rescued with the skeletal muscle isoform of DHPR (7, 8).

An unexpected observation was that chimera C3 was bound to a GST fusion protein affinity column made up from the loop between domains II and III of the homologous skeletal muscle voltage-gated Na\(^{+}\) channel (skNa in Fig. 3, A and B). We extended this observation by aligning sequences that include 14 highly charged amino acids in the II-III loop of skeletal and cardiac DHPRs, shown to be important for activation of RyR1 (11), with the corresponding sequence in the skeletal Na\(^{+}\) channel sequences (Fig. 3C). A comparison shows that net charge ranges from 0 to +6, that lysine occurs at positions 3 and 8 of the 14-amino acid sequence in the skeletal DHPR and the Na\(^{+}\) channel (both of which bind to RyR1), and that glutamate occurs at positions 3 and 8 in the cardiac DHPR (which does not bind to RyR1). The double mutation K677E/K682E in the skeletal DHPR II-III loop decreased chimera C3 binding by two-thirds (from 13% to 4%). Thus, the presence of positive charges at positions 3 and 8 seems to be an important feature for binding of RyR1, but net charge does not. Because phosphorylation of Ser667 (found at position 13 in both the skeletal muscle DHPR and the Na\(^{+}\) channel) has been shown to inhibit the interaction of the skeletal DHPR II-III loop with RyR1 (10), charged residues at the C-terminal end of this 14-amino acid sequence in the skeletal DHPR II-III loop may also be important.

Although this study does not deal directly with the functional interaction between the RyR1 and DHPR, numerous studies have implicated the II-III loop of the DHPR in E-C coupling (25, 26). The loss of interaction that we observed when two residues were mutated in a region of the DHPR II-III loop, previously implicated in the triggering of Ca\(^{2+}\) release, makes it likely that this interaction site is functional.

Monnier et al. (27) have demonstrated that an Arg to His mutation in the loop linking DHPR domains III and IV in the α-subunit of the skeletal muscle DHPR can cause susceptibility to malignant hyperthermia (MH). The physiological basis of MH is an abnormality in the regulation of sarcoplasmic Ca\(^{2+}\) concentration, and the RyR1 gene has been linked to MH in human and pigs (28). The involvement of both RyR1 and DHPR in MH suggests that the pathology of MH is due to aberrant E-C coupling. These observations, together with the observations that a C-terminal peptide of the DHPR can inhibit the Ca\(^{2+}\) release function of RyR1 (14) and that there is a retrograde signal by which RyR1 enhances slow Ca\(^{2+}\) channel function (6, 29), suggest that interactions between the RyR1 and DHPR involve many sites in the two proteins.

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