The Cytoplasmic Loops between Domains II and III and Domains III and IV in the Skeletal Muscle Dihydropyridine Receptor Bind to a Contiguous Site in the Skeletal Muscle Ryanodine Receptor*  

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Excitation-contraction coupling in skeletal muscle is a result of the interaction between the Ca2+ release channel of skeletal muscle sarcoplasmic reticulum (ryanodine receptor or RyR1) and the skeletal muscle L-type Ca2+ channel (dihydropyridine receptor or DHPR). Interactions between RyR1 and DHPR are critical for the depolarization-induced activation of Ca2+ release from the sarcoplasmic reticulum, enhancement of DHPR Ca2+ channel activity, and repolarization-induced inactivation of RyR1. The DHPR III–IV loop was fused to glutathione S-transferase (GST) or His-peptide and used as a protein affinity column for 35S-labeled, in vitro translated fragments from the N-terminal three-fourths of RyR1. RyR1 residues Leu922–Asp1112 bound specifically to the DHPR III–IV loop column, but the corresponding fragment from the cardiac ryanodine receptor (RyR2) did not. Construction of chimeras between RyR1 and RyR2 showed that amino acids Lys954–Asp1112, previously shown to interact with the DHPR III–IV loop (14), were required for half-maximal copurification of the longer RyR1 sequence, Lys954–Asp1112. These data suggest that the site of DHPR III–IV loop interaction contains elements from both the RyR1-Phe1075 and Arg1076–Asp1112 fragments. The presence of 4 ± 0.4 μM GST-DHPR II–III or 5 ± 0.1 μM His-peptide-DHPR III–IV was required for half-maximal co-purification of 35S-labeled RyR1 Leu922–Asp1112 on glutathione-Sepharose or Ni2+-nitrilotriacetic acid. Dose-dependent inhibition of 35S-labeled RyR1 Leu922–Asp1112 binding to GST-DHPR II–III and GST-DHPR III–IV by His1075-DHPR II–III and His-peptide-DHPR III–IV was observed. These studies indicate that the DHPR II–III and III–IV loops bind to contiguous and possibly overlapping sites on RyR1 between Lys964 and Asp1112, leading from electrical stimulation of muscle to the release of Ca2+ from the sarcoplasmic reticulum. Closure of the Ca2+ release channel and activation of the sarco(endoplasmonic reticulum Ca2+ ATPase (SERCA) return intracellular Ca2+ to resting levels (1). Extracellular Ca2+ is not necessary for E-C coupling in skeletal muscle, but is required in cardiac muscle, distinguishing the mechanism of E-C coupling in these muscles (2). E-C coupling in RyR1 knockout mice can be restored in primary cultures of myotubes isolated from these mice by injection with RyR1 cDNA (3). During E-C coupling, Ca2+ release from the Ca2+ release channel of skeletal muscle sarcoplasmic reticulum (ryanodine receptor or RyR1) is activated by the L-type Ca2+ channel of skeletal muscle (dihydropyridine receptor or DHPR) (4). A retrograde signal from RyR1, but not from the cardiac ryanodine receptor (RyR2), enhances DHPR Ca2+ channel activity (3, 5). In addition, skeletal DHPR is critical for terminating Ca2+ release through RyR1 upon membrane repolarization (6–8). Regions of RyR1 important for interactions with DHPR, leading to activation of Ca2+ release during E-C coupling and for retrograde enhancement of DHPR Ca2+ channel activity by RyR1, have been identified through the use of a glycine chimeras in RyR1 knockout mice (9). The complex physiology involving the multiple interactions between RyR1 and DHPR suggests that the structural interaction between RyR1 and DHPR may involve more than one site in the two proteins.

The importance of the DHPR II–III loop in E-C coupling has been demonstrated by Tanabe et al. (10, 11) through the study of dysgenic myotubes lacking DHPR. Skeletal-type E-C coupling can be reconstituted in these myotubes by injecting cDNA encoding chimeras between skeletal and cardiac DHPRs with only the II–III loop retaining the skeletal sequence. The DHPR II–III loop has been shown to activate the skeletal muscle ryanodine receptor in studies measuring Ca2+ currents across planar lipid bilayer carried by RyR1 (12, 13). Ca2+ release has also been elicited from muscle triad vesicle preparations by the addition of the DHPR II–III loop peptide (14). A protein affinity chromatography approach was used to identify an interaction site on RyR1 for the DHPR II–III loop (15). Malignant hyperthermia is an abnormality of Ca2+ regulation that is linked to RyR1 on human chromosome 19 in at least 50% of cases (16). A mutation in the DHPR III–IV loop has also been linked to susceptibility to malignant hyperthermia (17). Thus, the DHPR III–IV loop may play a role in E-C coupling, and a mutation in this loop could modify the interaction between the dihydropyridine and ryanodine receptors.
mimicking the effects of mutations of RyR1 previously found to be causative for malignant hyperthermia susceptibility. The studies in dyogenic mice (11) that identify the DHPR II–III loop as being critical for RyR1 activation neither rule out nor implicate the III–IV loop in E-C coupling. In this study, we used protein affinity chromatography to demonstrate that the DHPR III–IV loop can interact with RyR1.

MATERIALS AND METHODS

Chemicals and Reagents—All chemicals were of molecular biology grade. N2+–NTA resin was purchased from Qiagen Inc. and glutathione-Sepharose 4B and transglutathione bond was purchased from Amersham Pharmacia Biotech. The coupled in vitro transcription and translation kit (TNT Quick) was from Promega. Rabbit skeletal and cardiac muscle cDNAs were purified from μl of phage cDNA libraries (18, 19) using mediprep columns from Qiagen Inc. The rat N-type Ca2+ channel α1I 117 loop of the DHPR III–IV loop, was amplified and cloned using the polymerase chain reaction (PCR) as described (15). Nucleotides 3469–3861 of the DHPR III–IV loop, were amplified using PCR, with the skeletal muscle cDNA library as template, and cloned; nucleotides 3492–3861 of the cardiac DHPR III–IV loop (Pro1164-Leu1207) were amplified, with the cardiac cDNA library as template, and cloned. 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 pTrcHis-C vector or the pGEXXX vector (Amersham Pharmacia Biotech). Flanking BamHI and EcoRI restriction endonuclease sites for in-frame cloning of the RYRI sequence encoding amino acid residues 922–1112 into the pTrcHis-C vector were added to oligonucleotide primers used in PCR amplification of RYRI nucleotides 2766–3335. All cloned fragments were verified by DNA sequence analysis. The modified pGEX vector, which encodes sequence of RyR1 nucleotides 2766–3335, and pBS-RYR2-(923–1075)/RYR1-(1076–1112), and pBS-RYR2-(922–953)/RYR1-(954–1112). [35S]Methionine-labeled RyR1 fragments were synthesized by coupled in vitro transcription and translation (TNT Quick) and quantified by trichloroacetic acid precipitation and scintillation counting (21).

Preparation of Ryanodine Receptor Fragments for in Vitro Transcription/Translation—Fragments F1–F10 (see Fig. 1A) for in vitro transcription and translation were prepared from pBS-SRR10 (20) downstream of a Kozak consensus start sequence (22) and an ATG for translation initiation (15). pBS-RYRI F3a, containing RyR1 nucleotides, 2766–3335, and pBS-RYRI F3a, containing RyR1 nucleotides 2766–3335, were amplified by PCR from pCR YRI cDNA. Chimeric between RYRI and RyR2 were made using pBS-RYRI F3a and pBS-RYR2 F3a through the introduction of restriction endonuclease sites by PCR-based mutagenesis (QuickChange kit, Stratagene) (see Fig. 2A) as described previously (15). The chimera constructs are as follows with all numbering based on RYRI, not RyR2: pBS-RYRI (923–1075)/pRyR2 (1076–1112), pBS-RYRI (923–1075)/pRyR1 (1076–1112), and pBS-RYR2 (922–953)/

RESULTS

Identification of an RyR1 Sequence Interacting with the DHPR III–IV Loop—We scanned 35S-labeled, in vitro translated RyR1 fragments for interactions with the DHPR III–IV loop immobilized on a N2+ column as a His-peptide fusion protein (Fig. 1). We found that 18 ± 4.3% of in vitro translated fragment F3 (amino acids 922–1220) was retained on the DHPR III–IV loop affinity column (Fig. 1, B and C). When the RyR1 fragment was reduced in size to 191 amino acids, spanning residues 922–1112, the proportion of the in vitro trans-
lated fragment retained on DHPR III–IV loop columns remained high at 13 ± 1.3% (Fig. 2).

We observed that the nonspecific binding to GST-His<sub>10</sub> of 35S-labeled RyR1 F5, F8, and F9 fragments was >15% of column input compared with <5% for the other RyR1 fragments (Fig. 1B). This raised the possibility that some in vitro transcribed/translated fragments may form nonspecific aggregates with the affinity matrix and/or other proteins. To determine whether DHPR II–III loop, DHPR III–IV loop, or RyR1-(922–1112) fusion proteins form aggregates, we analyzed their dynamic light scattering characteristics. Assuming the proteins to be globular in nature, software-based conversion of hydrodynamic radii measurements to molecular mass (Dynamics) showed that 99% of the fusion proteins did not form high molecular mass aggregates. GST-DHPR II–III was estimated to be 94.8 kDa, which corresponds to the predicted molecular mass of a dimer of two 49.5-kDa GST-DHPR II–III fusion proteins, and similarly, GST-DHPR III–IV was estimated to be 65.2 kDa, which corresponds to the predicted molecular mass of a dimer of two 33.1-kDa GST-DHPR III–IV fusion proteins. GST fusion proteins are known to dimerize in solution (23). Therefore, dimerization of the DHPR II–III and III–IV loop fusion proteins was expected. His-peptide-RyR1-(922–1112) was estimated to be 26.9 kDa, which corresponds to its predicted molecular mass of 24.5 kDa. In addition, no high molecular mass aggregates were observed when GST-DHPR II–III and His-peptide RyR1-(922–1112) or GST-DHPR III–IV and His-peptide RyR1-(922–1112) were mixed together in a 1:1 ratio.

To test the isoform specificity of the RyR1 interaction with DHPR, we passed 35S-labeled, in vitro translated fragments of RyR1 over the affinity column. We did not detect specific binding of RyR2-(933–1126) (corresponding to RyR1-(922–1112)) to the skeletal DHPR III–IV loop affinity column (Fig. 2). Because further reduction in the size of the RyR1 fragment resulted in loss of synthesis of the in vitro translated product, we reduced the size of the RyR1 sequence that binds to the DHPR III–IV loop by making chimeras between RyR1 and RyR2, as illustrated in Fig. 2A. The RyR1-(922–1075)/RyR2-(1076–1112) (C1) chimera did not bind to the III–IV loop, but 13 ± 1.7% of the RyR2-(922–953)/RyR1-(954–1112) (C3) chimera and 6 ± 1.1% of the RyR2-(922–1075)/RyR1-(1076–1112) (C2) chimera were retained on a 0.5-mg/ml DHPR III–IV loop affinity column (Fig. 2, B and C). These results indicate that the 37 RyR1 amino acids Arg<sup>1076</sup>–Asp<sup>1112</sup>, previously shown to interact with the DHPR II–III loop (15), do not by themselves form the binding site for both the DHPR II–III and III–IV loops, but do not rule out the possibility that they might con-
RyR1/DHPR Interaction Site

RyR1 residues between Lys954 and Val1075 contribute to the binding site for the DHPR III–IV loop.

Co-purification of RyR1-(922–1112) with DHPR Loops—Greater than 6% of two adjacent RyR1 fragments, 1220–1614 (F4) and 1614–1861 (F5), was also retained on the His-peptide-DHPR III–IV column compared with 2% for any of the other fragments of RyR1 lying between amino acids 1 and 3724 (Fig. 1). A complementary test of protein/protein interactions is to mix two potentially interacting proteins in solution and to then purify complexes containing the two proteins. We found that 6.6% of 35S-labeled RyR1-(922–1112) co-purified with His-peptide-DHPR III–IV (3/4) on Ni2+-NTA representing 5% of input and 20% of fragments co-purified with the DHPR II–III and III–IV loops; B, percent specific co-purification of in vitro translated RyR1 fragments with DHPR loops, quantified by densitometry and expressed as the mean ± S.E. from at least four separate experiments. Specific binding was defined as total binding to glutathione-Sepharose and Ni2+-NTA columns less nonspecific binding in the absence of DHPR loop fusion proteins.

Competitive Inhibition of RyR1-(922–1112) Binding to DHPR Loops—Binding of picomolar concentrations of 35S-labeled RyR1 to a GST-DHPR II–III affinity column was inhibited in a dose-dependent manner by both His10-DHPR II–III and His-peptide-DHPR III–IV at a concentration of 0.05–25 μM (Fig. 5A). Similarly, binding of 35S-labeled RyR1 to a GST-DHPR III–IV affinity column was inhibited in a dose-dependent manner by 0.05–25 μM His10-DHPR II–III or His-peptide-DHPR III–IV (Fig. 5B). The concentration of His10-DHPR II–III remained at 4 ± 0.4 μM GST-DHPR II–III fusion protein. Half-maximal purification of RyR1-(922–1112) by GST-DHPR II–III fusion protein and His-peptide-DHPR III–IV fusion protein A and C, autoradiograms of the in vitro translated RyR1-(922–1112) fragment representing 5% of input and 20% of fragments co-purified with GST-DHPR II–III on glutathione-Sepharose or with His-peptide-DHPR III–IV on Ni2+-NTA, respectively, as described under "Materials and Methods"; B and D, percent specific co-purification of in vitro translated RyR1 fragments with the DHPR II–III and III–IV loops, quantified by densitometry and expressed as the mean ± S.E. from at least four separate experiments. Specific binding was defined as total binding to glutathione-Sepharose or Ni2+-NTA less nonspecific binding in the absence of DHPR loop fusion proteins.
from at least three separate experiments; D, or GST-DHPR III–IV (squares) binding by the DHPR III–IV loop to GST-DHPR II–III (and expressed as the mean ± S.E. from at least three separate experiments.

A: Binding to GST DHPR II–III Column

| Input RyR1 (922–1112) | G | DHPR II–III | DHPR III–IV
|------------------------|---|-------------|-------------|
| Competitor (μM)       |   |             |             |
| 0                      | 0.050.05 | 7.5 | 0.05 1.0 20 |

B: Binding to GST DHPR III–IV Column

| Input RyR1 (922–1112) | G | DHPR III–IV |
|------------------------|---|-------------|
| Competitor (μM)       |   |             |
| 0                      | 0.050.05 | 7.5 | 0.05 1.0 20 |

C: Inhibition of RyR (922-1112) Binding by DHPR II–III

![Graph](image1)

D: Inhibition of RyR (922-1112) Binding by DHPR III–IV

![Graph](image2)

FIG. 5. Competitive inhibition of RyR1-(922–1112) binding to DHPR loops. A and B, autoradiograms of in vitro translated RyR1-(922–1112) mixed with the indicated concentrations of DHPR II–III and III–IV loops and passed through GST-DHPR II–III and GST-DHPR III–IV columns, respectively, as described under “Material and Methods”. C, inhibition of RyR1-(922–1112) binding by the DHPR II–III loop to GST-DHPR II–III (squares) or GST-DHPR III–IV (diamonds) columns, quantified by densitometry and expressed as the mean ± S.E. from at least three separate experiments; D, inhibition of RyR1-(922–1112) binding by the DHPR III–IV loop to GST-DHPR II–III (squares) or GST-DHPR III–IV (diamonds) columns, quantified by densitometry and expressed as the mean ± S.E. from at least three separate experiments.

required to inhibit binding of RyR1-(922–1112) to GST-DHPR II–III and GST-DHPR III–IV was comparable (Fig. 5C), as was the concentration of His-peptide-DHPR III–IV required to inhibit binding to GST-DHPR II–III and GST-DHPR III–IV (Fig. 5D).

DHPR Specificity in the RyR1/DHPR Interaction—A GST fusion protein affinity column of the skeletal muscle DHPR III–IV loop (skDHPR) retained 13 ± 1.1% of the input of the in vitro translated RyR1 fragment; the corresponding cardiac muscle DHPR III–IV loop (cdDHPR) retained 13 ± 0.2%; and the N-type Ca2+ channel III–IV loop (NCa) retained 5 ± 1.4% (Fig. 6, A and B). Skeletal and cardiac muscle DHPR III–IV loops are identical in 46 of 54 amino acids. The fact that only 24 of 54 amino acids are identical between the N-type Ca2+ channel III–IV loop and the skeletal DHPR III–IV loop is consistent with the reduced binding of RyR1-(922–1112) to N-type Ca2+ channel III–IV loop-GST.

FIG. 6. Binding of RyR1-(922–1112) constructs to skeletal and cardiac fused Ca2+ and N-type Ca2+ channel III–IV loop-GST fusion proteins. A, autoradiogram of the in vitro translated RyR1-(922–1112) fragment representing 5% of input and 20% of fragments eluted from different 0.1 mg/ml-GST fusion protein affinity columns: GST (G), skeletal DHPR III–IV loop (skDHPR), cardiac DHPR III–IV loop (cdDHPR), and N-type Ca2+ channel III–IV loop (NCa). B, percent specific binding of the in vitro translated fragment to the III–IV loop affinity columns, quantified by densitometry and expressed as the mean ± 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 Ca2+ and N-type Ca2+ channel III–IV loops with residues different from the skeletal DHPR III–IV loop underlined and the proportion of residues identical to the skeletal DHPR III–IV loop shown on the right.

DISCUSSION

In this study, we localized a binding site for the skeletal muscle DHPR III–IV loop to F3-(922–1112), a 191-amino acid sequence between Leu922 and Asp1112 in in vitro transcribed and translated fragments of RyR1 (Figs. 1 and 2). About 13% of this fragment applied to a DHPR III–IV loop affinity column was bound specifically. We also noted binding of ~6–7% of fragments F4 and F5 to the same column. If complexes between the DHPR II–III or III–IV loop and radioactive fragment F3-(922–1112), F4, or F5 were made in solution and then passed over columns that would bind the DHPR loops, co-purification of F3 was observed for both the II–III and III–IV loops (Fig. 3), but very little co-purification of F4 or F5 was observed under comparable conditions. Thus, RyR1 fragments F4 and F5 may have been retained on His-peptide-DHPR III–IV columns due to nonspecific interactions with high matrix concentrations of His-peptide-DHPR III–IV (Fig. 3). RyR1 F5 was observed to interact with proteins unrelated to DHPR, including GST, supporting the view that RyR1 F5 can interact nonspecifically with proteins. The interaction between F3-(922–1112) and DHPR loops is not due to the formation of large aggregates since analysis of dynamic light scattering determined that high molecular mass aggregates made up <1% by mass of His-peptide-RyR1-(922–1112). GST-DHPR II–III, and GST-DHPR III–IV fusion protein preparations, either alone or in combination with each other.

Decreasing the size of the RyR1-(922–1112) fragment resulted in loss of in vitro translation, and His-peptide fusion proteins of RyR1 fragments shorter than 191 amino acids were
unstable, suggesting that the complete sequence might form a stable structural domain. We made use of repeat domain boundaries in RyR (18, 19, 24) to create three RyR1/RyR2 chimeras (Fig. 2A). Chimera C2, which retained partial binding to the DHPR III–IV loop column, and chimera C3, which retained full binding, both contained the 37-amino acid sequence Arg1076–Asp1112, previously identified as being important for interaction with the DHPR II–III loop (15). However, the sequence Arg1076–Asp1112 was absent from chimera C1, which did not bind the DHPR III–IV loop. The RyR1 repeat sequence 2, which is included in chimera C3, contributes to the DHPR III–IV loop interaction site and may form part of a structural domain and be important for proper folding.

An estimate of the affinity for RyR1-(922–1112) of the DHPR II–III and III–IV loops can be derived from the concentration of DHPR loops required for half-maximal co-purification of picomolar 53S-labeled RyR1-(922–1112) with GST-DHPR II–III on glutathione-Sepharose and with His-peptide-DHPR III–IV on Ni2+–NTA (Fig. 4). The concentration of the DHPR II–III loop required was 4 ± 0.4 μM, and the concentration of the DHPR III–IV loop was 5 ± 0.1 μM. Unfortunately, the affinity was not high enough to permit resolution of RyR1-DHPR complexes using dynamic light scattering.

We attempted to verify that the RyR1 region that we identified as interacting with the DHPR II–III loop also constituted part of the interaction site of the DHPR III–IV loop (Fig. 2) through the use of a competition binding assay (Fig. 5). As expected, the complex between the DHPR II–III loop and RyR1-(922–1112) was inhibited from binding to a DHPR II–III loop affinity column, and the complex between the DHPR III–IV loop and RyR1-(922–1112) was inhibited from binding to the DHPR III–IV loop affinity column. We also observed the inhibition of binding of the complex between soluble His-peptide-DHPR III–IV and RyR1-(922–1112) to a GST-DHPR II–III affinity column, and the inhibition of binding of the complex between His10-DHPR II–III and RyR1-(922–1112) to a DHPR III–IV loop column. These results can be interpreted in two ways. One possibility is that the DHPR II–III loop binds to a site on RyR1-(922–1112) that overlaps with the binding site for the DHPR III–IV loop. Alternatively, the accessibility of a GST-DHPR loop on protein affinity columns to its binding site on RyR1 bound to a DHPR loop in solution may be restricted by the size of the fusion proteins already bound to RyR1-(922–1112). This is particularly relevant considering that the GST portion of the fusion contains ~250 amino acids. The close relationship between the concentration of the DHPR II–III loop required for half-maximal inhibition of RyR1-(922–1112) binding to GST-DHPR II–III or GST-DHPR III–IV (Fig. 5c) suggests that soluble complexes form in solution between the DHPR II–III loop and RyR1-(922–1112). These concentrations are equivalent to the 4 μM DHPR II–III loop required for half-maximal saturation of binding to RyR1 in the RyR1-(922–1112) co-purification studies (Fig. 4, A and B). The concentration of the DHPR III–IV loop required for half-maximal inhibition of RyR1-(922–1112) binding to GST-DHPR II–III and GST-DHPR III–IV affinity columns was also similar (Fig. 5D). These concentrations are also equivalent to the 5 μM DHPR III–IV loop required for half-maximal saturation of formation of soluble complexes between the DHPR III–IV loop and RyR1-(922–1112) (Fig. 4, C and D).

Since skeletal and cardiac DHPR III–IV loops are highly conserved, it was not surprising that RyR1-(922–1112) bound to GST fusion protein affinity columns made up from both the skeletal and cardiac DHPR III–IV loops (Fig. 6). The decrease in binding to a GST fusion protein of the loop linking domains III and IV of the rat N-type Ca2+ channel corresponds to the decrease in amino acid identity with the DHPR III–IV loop.

Yamazawa et al. (25) reported that deletion of RyR1-(1303–1406) abolishes the ability of RyR1 to mediate skeletal E-C coupling, although E-C coupling is preserved when the RyR1-(1303–1406) sequence is converted to the RyR2 sequence. The deletion of RyR1-(1303–1406) may alter the structure of RyR and thus disrupt interactions with DHPR. This is consistent with the finding that the conversion of the RyR1-(1303–1406) sequence to the corresponding RyR2 sequence does not disrupt E-C coupling since the homology between RyR1 and RyR2 is likely sufficient to preserve native RyR structure (25). Nakai et al. (9) used chimeras between RyR1 and RyR2 to show that RyR1-(1635–2636) is sufficient for skeletal-type versus cardiac muscle-type E-C coupling and that RyR1-(1635–2636) and RyR1-(2659–3720) are sufficient for enhancement of DHPR Ca2+ channel activity. It is possible that the sites defined in these studies of RyR1/RyR2 chimeras may reflect an interaction resulting from the folding of RyR that brings together amino acids that are very distant in the linear sequence. Thus, the comparatively short sequences that we used to test for interactions may not contain all of the amino acids required for a high affinity interaction site. The high levels of nonspecific binding of some RyR1 fragments to GST-His10, including RyR1 F4 and RyR1 F5, may mask weaker binding interactions with DHPR loops. The possibility that some RyR1 fragments may aggregate, thereby preventing their interaction with DHPR, may also mask weaker binding interactions.

Monnier et al. (17) have demonstrated that an A1086H mutation in the loop linking DHPR domains III and IV in the α1-subunit of skeletal muscle DHPR can be linked to susceptibility to malignant hyperthermia. A mutation in DHPR that disrupts the termination of Ca2+ release through RyR1 upon repolarization (7, 26–28) might display the same clinical phenotype as a mutation in RyR1 that alters the regulation of Ca2+ release (29–32). The Ca2+ flux through wild-type DHPR is slow and of small magnitude (10), and the mutant is unlikely to be different. A mutation in the DHPR III–IV loop is unlikely to alter Ca2+ conductance since this loop does not form part of the channel pore and has not been identified as being important for either regulation or inactivation of DHPR (33). The DHPR mutation does not lead to other symptoms of ion channel disease, suggesting that it does not affect L-type Ca2+ channel function (17). The chimera studies in dysgenic mice (11) identifying the II–III loop as being critical to E-C coupling were not used either to rule out or to implicate the III–IV loop in E-C coupling. Thus, we propose that a possible function arising from the interaction between RyR1-(954–1112) and the DHPR III–IV loop is to terminate Ca2+ release through RyR1 during repolarization. A C-terminal peptide from amino acids 1487 to 1506 of skeletal DHPR has been shown to inhibit the Ca2+ release function of RyR1 (34), but the RyR1 interaction site is not known. It is possible that amino acids 1487–1506 in the C-terminal end of skeletal DHPR act in conjunction with amino acids in the DHPR III–IV loop.

The region we have identified may be important in the DHPR interaction with RyR1 at the junction between T-tubules and the terminal cisternae of the sarcoplasmic reticulum. In skeletal muscle, but not cardiac muscle, DHPRs are clustered in groups of four and are positioned in exact correspondence with RyR1 tetramers so that each DHPR is located immediately opposite to one of the RyR1 subunits (35, 36). Structural studies of mice with a targeted disruption of the skeletal muscle RyR1 gene have shown that the arrangement of DHPRs into groups of four is dependent on the presence of RyR1 (37). Thus, another possible function for the interaction between
RyR1 (954–1112) and the DHPR III–IV loop that we have identified might be clustering of DHPRs into groups of four in skeletal muscle. The absence of interaction with DHPR that we observed with the RyR2 sequence corresponding to RyR1 (922–1112) would be in line with this proposal (Fig. 2).

Alignment of inositol triphosphate and ryanodine receptor sequences reveals that the Ca²⁺ channel-forming domain and the domains that are important for regulation of channel opening are conserved between inositol triphosphate receptors and RyR. The region that we have identified as an interaction site between RyR1 and both the DHPR II–III and II–III–IV loops is not present in inositol triphosphate receptors (38, 39) and is therefore a domain unique to RyR that might be important for E-C coupling.

Site-directed mutagenesis of residues between Leu⁹⁵⁴ and Asp¹¹¹² that differ between RyR1 and RyR2 is likely to yield information on the amino acids that constitute the interaction site. The characterization of a domain unique to RyR1-(954–1112) would be in line with this proposal (Fig. 2).

The introduction of RyR1/RyR2 chimeras into myoblasts lacking RyR1 will allow characterization of a domain unique to RyR.

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