Residues Leu720-Leu764 within the II-III loop of the skeletal muscle dihydropyridine receptor (DHPR) α1S subunit represent a critical domain for the orthograde excitation-contraction coupling as well as for retrograde DHPR L-current-enhancing coupling with the ryanodine receptor (RyR1). To better understand the molecular mechanism underlying this bidirectional DHPR-RyR1 signaling interaction, we analyzed the critical domain to the single amino acid level. To this end, constructs based on the highly dissimilar housefly DHPR II-III loop in an otherwise skeletal DHPR as an interaction-inert sequence background were expressed in dystrophic (α1S-null) myotubes for simultaneous recordings of depolarization-induced intracellular Ca²⁺ transients (orthograde coupling) and whole-cell Ca²⁺ currents (retrograde coupling). In the minimal skeletal II-III sequence (Asp784-Asp786) required for full bidirectional coupling, eight amino acids heterologous between skeletal and cardiac DHPR were exchanged for the corresponding cardiac residues. Four of these skeletal-specific residues (Ala739, Phe741, Pro742, and Asp744) turned out to be essential for orthograde and two of them (Ala729 and Phe741) for retrograde coupling, indicating that orthograde coupling does not necessarily correlate with retrograde signaling. Secondary structure predictions of the critical domain show that an α-helical (cardiac sequence-type) conformation of a cluster of negatively charged residues (Asp744-Glu751 of α1S) corresponds with significantly reduced Ca²⁺ transients. Conversely, a predicted random coil structure (skeletal sequence-type) seems to be prerequisite for the restoration of the critical domain is an essential determinant of the tissue-specific mode of EC coupling.

Excitation-contraction (EC) coupling in skeletal muscle is understood as a protein-protein or “mechanical” interaction of two distinct Ca²⁺ channels, the voltage-gated L-type Ca²⁺ channel or dihydropyridine receptor (DHPR) and the Ca²⁺ release channel or ryanodine receptor (RyR1) in the sarcoplasmic reticulum (1, 2; reviewed in Refs. 3 and 4). Therefore, skeletal-type EC coupling is independent from the influx of extracellular Ca²⁺ (5–7), in contrast to cardiac EC coupling where Ca²⁺ influx is required to trigger the release of intracellular Ca²⁺ from the sarcoplasmic reticulum stores (8), which in turn activates contraction. In skeletal muscle EC coupling the voltage-sensing DHPR undergoes voltage-driven conformational changes that are allosterically communicated to RyR1 via the cytoplasmic loop connecting the homologous repeats II and III of the pore-forming DHPR α1S subunit (2, 9). The II-III loop is not only important for transmitting this orthograde EC coupling signal to the RyR1, it also receives a retrograde, current-enhancing signal from the RyR1 to the DHPR (10, 11). Beside unequivocally strong indications for an essential role of the II-III loop for this bidirectional coupling mechanism, accumulating evidence suggests an additional influence of other regions of the DHPR α1S subunit and/or of the accessory β subunit (12, 13).

Nevertheless, one sequence portion of the skeletal DHPR α1S II-III loop (Leu720-Leu764) was previously shown to be essential for bidirectional coupling (11, 14, 15). These 45 residues inserted into the corresponding regions of α1S subunit chimeras that contain II-III loops incapable of direct skeletal-type (Ca²⁺-independent) coupling, like the cardiac II-III loop (11, 14) or the highly heterologous II-III loop of the housefly (Musca domestica) DHPR (15), fully restored orthograde and retrograde signaling when expressed in dystrophic (α1S-null) myotubes. Based on the observation that bidirectional coupling was unaffected by drastic alterations of the sequence surrounding skeletal residues Leu720-Leu764 in the chimeric Musca loop, it was concluded (15) that the critical domain Leu720-Leu764 of the skeletal DHPR II-III loop represents a potential site of protein-protein interaction necessary for the functional coupling of DHPR and RyR1, whereas adjacent II-III loop sequences play little or no role.

However, the mechanism by which this domain activates the RyR1 upon depolarization or receives the retrograde signal is still poorly understood. In an effort to elucidate the structural bases of this bidirectional DHPR-RyR1 signaling interaction, we fine-mapped the critical DHPR-RyR1 interaction domain down to the single amino acid level in chimeras with the highly dissimilar Musca II-III loop (15) as a protein-protein interaction-inert neutral sequence background. Our results indicate a striking structure-function correlation between EC coupling properties of the individual chimeras or point mutants and the predicted secondary structure of the interaction domain. Whenever amino acid exchanges resulted in conversion of a predicted random coil structure (skeletal sequence-type) to an α-helical structure (cardiac sequence-type) of a negatively charged
Structure of the DHPR II-III Loop Decisive for EC Coupling

EXPERIMENTAL PROCEDURES

Construction of DHPR Chimeras—The design of the DHPR II-III loop chimeras used in the present study was based on the sequence of chimeras GFP-SkLM and GFP-SkLMS15C16 (15). Briefly, GFP-SkLM is composed of rabbit skeletal muscle DHPR αII subunit sequence (16) except for a II-III loop derived from the housefly body wall muscle DHPR αII subunit(17) (residues Asp734–Ser872). For GFP-SkLMS15, 45 rabbit αII residues (Leu720–Leu764) were introduced into the Musca II-III loop of GFP-SkLM, thus substituting for Musca αII residues Glu724–Thr765. Sequence portions of rabbit skeletal muscle (Sk) 18, or carp white skeletal muscle (K) 19 DHPR αII subunits were introduced into the Musca (M) II-III loop of "L" (GFP-SkLM). Chimeras were N-terminal fused to a modified GFP as described previously (19). Chimeras had amino acid compositions in their II-III loops as follows:

- **GFP-SkLMS15**: Sk sequence (Leu720–Glu876) replaced a portion of the Musca αII sequence of GFP-SkLM (residues Glu724–Gly860). For the succeeding chimeras, a different portion of Musca αII sequence of GFP-SkLM (residues Leu734–Thr755) was exchanged by the following sequence: Sk (Asp734–Asp748), C (Glu880–Leu895); GFP-SkLMC13, Sk (Asp734–Asp748), C (Glu880–Leu895); GFP-SkLMC31, Sk (Asp734–Asp748), C (Glu880–Leu895); GFP-SkLMC31, K (Asp876–Leu895).

Point mutants D347A, A393P, D406E, D421T, P422T, D424E, and E467D were created by SOE-PCR to yield SkLMS15C16 and the corresponding carboxy-terminal residues: D734S: Sk (Asp734) by C (Ser785); A739P: Sk (Ala739) by C (Asp880). The DHPR II-III loop of SkLMS15C16 was cloned into a proprietary mammalian expression vector (20) and were constructed as follows (nucleotide numbers are given in parenthesis, and asterisks indicate restriction enzyme (RE) sites introduced by PCR technique using proofreading Pfu DNA polymerase (Stratagene)).

**GFP-SkLMS15**: The SkM transition site (nt M2245) was introduced by the corresponding carboxy-terminal residues: D734S: Sk (Asp734) by C (Ser785); A739P: Sk (Ala739) by C (Asp880). For each of the subsequent chimeras and point mutants SOE-PCR fragments, which were coligated with the Musca II-III loop of GFP-SkLM, were used as the respective cDNA templates to introduce the M/K transition site (nt M2211/C2593) as well as a ClaI RE site (nt C2985) to generate the corresponding MfeI-ClaI PCR fragment (nt M2024–C2985).

**GFP-SkLMC13**: GFP-SkLMS15 and αII were used as the respective cDNA templates to introduce the M/C transition (nt M2211/C2593) as well as a ClaI RE site (nt C2985) to generate the corresponding MfeI-ClaI PCR fragment (nt M2024–C2985).

**GFP-SkLMC31**: SOE-PCR technique was used to create the C/Sk transition (nt C2622/Sk2230) with GFP-SkLMC13, as the upstream and GFP-SkLMS15C16 as the downstream cDNA template. Hence, the downstream Sk/C transition (nt M2244/C2388) is derived from clone GFP-SkLMC13.

**GFP-SkLMK31**: GFP-SkLM and carp αII cDNA were used as the respective cDNA templates to introduce the M/K transition site (nt M2211/K2339) as well as a ClaI RE site (nt K2339) to generate the MfeI-ClaI PCR fragment (nt M2024–K2339).

**GFP-SkLMS15C16**: Point mutants D734A, A739P, D406E, F421T, P422T, D424E, and E467D were codon-optimised and introduced intracellular Ca2⁺ transients (24). Point mutants D734A, A739P, D406E, F421T, P422T, D424E, and E467D were created by SOE-PCR and used to generate SkLMS15C16 as the downstream cDNA template. The PCR antisense primer was used to introduce these changes into the DHPR construct. Chimeras had amino acid compositions in their II-III loops as follows:

- **GFP-SkLMS15**: Sk sequence (Leu720–Glu876) replaced a portion of the Musca αII sequence of GFP-SkLM (residues Glu724–Gly860). For the succeeding chimeras, a different portion of Musca αII sequence of GFP-SkLM (residues Leu734–Thr755) was exchanged by the following sequence: Sk (Asp734–Asp748), C (Glu880–Leu895); GFP-SkLMC13, Sk (Asp734–Asp748), C (Glu880–Leu895); GFP-SkLMC31, Sk (Asp734–Asp748), C (Glu880–Leu895); GFP-SkLMK31, K (Asp876–Leu895).

- **GFP-SkLMS15**: Sk sequence (Leu720–Glu876) replaced a portion of the Musca αII sequence of GFP-SkLM (residues Glu724–Gly860). For the succeeding chimeras, a different portion of Musca αII sequence of GFP-SkLM (residues Leu734–Thr755) was exchanged by the following sequence: Sk (Asp734–Asp748), C (Glu880–Leu895); GFP-SkLMC13, Sk (Asp734–Asp748), C (Glu880–Leu895); GFP-SkLMC31, Sk (Asp734–Asp748), C (Glu880–Leu895); GFP-SkLMK31, K (Asp876–Leu895).

- **GFP-SkLMS15**: Sk sequence (Leu720–Glu876) replaced a portion of the Musca αII sequence of GFP-SkLM (residues Glu724–Gly860). For the succeeding chimeras, a different portion of Musca αII sequence of GFP-SkLM (residues Leu734–Thr755) was exchanged by the following sequence: Sk (Asp734–Asp748), C (Glu880–Leu895); GFP-SkLMC13, Sk (Asp734–Asp748), C (Glu880–Leu895); GFP-SkLMC31, Sk (Asp734–Asp748), C (Glu880–Leu895); GFP-SkLMK31, K (Asp876–Leu895).

For the construction of subclone pMax, three artificial RE sites were PCR-introduced into the Musca II-III loop of GFP-SkLM to be later used for cassette cloning: AflII* (nt M2171), XbaI* (nt M2177) and ClaI* as the “helper” RE site, and Clal* (nt M2265). In detail, the MfeI XbaI* fragment from the Musca DHPR αII subunit (nt M2024–2177) was coligated with the XbaI* Xhol fragment of GFP-SkLM (nt M2258–2654) into the MfeI Xhol-cleaved GFP-SkLM cDNA. The PCR antisense primer was used to introduce together with the XbaI* ClaI* helper RE site (nt M2177) an upstream AflII* site (nt M2171). Similarly, the sense primer introduced additional ClaI* RE site (nt M2265) downstream of the XbaI* (nt M2258) helper site.

**GFP-SkLMS15**: SOE-PCR was used to generate the M/Sk transition site (nt M2211/Sk2200) with GFP-SkLM as the upstream and GFP-SkLMS15C16 as the downstream cDNA template. The PCR antisense primer was used to introduce together with the XbaI* ClaI* helper RE site (nt M2177) an upstream AflII* site (nt M2171). Similarly, the sense primer introduced additional ClaI* RE site (nt M2265) downstream of the XbaI* (nt M2258) helper site.

**GFP-SkLMS15**: SOE-PCR was used to generate the M/Sk transition site (nt M2211/Sk2200) with GFP-SkLM as the upstream and GFP-SkLMS15C16 as the downstream cDNA template. The PCR antisense primer was used to introduce together with the XbaI* ClaI* helper RE site (nt M2177) an upstream AflII* site (nt M2171). Similarly, the sense primer introduced additional ClaI* RE site (nt M2265) downstream of the XbaI* (nt M2258) helper site.

**GFP-SkLMS15**: SOE-PCR was used to generate the M/Sk transition site (nt M2211/Sk2200), where the corresponding carboxy-terminal residues were controlled by pClamp software (version 7.0, Axon Instruments). Test pulses were preceded by a 1-s prepulse to −30 mV to inactivate endogenous T-type Ca2⁺ channels (25). Currents were determined with 200-ms depolarizing steps from a holding potential of −80 mV to test potentials between −50 and 80 mV in 10-mV increments. Leak currents were digitally subtracted by a PD3 pulse protocol. Recordings were low-pass Bessel-filtered at 2 kHz and sampled at 5 kHz. As shown in Equation 1, peak currents were normalized to total cell capacitance (pa/pF), plotted as a function of test potential, and fitted according to

\[ I = I_{\text{max}} \left[ V - V_{1/2} \right] \cdot \exp \left[ -1 \times \left( V - V_{\text{rev}} \right) / k \right] \]  
\[ V \text{max} \text{ is the maximal L-channel conductance of the cell, } V_{1/2} \text{ is the potential for half-maximal activation of } V_{\text{max}}, \text{ and } k \text{ is a slope factor. Only currents with a maximal voltage error } < 0.1 \text{ mV attributable to series resistance were analyzed. } \]
RESULTS

Skeletal Muscle DHPR II-III Loop Residues Asp\(^{734}\)-Leu\(^{764}\) Are Required for the Bidirectional Signaling Interaction with RyR1—In previous studies, residues Leu\(^{720}\)-Leu\(^{764}\) of the rabbit skeletal muscle DHPR \(\alpha_{1S}\) II-III loop were demonstrated to be essential for the full restoration of skeletal-type EC coupling (11, 12, 14, 15) as well as for retrograde coupling, which is the RyR1-mediated enhancement of Ca\(^{2+}\) influx through the DHPR (11, 12, 15). To better understand the molecular mechanism underlying the direct skeletal DHPR-RyR1 interaction, we identified the minimum sequence within \(\alpha_{1S}\) residues Leu\(^{720}\)-Leu\(^{764}\) required for the bidirectional coupling. Because chimera GFP-SkLMS\(_{45}\) (Fig. 1A) had coupling properties comparable with wild-type GFP-\(\alpha_{1S}\) (15), it was used as the reference for skeletal-type EC coupling and enhancement of Ca\(^{2+}\) currents. On the other hand, chimera GFP-SkLM (Fig. 1B), which contains the entire Musca DHPR II-III loop, was used as the reference for constructs not supporting orthograde or retrograde signaling (15). First, we further restricted the bidirectional signaling domain by generating two daughter chimeras of chimera GFP-SkLMS\(_{45}\): SkLMS\(_{30}\) containing the upstream sequence Leu\(^{720}\)-Glu\(^{749}\) and SkLMS\(_{31}\) containing the downstream Asp\(^{750}\)-Leu\(^{780}\) portion of the S\(_{31}\) insert with a 15-amino acid overlap (Fig. 1B). Simultaneous recordings of depolarization-induced intracellular Ca\(^{2+}\) transients and whole-cell Ca\(^{2+}\) currents from GFP-fluorescing cells revealed that the Ca\(^{2+}\) current densities as well as the amplitude and voltage dependence of Ca\(^{2+}\) transients of SkLMS\(_{31}\) were similar to those of SkLMS\(_{45}\), whereas the values of SkLMS\(_{30}\) were similar to SkLM (Fig. 1, C and D). As previously described (15), the small Ca\(^{2+}\) transients of SkLM and hence those of SkLMS\(_{30}\) appeared to be a direct consequence of Ca\(^{2+}\) influx through the DHPR. In contrast, SkLMS\(_{31}\) fully restored both orthograde and retrograde coupling with RyR1.

Fine-mapping of the Skeletal DHPR II-III Loop Sequence Asp\(^{734}\)-Leu\(^{764}\) Important for Bidirectional Coupling—To identify individual amino acids of the skeletal muscle DHPR II-III loop essential for bidirectional signaling, sequence portions within the S\(_{31}\) insert of chimera SkLMS\(_{31}\) were exchanged for the corresponding rabbit cardiac (18) or for the evolutionary distant carp skeletal (19) DHPR sequences (Fig. 2A). The latter diverges from the S\(_{31}\) insert in six amino acids; nonetheless, bidirectional coupling was fully restored by the carp sequence portion Asp\(^{759}\)-Leu\(^{780}\) in chimera SkLMC\(_{31}\) (Fig. 2B). Next, we analyzed the similarities and differences between the S\(_{31}\) insert and the corresponding cardiac sequence Ser\(^{765}\)-Leu\(^{805}\). As depicted in Fig. 2A, the two \(\alpha_{1}\) subunits differ in 13 of the 31 residues, 5 of which are conservative Asp/Glu and Lys/Asp exchanges. In both sequences Asp and Glu residues form a central cluster of 7 negative charges. Among the total of 13 amino acid exchanges, 10 are positioned in the N-terminal half, and only 3 are located in the C-terminal half. Neither skeletal-type EC coupling nor Ca\(^{2+}\) current enhancement was restored when the entire 31-residue sequence was cardiac. Chimera SkLMC\(_{31}\) yielded small Ca\(^{2+}\) currents and marginal Ca\(^{2+}\) transients similar to SkLM (Fig. 2B). Interestingly, when only the C-terminal half of the 31-amino acid insert Glu\(^{806}\)-Leu\(^{865}\) was a Boltzmann distribution as shown in Equation 2

\[
A = A_{\text{max}}/[1 + \exp\left(-\frac{(V - V_{1/2})}{k}\right)]
\]

(Eq. 2)

where \(A\) is \(G\), \(\Delta F/F\), or \(Q\) and \(A_{\text{max}}\) is \(G_{\text{max}}\), \(\Delta F/F_{\text{max}}\), or \(Q_{\text{max}}\). \(V_{1/2}\) is the potential at which \(A = A_{\text{max}}/2\), and \(k\) is a slope factor. Recordings were performed at room temperature (~23 °C), and data are presented as mean ± S.E. Statistical significance was determined by unpaired Student’s t test. Data were analyzed using Clampfit® 8.0 (Axon Instruments, Foster City, CA) and SigmaPlot® 6.0 (SPSS Science, Chicago, IL) software.

**FIG. 1.** Chimera GFP-SkLMS\(_{31}\) but not GFP-SkLMS\(_{30}\) restores bidirectional coupling in dysgenic myotubes. A, transmembrane model of the GFP-tagged skeletal/Musca II–III loop DHPR chimera GFP-SkLMS\(_{45}\) (15). I–IV, homologous repeats of \(\alpha_{1}\) subunits. B, II–III loops of the two reference chimeras, GFP-SkLM and GFP-SkLMS\(_{45}\) (15), and of the daughter chimeras, GFP-SkLMS\(_{30}\) and GFP-SkLMS\(_{31}\). Rabbit skeletal muscle \(\alpha_{1S}\) sequence, gray; Musca domestica (housefly) muscle (\(\alpha_{1M}\)) sequence, black. C, depolarization-induced intracellular Ca\(^{2+}\) transients (upper) and whole-cell Ca\(^{2+}\) currents (lower) recorded simultaneously from dysgenic myotubes expressing SkLMS\(_{30}\) or SkLMS\(_{31}\). The holding potential was −80 mV; 200-ms test pulses to potentials between −50 and +80 mV were applied in 10-mV increments. The upper vertical scale indicates \(\Delta F/F\), Ca\(^{2+}\)-induced Fluor-4 fluorescence increments (\(\Delta F\)) with respect to basal fluorescence (F). D, voltage dependence of depolarization-induced Ca\(^{2+}\) transients (\(\Delta F/F\), upper) and of peak current densities (\(pA/pF\), lower) recorded from dysgenic myotubes expressing SkLMS\(_{30}\) (○), SkLM (○), SkLMS\(_{31}\) (●), and SkLMS\(_{30}\) (▲). Values represent the mean ± S.E. of 7–35 recordings.
cardiac sequence (chimera SkLMS$_{15C_{16}}$) full bidirectional coupling was restored. Thus, the N-terminal half (Asp$^{734}$-Asp$^{748}$) of the S$_{15}$ insert was sufficient to restore both orthograde and retrograde signaling with RyR1, regardless of whether cardiac or skeletal sequence was positioned downstream. This was not surprising because this downstream sequence is highly conserved between the two isoforms. By contrast, if the highly surprising because this downstream sequence is highly conserved, the cluster of negatively charged residues was positioned downstream. This was not retrograde signaling with RyR1, regardless of whether cardiac or skeletal sequence was located downstream of the S$_{15}$ signaling domain cassette and impact on bidirectional coupling. Musca $\alpha_{1C}$ sequence, black; rabbit $\alpha_{1C}$, blue; rabbit $\alpha_{1C}$, red; carp $\alpha_{15C_{16}}$, green. Residues of the central negatively charged amino acid cluster are indicated by an encircled minus sign. Chou-Fasman secondary structure predictions (p-turn probability, 0.75 $\times$ 10$^{-2}$) are represented underneath the sequence by loops for $\alpha$-helical, a dashed line for random coil, and a zig-zag line for $\beta$-sheet conformation. Bar graphs indicate the mean $\pm$ S.E. of the maximal depolarization-induced intracellular Ca$^{2+}$ transients ($\Delta F/F_{\text{max}}$) and were normalized to the reference chimera SkLMS$_{45}$. High values like those of SkLMS$_{45}$ and SkLMS$_{31}$ represent skeletal-type EC coupling. The mean $\pm$ S.E. of the maximal whole-cell Ca$^{2+}$ conductance $G_{\text{max}}$ is indicated at right; number of recordings in parentheses. ** indicates high significant difference (p < 0.001) compared with chimera SkLMS$_{45}$, * indicates that chimera GFP-SkLCS$_{18}$ had been analyzed for bidirectional coupling in a previous study (11) and is listed here for comparison.

To exclude the possibility that the reduction of EC coupling and of Ca$^{2+}$ currents observed for chimeras SkLMC$_{31}$ and SkLMCSC resulted from reduced membrane expression, we recorded immobilization-resistant charge movements (gating currents) as an accurate measure of expression density of voltage-gated ion channels in the surface membrane. The maximum charge movements ($Q_{\text{max}}$) obtained from SkLMC$_{31}$ and SkLMCSC were compared with that of SkLMS$_{15C_{16}}$, which fully restored bidirectional coupling. Regardless of the ability to restore bidirectional coupling or not, $Q_{\text{max}}$ values of chimeras SkLMS$_{15C_{16}}$ (5.8 $\pm$ 0.6 nC/\mu F, n = 15), SkLMC$_{31}$ (6.1 $\pm$ 0.8 nC/\mu F, n = 10), and SkLMCSC (5.6 $\pm$ 0.6 nC/\mu F, n = 15) were statistically indistinguishable (p > 0.7). $Q_{\text{max}}$ values of chimeras SkLM, SkLMS$_{31}$, and SkLCS$_{18}$ had been determined in previous studies (11, 15) and are highly comparable with our recent data. Thus, the reduction of Ca$^{2+}$ transients and Ca$^{2+}$ currents of our II-III loop chimeras cannot be simply explained by reduced membrane expression levels but rather by an impairment of bidirectional coupling.

**Fig. 2. Restoration of bidirectional coupling correlates with the predicted random coil structure of the DHPR-RyR1 interaction domain.** A, alignment of the II-III loop sequence portions corresponding to the S$_{15}$ signaling domain cassette of chimera SkLMS$_{31}$ from Musca muscle ($\alpha_{13C}$), rabbit cardiac ($\alpha_{1C}$), rabbit skeletal ($\alpha_{1C}$), and carp skeletal ($\alpha_{15C_{16}}$) DHPRs. Identical amino acids are boxed in gray and black. A cluster of negatively charged residues present in all sequences except for Musca $\alpha_{13C}$ is indicated. B, sequence recombination corresponding to the S$_{15}$ signaling domain cassette and impact on bidirectional coupling. Musca $\alpha_{13C}$ sequence, black; rabbit $\alpha_{13C}$, blue; rabbit $\alpha_{1C}$, red; carp $\alpha_{15C_{16}}$, green. Residues of the central negatively charged amino acid cluster are indicated by an encircled minus sign. Chou-Fasman secondary structure predictions (p-turn probability, 0.75 $\times$ 10$^{-2}$) are represented underneath the sequence by loops for $\alpha$-helical, a dashed line for random coil, and a zig-zag line for $\beta$-sheet conformation. Bar graphs indicate the mean $\pm$ S.E. of the maximal depolarization-induced intracellular Ca$^{2+}$ transients ($\Delta F/F_{\text{max}}$) and were normalized to the reference chimera SkLMS$_{45}$. High values like those of SkLMS$_{45}$ and SkLMS$_{31}$ represent skeletal-type EC coupling. The mean $\pm$ S.E. of the maximal whole-cell Ca$^{2+}$ conductance $G_{\text{max}}$ is indicated at right; number of recordings in parentheses. ** indicates high significant difference (p < 0.001) compared with chimera SkLMS$_{45}$, * indicates that chimera GFP-SkLCS$_{18}$ had been analyzed for bidirectional coupling in a previous study (11) and is listed here for comparison.

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Up to this point the results suggested that the 15 skeletal residues Asp734-Asp748 cannot be further reduced without significantly impairing bidirectional signaling. Downstream of this skeletal sequence the adjacent 16-residue sequence is also essential for bidirectional coupling. However, this sequence is highly homologous between skeletal and cardiac DHPR and could be of either origin as long as the upstream domain was skeletal.

The Predicted Secondary Structure of the Negatively Charged Cluster Correlates with Bidirectional Coupling Properties—Beyond the search for a putative interaction motif determined by the primary structure, we also considered the primary structure-dependent differences in the secondary structure of the S31 II-III loop region. The secondary structure of this domain predicted by Chou and Fasman (27) revealed a striking structure-function correlation in our chimeras. According to this algorithm (and others; not shown) the skeletal sequence (rabbit and carp) forms a random coil in all but the very last residues, which are α-helical. In the cardiac channel the centrally located negatively charged cluster is predicted to form an α-helix. This sequence contains a conserved proline (in position Pro750 corresponding to Pro881 of the cardiac sequence), a residue that is commonly considered to be a helix breaker. However, because a large number of α-helical domains in membrane and globular proteins contain prolines (28), it is not necessary to assume that the predicted α-helix terminates at Pro881. In the Musca channel, which lacks the negatively charged cluster, a short β-sheet exists in the corresponding region. Most interestingly, in all chimeras in which the central negative amino acid cluster was lacking, as in the Musca II-III loop sequence of chimera SkLM, or was predicted as an α-helix, as in chimeras SkLMS31, SkLMS30, SkLCS16, and SkLSCSC (Fig. 2B), bidirectional signaling was significantly reduced (p < 0.001) compared with SkLMS31 or SkLMS15C16. In contrast, when this negatively charged amino acid cluster was predicted to form a random coil, as in chimeras SkLMS31, SkLMK31, and SkLMS15C16, depolarization-induced intracellular Ca2+ transients and Ca2+ currents were statistically indistinguishable (p > 0.05) from those of SkLMS45 and hence from wild-type GFP-SkLMS15C16 (15) in which the entire critical domain or the entire II-III loop was skeletal, respectively. Thus, secondary structure appears to be an important determinant of the tissue-specific mode of DHPR-RyR interaction.

Four Individual Skeletal DHPR Residues Are Essential for Skeletal-type EC Coupling—To test whether this structure-function correlation could also be observed on the level of point mutants, we performed skeletal to cardiac amino acid exchanges in the S31 insert of chimera SkLMS15C16 and compared the effect of these substitutions on secondary structure and function. First we established that SkLMS15C16 had full skeletal coupling properties by comparing its intracellular Ca2+ release properties and characteristics of Ca2+ current enhancement with those of SkLMS45, which has previously been demonstrated to resemble wild-type α1s in EC and retrograde coupling (15). SkLMS45 and SkLMS15C16 not only had identical (ΔF/F)max and Gmax values (Fig. 2B) but were also statistically indistinguishable (p > 0.05) in terms of voltage-dependence of intracellular Ca2+ transients and of the whole-cell inward currents (Fig. 3). Consequently, chimera SkLMS15C16 fully restored bidirectional coupling.

Among the 15 residues of the S31 insert (Asp734-Asp748) required for full restoration of bidirectional coupling in chimera SkLMS15C16, 10 residues were different between the skeletal and cardiac DHPR sequences (Fig. 4A). Two of these 10 amino acids (Ser738 and Asp746) could be omitted from this analysis because they are already mutated in the corresponding carp sequence of chimera SkLMK31 to Pro754 and Val764, respectively, without any significant reduction of bidirectional coupling (Fig. 2). The presence of Val764 within the negatively charged amino acid cluster of the carp sequence is of particular interest because this resulted in the loss of one negative charge without any significant impact on skeletal-type EC coupling (Fig. 2).

The comparison of intracellular Ca2+ release of each one of the remaining eight single amino acid substitutions with the reference chimera SkLMS15C16 showed that the exchange of the four skeletal residues, Ala739, Phe741, Pro742, and Asp744, to their cardiac counterparts significantly (p < 0.01) reduced skeletal-type EC coupling (Fig. 4). Therefore, these four skeletal amino acids (Fig. 4A, boxed) are essential for skeletal-type EC coupling. Three of the eight point mutants were conservative Asp/Glu exchanges. Even though two of them are adjacent to each other (D744E and D745E), the impact of these substitutions on orthograde signaling differed considerably. Point mutant D744E with a predicted α-helix in the central negatively charged amino acid cluster showed significantly reduced Ca2+-transients. In contrast, mutations D745E as well as D740E, which had no impact on the random coil structure, restored skeletal-type EC coupling.
Fig. 4. Four individual skeletal DHPR residues are essential for skeletal-type EC coupling. A, alignment of the II-III loop sequence portions of rabbit skeletal (α1S, blue) and cardiac (α1C, red) sequences corresponding to the 31-amino acid insert of chimera GFP-SkLMS15C16. Identical amino acids are indicated by asterisks and conservative Asp/Glu exchanges by dots. B, sequence of chimera SkLMS15C16 and point mutants thereof. Color code identical to panel A. Indication of negatively charged residues of the central amino acid cluster and symbols for random coil, α-helix, β-sheet as described in Fig. 2B. Bar graphs indicate the mean ± S.E. of the maximal depolarization-induced intracellular Ca2⁺ transients (ΔF/ΔFmax) normalized to the reference chimera SkLMS15C16, which showed full skeletal-type EC coupling. The corresponding values of Gmax, the maximal whole-cell Ca2⁺ conductance, are indicated on the right (mean ± S.E.) for two separate sets of experiments conducted at different time periods (Group A and Group B); number of recordings in parentheses. **, p < 0.001; *, p < 0.01; #, p < 0.05; and no symbol, lack of significance compared with SkLMS15C16.

Next we examined whether this structure-function correlation is also valid for the point mutants A739P, F741T, and P742T, which also failed to restore full skeletal-type EC coupling like point mutant D744E. In fact, F741T, which showed the strongest reduction of Ca2⁺ transients, and P742T, which showed a more moderate reduction of orthograde signaling, have a central α-helix and thus clearly support this hypothesis. Only in A739P was the impairment of orthograde signaling not correlated to an α-helix, thus appearing to be an exception to this general rule. However, in this case not the presence of an α-helical structure but an additional bend introduced by the proline might weaken the signaling interaction of the DHPR with RyR1. On the other hand, in SkLMK31, a proline occurs in the adjacent position (corresponding to the rabbit skeletal Ser738) but had no adverse effect on EC coupling. Thus, our present study, a significant reduction in orthograde signaling correlated with a significant reduction in retrograde signaling (10, 11, 15, and see Fig. 2). This strict correlation was not observed with all SkLMS15C16 point mutants. P742T and D744E showed significantly impaired skeletal-type EC coupling but revealed Ca2⁺ current enhancement comparable with SkLMS15C16 (Fig. 4B). Taken together, our data obtained by the analysis of single amino acid substitutions reveal that among the four skeletal residues (Ala739, Phe741, Pro742, and Asp744) essential for skeletal-type EC coupling, two (Ala739 and Phe741) are also essential for the reception of retrograde signaling. Thus our results indicate that some individual amino acids are involved in an unidirectional interaction with RyR1. Moreover, the correlation between the secondary structure and the mode of DHPR-RyR1 interaction was also valid for single amino acid substitutions.

**DISCUSSION**

The results reported here characterize the minimal 31-amino acid sequence in the II-III loop of the α1S subunit that supports skeletal muscle EC coupling and the RyR1-dependent Ca2⁺ current amplification. This sequence contains three structural characteristics important for the bidirectional interaction of the α1 subunit and the RyR1: 1) a motif of four essential skeletal residues in the N-terminal half of the 31-amino acid sequence, 2) a cluster of negatively charged residues in the center of this sequence, and 3) the secondary structure of this negative amino acid cluster. Because this critical EC coupling sequence was analyzed in the heterologous Musca II-III loop inserted in the α1S background, this study does not address the possible contributions of additional sites involved in the reciprocal interaction with the RyR1. In fact, a recent study shows that, whereas deletion of amino acids 720–765 abolished EC coupling, a construct that restored 17% of control skeletal-type Ca2⁺ transients (12) after the II-III loop sequence 765–780 was inserted in addition to the 720–765 sequence. Moreover, resto-
ration of retrograde coupling originally assessed in dyspedic (RyR1-null) myotubes by expression of recombinant RyR1 seems to be always larger (~5-fold; Refs. 10 and 29) than that observed with II-III loop chimeras in dysgenic myotubes (~2-fold; Refs. 11, 15, and this work). Finally, transferring the αs15 II-III loop sequence 720–764 into the heterologous low voltage-activated Ca2+ channel αs11 was not sufficient to restore skeletal EC coupling (30). Together, these and other studies (9, 13, 31) suggest that parts of αs15 outside the II-III loop and/or auxiliary DHPR subunits also participate directly or indirectly in the specific interaction with the RyR1. However, within the II-III loop the 31-amino acids (734–764) in which the upstream 15 amino acids need to be skeletal but the downstream 16 residues can be of either skeletal or cardiac origin is the only critical sequence, and each of the three structural characteristics mentioned above are decisive determinants for skeletal muscle EC coupling.

Single amino acid substitution showed that in this sequence only four of the residues heterologous in αs15 and αc1c were essential for skeletal muscle EC coupling. Other residues may also contribute to the signaling but not in the tissue-specific manner, because their replacement for the cardiac counterparts did not significantly affect EC coupling. The importance of one of these essential residues, Asp744, has already been suggested by previous work. An insert of 18 skeletal residues, PheE255-ProT425, including three of the four essential skeletal residues but not Asp744, inserted in either αc1c (CSk58; Ref. 14) or in the cardiac II-III loop within αs15 (SkLCS16; Ref. 11 and Fig. 2B) resulted in only marginal restoration of EC coupling. Interestingly, of the four residues essential for skeletal EC coupling only two are also essential for the retrograde interaction of the RyR1 with the αs1 subunit. Thus, the two signaling mechanisms utilize overlapping but not identical II-III loop sequences. A similar unidirectional coupling has been observed in studies of RyR1/RyR2 chimeras expressed in dyspedic myotubes. Nakai et al. (32) described a region in the RyR1 (R9) that was solely responsible for retrograde coupling, whereas an upstream region was required for signaling in both directions. If multiple domains of the RyR1 and the DHPR cooperate in the signaling (32–34), a possible explanation for the divergence of orthograde and retrograde coupling in the point mutants P742T and D744E could be that conformational changes induced by these substitutions impaired their orthograde signaling, whereas the physical interaction for the reception of retrograde signaling was still intact.

The second characteristic of the critical 31-amino acid sequence is the central cluster of negatively charged amino acids. This cluster is found in all L-type Ca2+ channels of vertebrates but not in that of Musca; its presence is necessary but not sufficient for skeletal muscle EC coupling. Exchanges of an aspartic acid (AspT425) in this cluster for either a glutamic acid in zebrafish2 or an uncharged valine in carp (19) had no adverse effect on skeletal EC coupling. On the other hand, the mere presence of this negatively charged amino acid cluster is not sufficient for EC coupling if its secondary structure is predicted to be α-helical, as in the case of the cardiac sequence. Thus, the secondary structure of this cluster of negatively charged residues appears to be a third determinant of skeletal muscle EC coupling.

This hypothesis was supported by a striking correlation of the secondary structure of all analyzed chimeras and point mutants predicted with the Chou-Fasman algorithm (27) and their EC coupling properties. Whenever exchanges of sequence portions or single amino acids within the DHPR II-III loop interaction domain induced an α-helical conformation of the central negatively charged amino acid cluster, signaling interaction with RyR1 was impeded or at least significantly reduced. Conversely, a secondary structure predicted as random coil seemed to be a prerequisite for skeletal-type EC coupling. Whereas empirical statistical methods of secondary structure predictions are of limited predictive value, the Chou-Fasman secondary structure prediction is conceptually the simplest model and the most widely used (35). However, their predictions need to be tested with more experimental structural methods. The random coil structure of this region in the skeletal DHPR II-III loop was recently confirmed by NMR and CD (36); however, NMR analysis of the corresponding cardiac region is still elusive. If our structure predictions are correct, it is likely that not only the presence or absence of individual residues but also the consequences of such substitutions on the secondary structure of the adjacent negatively charged cluster determine the tissue-specific mode of interaction between the DHPR and the RyR1.

Taken together, the analyses of primary and secondary structures of the minimal essential EC coupling domain in the II-III loop of αs15 allows drafting of the following alternative models of II-III loop-RyR1 interactions (Fig. 5). First, the motif of critical residues may specifically interact with a corresponding sequence of RyR1 and the adjacent amino acids, including the negatively charged cluster, are important in that they enable this interaction. Any changes of the four critical amino acids into their cardiac homologues abolish the specific interaction with the RyR1 because those changes lead to an α-helical structure of the negative cluster that now masks the specific interaction site (Fig. 5A). Alternatively, not the motif of

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A GenBank™ accession number AY495688.
specific residues but the cluster of negatively charged amino acids may be the site of interaction with the RyR1 (Fig. 5B). In this case the interaction is likely to be electrostatic attraction or repulsion, and the adjacent critical residues determine the secondary structure and consequently the function of the interaction site. In either case, our present findings demonstrate that both the primary and the secondary structures of the participating sequences of the α1 subunit and the RyR1 need to be considered to understand the protein-protein interactions involved in skeletal muscle EC coupling.

Acknowledgments—We thank D. Kohler for experimental help and Dr. H. Glossmann for continuous support.

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Structural Requirements of the Dihydropyridine Receptor α₁₅ II-III Loop for Skeletal-type Excitation-Contraction Coupling
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J. Biol. Chem. 2004, 279:4721-4728.
doi: 10.1074/jbc.M307538200 originally published online November 18, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M307538200

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