The skeletal muscle dihydropyridine receptor $\alpha_{1S}$ subunit plays a key role in skeletal muscle excitation-contraction coupling by sensing membrane voltage changes and then triggering intracellular calcium release. The cytoplasmic loops connecting four homologous $\alpha_{1S}$ structural domains have diverse functions, but their structural arrangement is poorly understood. Here, we used a novel FRET-based method to characterize the relative proximity of these intracellular loops in $\alpha_{1S}$ subunits expressed in intact cells. In dysgenic myotubes, energy transfer was observed from an N-terminal-fused YFP to a FRET acceptor, ReAsH (resorufin arsenical hairpin binder), targeted to each $\alpha_{1S}$ intracellular loop, with the highest FRET efficiencies measured to the $\alpha_{1S}$ II-III loop and C-terminal tail. However, in HEK-293T cells, FRET efficiencies from the $\alpha_{1S}$ N terminus to the II-III and III-IV loops and the C-terminal tail were significantly lower, thus suggesting that these loop structures are influenced by the cellular microenvironment. The addition of the $\beta_{1a}$ dihydropyridine receptor subunit enhanced FRET to the II-III loop, thus indicating that $\beta_{1a}$ binding directly affects II-III loop conformation. This specific structural change required the C-terminal 36 amino acids of $\beta_{1a}$, which are essential to support EC coupling. Direct FRET measurements between $\alpha_{1S}$ and $\beta_{1a}$ confirmed that both wild type and truncated $\beta_{1a}$ bind similarly to $\alpha_{1S}$. These results provide new insights into the role of muscle-specific proteins on the structural arrangement of $\alpha_{1S}$ intracellular loops and point to a new conformational effect of the $\beta_{1a}$ subunit in supporting skeletal muscle excitation-contraction coupling.

In skeletal muscle excitation contraction (EC) coupling, the dihydropyridine receptor (DHPR), an L-type voltage-gated Ca$^{2+}$ channel, senses membrane depolarization and then initiates intracellular Ca$^{2+}$ release by activating the type 1 ryanodine receptor (RyR1) embedded in the sarcoplasmic reticum membrane. Of the five skeletal DHPR subunits, $\alpha_{1S}$ and $\beta_{1a}$ are absolutely required for skeletal type EC coupling (1–8). The 170-kDa $\alpha_{1S}$ subunit contains both the voltage sensor and Ca$^{2+}$ conduction pore and is composed of four homologous domains, each containing six transmembrane segments (9, 10). These domains are connected by intracellular loops with well-defined functions. For example, the I-II loop has an $\alpha_{1S}$ subunit interaction domain binding site for the $\beta_{1a}$ DHPR subunit (11, 12), whereas the II-III loop contains an essential determinant (amino acids 720–765) required to activate RyR1 during skeletal-type EC coupling (13, 14). Although the III-IV loop does not appear to have a direct role in RyR1 activation, a malignant hyperthermia mutation located in this loop (R1086H) has been reported to alter DHPR gating properties and EC coupling (15). Finally, the C-terminal tail has been implicated as binding Ca$^{2+}$/calmodulin (16) as well as mediating proper DHPR targeting to triad junctions (17). Although a recent high resolution cryo-EM reconstruction of the DHPR has resolved many of the membrane-spanning helices in the complex (18), these key $\alpha_{1S}$ intracellular loops are completely absent from the structure, most likely due to intrinsic flexibility. Thus, understanding the relative arrangement of these loops as well as how they change conformation due to binding of cell-specific factors remain as key questions.

Like the $\alpha_{1S}$ subunit, the $\beta_{1a}$ DHPR subunit also plays an essential role in skeletal muscle EC coupling. $\beta_{1a}$ is required to target $\alpha_{1S}$ to the cell surface and to support depolarization-induced intracellular Ca$^{2+}$ release (i.e. orthograde signaling) as well as to enhance DHPR inward Ca$^{2+}$ current (i.e. retrograde signaling) (2, 5, 19). $\beta_{1a}$ is also needed to assemble the DHPR complex into tetradic arrays visualized in freeze-fracture studies (20–22). An essential determinant between residues 489–503 of the $\beta_{1a}$ C-terminal tail is required for both bidirectional signaling and tetrad formation (22, 23).

Recent structural analysis of the DHPR intracellular loops has been achieved using fluorescence resonance energy transfer (FRET) measurements between cyan and yellow fluorescent proteins (CFP/YFP) fused into $\alpha_{1S}$ and $\beta_{1a}$ (24, 25). These studies have suggested a direct role of RyR1 in the structural organization of the $\alpha_{1S}$ subunit and have identified potential RyR-
interacting domains in $\alpha_{1S}$ and $\beta_{1a}$. Although these studies illustrate the potential of FRET-based approaches for structural analysis of the DHPR complex in situ, they have been hampered by the relative bulkiness of the fused CFP/YFP FRET probes, as suggested by functional impairments of $\alpha_{1S}$ subunits carrying a fluorescent protein fused into the III-IV loop (25). In addition, FRET data derived exclusively from fluorescent protein insertions can be difficult to interpret due to the distance between their chromophoric centers and their insertion sites (26) as well as the bulkiness of these inserted proteins.

In this report we used an innovative site-specific labeling method combined with FRET-based structural measurements to determine the spatial interrelationships between cytoplasmic loops of $\alpha_{1S}$ subunits expressed in cultured myotubes harboring the $\alpha_{1S}$ muscular dysgenesis mutation ($\text{mdg}$) (6, 7), which renders these dysgenic myotubes completely deficient in native $\alpha_{1S}$ expression (6). Energy transfer was measured from YFP fused to the N terminus of $\alpha_{1S}$ to the cell-permeant resorufin arsenical hairpin repressor (27) targeted to short tryptophan (Tc) motifs inserted into each of the $\alpha_{1S}$ intracellular loops. Our results suggest that within the triadic environment, the $\alpha_{1S}$ loops are clustered together, with the II–III loop and C terminus being closest to the $\alpha_{1S}$ N terminus. However, in a non-myogenic system (HEK-293T cells), FRET to the II–III and III–IV loops and the C-terminal tail is significantly lower, thus suggesting that cellular microenvironment influences $\alpha_{1S}$ structure. Finally, we found that binding of the $\beta_{1a}$ subunit affects the structure of the $\alpha_{1S}$ II–III loop in situ. This study reveals the influence of muscle-specific environmental factors on the arrangement of the $\alpha_{1S}$ cytosolic loops and provides the first direct evidence that the $\beta_{1a}$ subunit modulates the $\alpha_{1S}$ II–III loop conformation.

**Experimental Procedures**

$\alpha_{1S}$ DHPR cDNA Cloning—A full-length rabbit $\alpha_{1S}$ cDNA (amino acids 1–1873) (GenBank™ accession number M23919) was cloned in-frame downstream from the YFP-citrine gene (28) in the bicistronic retroviral vector pCMMP-MCS-1RES-Puro, containing a puromycin resistance gene (Addgene 36952; Ref. 29). Insertion of a Tc tag-encoding DNA fragment into FLNCCPGCCMEP (Addgene 36952; Ref. 29). Insertion of a Tc tag-encoding DNA fragment into MCS-IRES-Puro, containing a puromycin resistance gene (28) was cloned in-frame downstream from the YFP-citrine gene (29). Insertion of a Tc tag-encoding DNA fragment into the pCold-II expression vector (Takara™) to optimize protein folding. Protein induction and purification were performed using isopropyl 1-thio-β-D-galactopyranoside/arabinose/tetracycline and Strep-trap affinity chromatography columns as described (30).

Cell Culture and Immunocytochemistry—cDNAs were expressed in dysgenic myotubes after packaging into lentivirus using a set of three packaging vectors as described (22). Dysgenic myotubes were infected with lentiviral particles containing YFP-$\alpha_{1S}$ cDNAs at a multiplicity of infection = 0.5 and then selected with 1.5 $\mu$g/ml puromycin for 2 weeks. Individual myoblast colonies stably transduced with each clone were then isolated using glass rings.

DHPR-expressing myoblasts were grown and differentiated in 96-well plates as described (22). $\alpha_{1S}$ DHPR expression was confirmed by immunocytochemistry on methanol-fixed cultured myotubes using either anti-$\alpha_{1S}$ MA3–921 (Thermo Scientific) or anti-GFP G10362 (Life Technologies) monoclonal antibodies.

HEK-293T cells were grown and polyethyleneimine-transfected with Tc-tagged YFP-$\alpha_{1S}$ cDNAs as described (31). Two days after transfection, cells were tested in FRET-based assays.

Calcium Imaging—After differentiation for 4–5 days, myotubes expressing YFP-$\alpha_{1S}$ cDNAs were loaded with 5 $\mu$M Fura-2 AM (Molecular Probes) in imaging buffer (125 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1.2 mM MgSO$_4$, 6 mM glucose, and 25 mM HEPES/Tris, pH 7.4). Membrane depolarization was performed by a 5-s perfusion with 5–7 volumes of imaging buffer containing increasing concentrations of KCl. Cells were imaged with an intensified 10-bit digital CCD camera (XR-Mega-10, Stanford Photonics, Stanford, CA) using a DG4 multiwave-length light source (Sutter Instruments, Novato, CA). Fluorescent emission at 510 nm was captured from regions of interest within each myotube at 33 frames per second using Pioneer acquisition software (Stanford Photonics) and expressed as ratio of signal collected at alternating 340/380-nm excitation wave-lengths. Calcium transients quantified from the peak amplitudes were plotted as a function of added KCl and fit to a sigmoidal dose-response function (variable slope) to determine EC$_{50}$ values, which were then compared via analysis of variance.

Labeling with FRET Acceptors—ReAsH labeling of Tc-tagged $\alpha_{1S}$-expressing intact cells was performed as described (32) with some modifications. ReAsH was first complexed with ethane dithiol (EDT) for 10 min in a reaction consisting of 0.5 mM ReAsH and 12.5 mM EDT in DMSO. This reaction mixture was diluted 1:1000 in FRET buffer consisting of 125 mM NaCl, 5 mM KCl, 6 mM glucose, and 25 mM HEPES, pH 7.6, and then added to myotubes or HEK-293T cells expressing Tc-tagged YFP-$\alpha_{1S}$ constructs. Cells were incubated at 37 °C for 1.5 h followed by washing with 100 $\mu$M British anti-Lewesite for 15 min to reduce nonspecific ReAsH labeling. In some experiments HEK-293T cells expressing Tc-tagged YFP-$\alpha_{1S}$ constructs were permeabilized with 0.1% saponin and then incubated with 150 nM purified $\beta_{1a}$ protein for 2 h at 37°C. Cells were then tested in FRET experiments (see below).

FRET measurements between $\alpha_{1S}$ and $\beta_{1a}$ subunit were conducted using the His-tag-specific FRET acceptor, Cy3NTA, as described previously (31). HEK-293T cells expressing YFP-$\alpha_{1S}$ constructs were permeabilized using 0.1% saponin in FRET buffer containing 3 $\mu$M Cy3NTA with or without 150 nM purified $\beta_{1a}$ protein bearing 10 histidine residues (His$_{10}$) at its
N-terminal end. After incubation for 2 h at 37 °C, cells were analyzed for FRET.

FRET Imaging—FRET was measured using epifluorescence microscopy as described (31, 33). Briefly, YFP donor fluorescence was acquired using a 480/30-nm bandpass excitation filter and 535/40-nm bandpass emission filter as a series of 60 16-bit 672 × 516 pixel images across a z stack 60 μm in thickness. Acceptor fluorescence was photobleached for 4 min at maximum DG-4 light source intensity using a ReAsH cubeset composed of a 570/20-nm bandpass excitation filter and 620/60-nm bandpass emission filter. FRET was measured as an enhancement in donor fluorescence upon acceptor photobleaching using

\[
E = 1 - \left( \frac{F_{\text{prebleach}}}{F_{\text{postbleach}}} \right)
\]

where \( E \) represents FRET efficiency, and \( F_{\text{prebleach}} \) and \( F_{\text{postbleach}} \) indicate donor fluorescence intensities before and after acceptor photobleaching, respectively. Fluorescence was quantified using ImageJ version 1.43u as described (34).

Measurement of ReAsH Labeling Efficiency—For each Tc-tagged YFP-\( \alpha_{15} \) construct expressed in HEK-293T cells, YFP and ReAsH fluorescence was quantified both before and after ReAsH photobleaching. ReAsH labeling efficiency was then calculated as the ratio of ReAsH (pre-acceptor bleach) to YFP (post-acceptor bleach) fluorescence for each construct. Note that YFP fluorescence acquired after acceptor bleach was used for these calculations to eliminate any contribution of YFP/ReAsH FRET from these measurements.

Molecular Visualization and Distance Measurements—An atomic model (Protein Data Bank Accession code 3JBR) derived from a high resolution cryo-EM reconstruction of the full DHPR complex (18) was used for distance measurements and preparation of Fig. 7, which was created using Chimera image processing software version 1.10.1 (build 40427) (35).

Results

Expression and Functional Analysis of Tc-tagged YFP-\( \alpha_{15} \) DHPRs in Dysgenic Myotubes—To characterize the relative proximity of the cytoplasmic loops in the \( \alpha_{15} \) DHPR subunit using FRET, we fused citrine, a bright YFP variant (28), to the N terminus of \( \alpha_{15} \) to act as a FRET donor. Then we inserted short Tc motifs (FLNCCPGCCMEP; Ref. 27) separately into each \( \alpha_{15} \) cytoplasmic loop and domain (Fig. 1A) to act as binding sites for the FRET acceptor ReAsH. Proper targeting of these Tc-tagged YFP-\( \alpha_{15} \) fusion constructs to the junctional sarcoplasmic reticulum of stably transduced dysgenic myotubes was confirmed by immunocytochemistry (Fig. 1B). After staining with an anti-GFP antibody, both YFP-\( \alpha_{15} \) and Tc-tagged YFP-\( \alpha_{15} \) constructs displayed discrete fluorescent foci that closely resembled the immunofluorescent pattern observed in wild type myotubes labeled with anti-\( \alpha_{15} \) antibodies (Fig. 1B). This staining pattern is known to represent the peripheral couplings where the sarcoplasmic reticulum terminal cisternae interact with the surface membrane (36). These results suggest that Tc-tag insertions did not affect \( \alpha_{15} \) targeting to peripheral couplings.

To functionally characterize these Tc-tagged \( \alpha_{15} \) channels, we quantified depolarization-induced Ca\(^{2+} \) release (i.e. EC coupling) in Fura-2-loaded stably transduced dysgenic myotubes. Each Tc-tagged \( \alpha_{15} \) construct restored robust Ca\(^{2+} \) release in response to K\(^{+}\)-depolarization. This Ca\(^{2+} \) release was comparable with control myotubes expressing YFP-\( \alpha_{15} \) lacking a Tc tag (−Tc; Fig. 2A). A small but significant reduction in average peak Ca\(^{2+} \) transient was observed in myotubes expressing constructs Tc-tagged at the N terminus, I-II loop, and II-III loop (Fig. 2B). Because no changes in sensitivity to K\(^{+}\) depolarization were observed for these constructs compared with −Tc controls (\( p > 0.05 \)), it is conceivable that the smaller Ca\(^{2+} \) transients result from slight differences in \( \alpha_{15} \) expression between individual cell clones. Myotubes expressing a Tc-tagged III-IV loop \( \alpha_{15} \) construct restored robust EC coupling though with enhanced K\(^{+}\) sensitivity. Mutations in the III-IV loop region have been reported to affect the conductive properties of the \( \alpha_{15} \) subunit (15, 37). However, whether the insertion of the Tc tag in our study alters the voltage-sensing properties of the DHPR complex is currently unknown and will require further testing. Thus, even though small functional changes were observed in some cases, the overall targeting and function of these YFP-\( \alpha_{15} \) constructs was largely unaffected by the Tc-tag insertions.

FRET-based Structural Analysis of Tc-tagged YFP-\( \alpha_{15} \) DHPRs—FRET-based analysis was used to characterize the structural arrangement of the \( \alpha_{15} \) intracellular loops (Fig. 3). The cell-permeant biarsenical FRET acceptor, ReAsH (27), was targeted to the Tc-tagged YFP-\( \alpha_{15} \) constructs (Fig. 3A), and energy transfer was quantified from the increase in the N-terminally fused YFP donor fluorescence after acceptor photobleaching (34). As reported (38), untransfected dysgenic myotubes were strongly labeled by ReAsH, which persisted even
after washing with British anti-Lewisite (39) to remove nonspecific ReAsH labeling (data not shown). However, this background ReAsH labeling did not impede our ability to measure FRET as only very low background energy transfer (E = 0.10) was observed in ReAsH-labeled myotubes expressing non-Tc-tagged control YFP-α15 (−Tc). On the other hand, significant energy transfer above background was detected to ReAsH separately targeted to each intracellular domain (Fig. 3B, filled bars). As expected, a positive control construct consisting of a Tc-tag/YFP tandem fused at the α15 N terminus displayed the highest FRET efficiency (N-term, E = 0.35; Fig. 3B), similar to FRET values previously reported for this tandem inserted into RyR1 (34). FRET efficiencies measured to the I-II loop (E = 0.20), II-III loop (E = 0.25), and the C-terminal tail (E = 0.24) were also significantly higher compared with the −Tc negative control construct. Average FRET efficiency measured to the III-IV loop (E = 0.18) was slightly but significantly reduced compared with the II-III loop and C-terminal domain (p < 0.05), thus suggesting differences among the loops in either relative distance to the α15 N terminus and/or orientation of the Tc tags. Overall, these results indicate that FRET efficiencies measured between the α15 N terminus and each cytoplasmic domain are similar, and therefore, these loops might be tightly clustered in dysgenic myotubes.

Previous FRET studies have suggested that skeletal muscle-specific protein-protein interactions can affect the organization of the α15 cytoplasmic loops (25). Thus, we performed a parallel series of FRET measurements on these α15 constructs expressed in HEK-293T cells (Fig. 3B, open bars), which lack skeletal muscle-specific proteins including the DHPR and RyR1. ReAsH-labeled HEK-293T cells expressing YFP-α15 (−Tc) displayed background energy transfer (E = 0.10) unchanged compared with FRET measurements for this construct expressed in dysgenic myotubes (Fig. 3B, black bars). Similarly, FRET efficiencies measured in HEK-293T cells to ReAsH targeted to the α15 N terminus and I-II loop (E = 0.39, and E = 0.21, respectively) were unchanged compared with similar measurements conducted in dysgenic myotubes. However, in intact HEK-293T cells, average FRET efficiencies to the II-III loop (E = 0.20) and III-IV loop (E = 0.14) were significantly reduced compared with measurements in dysgenic myotubes (Fig. 3B). Most surprisingly, no significant energy transfer above background was detected to the α15 C-terminal tail in HEK-293T cells (E = 0.10) despite robust FRET to this position for measurements conducted in dysgenic myotubes. This striking difference in energy transfer was unrelated to myotube-specific post-translational cleavage of the C-terminal tail at position 1664. As shown in Fig. 3B, FRET efficiencies measured from HEK-293T cells expressing α15 containing a full-length
C-terminal tail were not different from those measured in cells expressing the C-terminal tail truncated at position 1668. Thus, these results suggest that the conformation of the α_{15} II-III and III-IV loops and particularly the C-terminal tail are influenced by the cellular microenvironment.

ReAsH Labeling Efficiency—To verify that differences in energy transfer observed between the various Tc-tagged α_{15} constructs were not due to differences in ReAsH labeling efficiency at the various Tc-tagged sites, we quantified ReAsH labeling to each construct expressed in HEK-293T cells (Fig. 4). In contrast to dysgenic myotubes, specific ReAsH labeling of each recombinant Tc-tagged α_{15} DHPR was readily observed after treatment with 100 μM British anti-Lewisite (Fig. 4A), as we have shown previously with Tc-tagged RyRs expressed in HEK-293T cells (34). However, no differences in ReAsH labeling efficiency (i.e. the ReAsH/YFP fluorescence ratio) were observed between the Tc-tagged YFP-α_{15} constructs (Fig. 4B). Because of high nonspecific ReAsH labeling in myotubes, these control experiments were not feasible. However, in these cells FRET efficiencies measured after labeling Tc-tagged α_{15} YFP constructs with either 0.5 μM ReAsH (i.e. the concentration used in all FRET experiments) or a 4-fold higher ReAsH concentration (2 μM) were identical (data not shown), thus indicating that 0.5 μM ReAsH is a saturating concentration for these measurements. These results indicate that, as in HEK-293T cells, differences in FRET efficiencies measured in dysgenic myotubes does not result from trivial differences in ReAsH accessibility.

Effect of β_{1α} Binding on α_{15} Loop Structure—The β_{1α} DHPR subunit is critical for α_{15} membrane targeting and Ca^{2+} channel function as well as EC coupling (2, 4, 5, 22). To determine whether this essential subunit was responsible for differences in energy transfer observed in the two cell expression systems, we assessed the effect of direct addition of exogenous β_{1α} subunit on FRET efficiencies measured in HEK-293T cells. For these experiments, saponin-permeabilized cells were incubated with and without 150 nM purified recombinant β_{1α} subunit, which saturates α_{15} expressed in HEK-293T cells (data not shown). In the absence of β_{1α}, measured energy transfer to each α_{15} cytoplasmic loop in permeabilized HEK-293T cells (Fig. 5B, open bars) was comparable to similar measurements in intact HEK-293T cells (Fig. 3B, open bars), suggesting that cell permeabilization did not significantly affect the structure of the cytosolic loops/domains. Upon incubation with β_{1α} (Fig. 5B, filled bars) no significant changes in energy transfer were observed to any position except the II-III loop, where β_{1α} addition significantly increased FRET efficiency from 0.22 to 0.30 (p < 0.05). This finding was confirmed using a second II-III loop Tc-tagged construct (at position 719), where β_{1α} enhanced FRET from 0.26 to 0.31 (Fig. 5B). Recombinant β_{1α} lacking its C-terminal 36 amino acids (β_{1αΔC}), which are required to support bidirectional signaling (9), did not enhance FRET from the N-terminal YFP to the II-III loop (Fig. 5B, gray bar), thus indicating that these C-terminal tail residues are required to mediate β_{1α}–induced conformational changes in the α_{15} II-III loop.

β_{1α} binding specificity was confirmed using a YFP-α_{15} Tc-tagged construct harboring a Y366S mutation, which disrupts β_{1α} binding to the α_{15} subunit interaction domain motif in the α_{15} I-II loop (11). This Y366S mutation completely prevented β_{1α}–mediated enhancement in FRET to the II-III loop (Fig. 5C), thus confirming that β_{1α} binding to its native α_{15} subunit interacting domain motif is required for its conformational effects on the II-III loop.
FRET between α₁S and β₁a—Direct binding of both wild type and truncated β₁a to α₁S in HEK-293T cells was confirmed by measuring FRET from the N-terminal-fused YFP of α₁S to the FRET acceptor Cy3NTA (31) targeted to a His₁₀ tag attached to the β₁a N terminus (Fig. 6A). Significant energy transfer was observed after incubation with β₁a subunit (E = 0.24) compared with background (E = 0.10). Incubation with truncated β₃₆ resulted in an even greater increase in energy transfer (E = 0.31), significantly higher than FRET measured with wild type β₁a (Fig. 6B), thus suggesting that deletion of the β₁a C terminus may affect the relative α₁S/β₁a orientation. This finding is consistent with the suggested role of the β₁a C-terminal tail in supporting domain cooperativity within the subunit (40). Binding specificity was confirmed using the Y366S-α₁S mutation, which prevented specific energy transfer between α₁S and β₁a (Fig. 6C). These findings indicate that both wild type and truncated β₁a bind to the I-III loop of α₁S expressed in HEK-293T cells.

Discussion

In this study using a unique FRET-based approach we have shown that the DHPR α₁S subunit intracellular loops have remarkably similar structural properties when expressed either in myotubes or HEK-293T cells. However, key differences exist. To our knowledge we are the first to show that the structure of the α₁S C-terminal tail is highly sensitive to muscle-specific protein-protein interactions. Our data also provide the first experimental evidence of a β₁a-mediated reorientation of the α₁S I-III loop domain, further supporting the idea that a synergistic α₁S/β₁a interaction could account for the conformational changes required to sustain skeletal muscle E-C coupling. The details of these findings and our unique FRET-based experimental system are outlined below.

Labeling System—FRET-based analysis of the DHPR using fused fluorescent proteins as FRET donors/acceptors has become a powerful tool for *in situ* studies of DHPR conformation and its structural interaction(s) with RyR1 (24, 25). Although these studies have revealed important structural aspects of DHPR/RyR interactions, they are limited by the exclusive use of fluorescent proteins, which can affect DHPR function and targeting as well as interpretation of the resulting FRET data (25, 26, 41). In the current study a small 12-residue peptide tag was used to target the FRET acceptor, ReAsH, to each of the α₁S, DHPR cytoplasmic loops and domains, thereby minimizing alteration of native protein conformation. Thus, we could measure FRET to the α₁S III-IV loop without compromising DHPR function or proper targeting, which are both severely disrupted by insertion of larger FRET probes (i.e. fluorescent protein fusions) in this loop (25). In addition, we could easily quantify energy transfer to a specific Tc tag via direct comparison with non—Tc-tagged controls. And because all DHPR loop positions were equally accessible to FRET acceptor, measured FRET efficiencies could more easily be related to differences in either donor/acceptor distance or orientation. Finally, nonspecific biarsenical labeling of myotubes reported previously (38) was not problematic for these studies, as ReAsH was used as FRET acceptor, and so only ReAsH fluorophores tar-

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**FIGURE 5.** The β₁a DHPR subunit specifically alters the structure of the α₁S II-III loop. A, schematic representation of the α₁S DHPR subunit showing the position of YFP (yellow oval), Tc tag (red squares), and the β₁a DHPR subunit (green oval) bound to its α₁S subunit interacting domain determinant in the I-III loop (blue rectangle). The location of the critical Y366 residue required for β₁a binding to α₁S is shown. B, average FRET efficiency values are shown from YFP in the α₁S N terminus to ReAsH targeted to permeabilized HEK-293T cells expressing the indicated Tc-tagged α₁S DHPR constructs in either the absence (open bars) or presence (filled bars) of 150 nM recombinant β₁a. The effect of β₁a lacking 36 C-terminal amino acids (β₃₆) on FRET efficiency to the II-III loop is shown (gray bar). Data points represent mean FRET efficiencies ± S.E. for the number of cells indicated in each bar. Asterisks indicate a significant difference in energy transfer (p < 0.001 using a paired two-tailed t test) between FRET measurements for a given construct conducted in either the absence or presence of β₁a. C, average FRET efficiency values are shown from YFP in the N terminus of α₁S to ReAsH targeted to Tc726 in the II-III loop of α₁S containing the Y366S mutation, which cannot bind β₁a (11). Data points represent mean FRET efficiencies ± S.E. for the number of cells indicated in each bar.

**FIGURE 6.** Both β₁a and β₃₆ bind to α₁S DHPR. A, strategy for direct FRET measurements between α₁S and β₁a DHPR subunits in HEK-293T cells. FRET from the fused N-terminal YFP in α₁S (yellow oval) to the His₁₀ tag-specific FRET acceptor, Cy3NTA (red octagon), targeted to β₁a (green circle) is indicative of binding between these two DHPR subunits. B and C, FRET efficiency values are shown from YFP to Cy3NTA targeted to HEK-293T cells expressing wild type YFP α₁S (B) or Y366S YFP α₁S (C) with and without the indicated β₁a constructs. Data represent the mean FRET values ± S.E. for the number of cells indicated in each bar. The asterisk indicates a significant difference in energy transfer (p < 0.001 using a paired two-tailed t test) between FRET measurements conducted using either wild type β₁a or truncated β₃₆.
**β_{1a}** Binding Alters α_{15} DHPR Conformation

**Comparison between Loop Conformations in Heterologous and Homologous Systems**—We observed remarkable similarities and differences in the structure of α_{15} expressed either in HEK-293T cells or dysgenic myotubes. For example, FRET measurements to the N terminus and the I-III loop were essentially identical between the two systems, thus suggesting that cell-specific factors do not affect FRET measured to these areas. In contrast, FRET efficiencies measured from the α_{15} N terminus to the α_{15} C-terminal loop in the I-III, III-IV loops and, most significantly, to the C-terminal tail were all quite different between the two systems. We can attribute some of these differences to specific protein factors, whereas other differences will require additional experiments to investigate. A summary of these differences follows.

**II-III Loop Structure**—Compared with identical measurements in HEK-293T cells, we observed a significant elevation in energy transfer from the N terminus to the α_{15} II-III loop for constructs expressed in dysgenic myotubes. This increase in FRET likely reflects a specific conformational effect in the II-III loop as structural changes in the vicinity of the N-terminal YFP FRET donor common to all constructs would almost certainly have altered FRET efficiencies to all DHPR loops. This enhanced energy transfer to the II-III loop most likely results from binding of β_{1a} to its I-III loop determinant, as it is the same degree of elevated energy transfer to the II-III loop in HEK-293T cells incubated with recombinant β_{1a}. Because β_{1a} enhanced FRET to two different Tc-tagged positions in the II-III loop, this effect is reproducible and meaningful. This specific conformational change in the II-III loop also requires the C-terminal 36 amino acids of β_{1a}, a determinant needed to support EC coupling and to organize DHPRs into tetrads (22, 23). This conformational change might reflect the natural structure of this II-III loop required to communicate with RyR1. Moreover, the II-III loop and the C-terminal tail of β_{1a} might form a larger structural domain that then interacts with RyR1 (a notion supported by recent cryo-EM reconstructions of the DHPR; see Fig. 7A below). Although these possibilities require testing, it should be stressed that the only specific conformational effect of β_{1a} binding we observed was within the II-III loop. Future FRET-based measurements to more defined determinants in the II-III loop may resolve more subtle structural changes occurring as a result of β_{1a} binding or during EC coupling.

**III-IV Loop Structure**—In this study we were able to make the first direct FRET measurements to the III-IV loop. We detected very slight differences in FRET to the III-IV loop when constructs were expressed either in HEK-293T cells or dysgenic myotubes. The origin of these differences is still difficult to discern but appear to be unrelated to β_{1a} binding. However, now that the III-IV loop can be labeled with FRET acceptors, future studies may detect structural changes in this loop that provide clues as to its function.

**C-terminal Tail Structure**—The largest difference we observed in FRET measurements conducted in the two systems was to the α_{15} C-terminal tail. Robust FRET was measured to this position when experiments were conducted in myotubes, whereas no significant FRET was detected in HEK-293T cells. These differences suggest changes in structural conformation of the C-terminal tail that could result from post-translational processing of the α_{15} subunit or differences in protein composition between the two systems as discussed below.

**Posttranslational Processing of α_{15}**—Differences in FRET efficiencies measured between the N terminus and the C-terminal tail may result from intrinsic differences in post-translational processing of α_{15} in dysgenic myotubes. For this study we used an α_{15} construct encoding the full 1873 amino acids of the protein. However, in myotubes, full-length α_{15} subunit is likely cleaved post-translationally at position 1664 (25, 42), right after our Tc-tag insertion, thus significantly shortening the C-terminal tail. To test whether post-translational processing of the C-terminal tail may have affected FRET efficiencies measured to this position, we conducted parallel FRET measurements on α_{15} YFP fusion constructs with either a full-length C-terminal tail or a tail shortened at position 1668. No differences in FRET for these variants were observed in either HEK-293T cells or dysgenic myotubes. Thus, it is unlikely that post-translational processing of the C-terminal tail results in the different FRET efficiency profiles to these different positions.

**Muscle-specific Proteins**—Differences in FRET efficiency to the C-terminal tail of α_{15} were significant and unrelated to the absence of β_{1a} subunit in HEK-293T cells. Similarly, differences in interactions with RyR1 most likely are not responsible for changes in FRET to the α_{15} C terminus, as previous studies using CFP/YFP fusions showed no difference in FRET between the N and C termini for α_{15} constructs expressed in either dysgenic (i.e. lacking RyR1) or dysgenic systems (25). Thus, it is possible that other proteins that make up the DHPR complex, such as the α_{6,1} and γ1 subunits, might account for this difference. Similarly, interactions between the α_{15} C terminus tail and other muscle-specific proteins should not be ruled out. Indeed, differences in protein interactions with various functional domains of the α_{15} C-terminal tail, like the triad targeting signal (position 1543–1661; Refs. 44 and 45) and the AKAP/ PKA binding domain (position 1724–1821; Ref. 42) might lead to conformational changes in the C-terminal tail evident in myotubes but not HEK-293T cells. These possibilities await...
further testing using co-expression of these proteins combined with our FRET-based measurements.

Effects of the $\beta_{1a}$ C-terminal Tail on DHPR Structure—In this study we have made the first direct FRET measurements between $\alpha_{1S}$ and $\beta_{1a}$. These measurements confirmed that both wild type and $\beta_{1a}$, bearing a 36-amino acid C-terminal truncation ($\beta$-36), bind to $\alpha_{1S}$ in our experimental system. In addition, relative to wild type $\beta_{1a}$, enhanced FRET was observed between the N termini of $\alpha_{1S}$ and the $\beta$-36 construct. This result suggests that this truncation results in a significant realignment of $\beta_{1a}$, thereby bringing its N terminus closer to $\alpha_{1S}$. Thus, deletion of the $\beta_{1a}$ C terminus, which prevents bidirectional signaling (22, 23), also may affect the conformation/orientation of both the $\alpha_{1S}$ II-III loop and the $\beta_{1a}$ subunit. These results are consistent with the hypothesis that the C terminus of the $\beta_{1a}$ subunit is required for inducing conformational changes in the $\alpha_{1S}$ subunit necessary to transmit the EC coupling signal (20, 40).

Comparison with Recent High Resolution DHPR Structures—Recently, a near-atomic 4.2 Å resolution model of the DHPR complex has been published (18). Although many parts of the DHPR complex are well defined, none of the intracellular loops tested in this study are localized in the model, most likely due to high intrinsic flexibility of the loops. However, several aspects of the model support conclusions derived from our FRET data. For example, from this new cryo-EM reconstruction, it is evident that the II-III loop is the closest of the $\alpha_{1S}$ cytoplasmic loops (except the I-II loop) to the $\beta_{1a}$ subunit (Fig. 7, A and B). Thus, it is conceivable that $\beta_{1a}$ binding could modulate the structure of the II-III loop via short range allosteric interactions and that these two elements could form a structural complex. In addition, a central placement of the fused YFP would result in a relatively uniform profile of FRET efficiencies measured to the various intracellular loops (Fig. 7A). Indeed, the inherent intrinsic disorder of these loops revealed by the high resolution structures makes them excellent targets for further FRET-based structural studies to reveal subtle conformational changes underlying other important $\alpha_{1S}$ functionalities.

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