Functional analysis of a frame-shift mutant of the dihydropyridine receptor pore subunit (α₁S) expressing two complementary protein fragments

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Abstract

Background: The L-type Ca²⁺ channel formed by the dihydropyridine receptor (DHPR) of skeletal muscle senses the membrane voltage and opens the ryanodine receptor (RyR1). This channel-to-channel coupling is essential for Ca²⁺ signaling but poorly understood. We characterized a single-base frame-shift mutant of α₁S, the pore subunit of the DHPR, that has the unusual ability to function voltage sensor for excitation-contraction (EC) coupling by virtue of expressing two complementary hemi-Ca²⁺ channel fragments.

Results: Functional analysis of cDNA transfected dysgenic myotubes lacking α₁S were carried out using voltage-clamp, confocal Ca²⁺ indicator fluorescence, epitope immunofluorescence and immunoblots of expressed proteins. The frame-shift mutant (fs-α₁S) expressed the N-terminal half of α₁S (M1 to L670) and the C-terminal half starting at M701 separately. The C-terminal fragment was generated by an unexpected restart of translation of the fs-α₁S message at M701 and was eliminated by a M701I mutation. Protein-protein complementation between the two fragments produced recovery of skeletal-type EC coupling but not L-type Ca²⁺ current.

Discussion: A premature stop codon in the II-III loop may not necessarily cause a loss of DHPR function due to a restart of translation within the II-III loop, presumably by a mechanism involving leaky ribosomal scanning. In these cases, function is recovered by expression of complementary protein fragments from the same cDNA. DHPR-RyR1 interactions can be achieved via protein-protein complementation between hemi-Ca²⁺ channel proteins, hence an intact II-III loop is not essential for coupling the DHPR voltage sensor to the opening of RyR1 channel.

Background

The dihydropyridine receptor (DHPR) of skeletal muscle consists of α₁S, α₂, β₁a and γ₁ subunits [1]. The α₁ subunit is a large four-repeat transmembrane protein of ~220 kDa that contains the basic functional elements of the L-type Ca²⁺ channel, including the Ca²⁺ selective pore and S4 "voltage-sensing" transmembrane segments in each of the four internal repeats [2]. β subunits are ~65 kDa cytosolic proteins essential for membrane trafficking, modulation of channel kinetics, and for excitation-contraction (EC) coupling [3,4]. The α₂ subunit is a highly glycosylated ~175 kDa protein formed by two disulfide-linked pep-
tides [5], whereas the γ1 subunit is a ∼32 kDa skeletal muscle-specific protein of four presumptive transmembrane domains with almost unknown function [6,7].

Skeletal muscle cells utilize the voltage sensors formed by the S4 segments to trigger a rapid elevation of cytosolic Ca2+, thus coupling membrane excitation to muscle cell contraction. Subsequent to charge movements in the voltage sensors, a conformational change in the DHPR is transmitted to the ryanodine receptor (RyR1), presumably, via protein-protein interactions [8]. Ultimately, there is a brief opening of the RyR1 channel resulting in the release of Ca2+ from the sarcoplasmic reticulum (SR). Numerous observations have lent support to this view, and especially significant are the functional expression studies in dysgenic myotubes lacking α1S. The dysgenic myotube is devoid of L-type Ca2+ current, charge movements and EC coupling. All three are restored in the dysgenic myotube by expression of α1S[9–11]. These results corroborated the essential role of α1S in the mechanism of EC coupling of skeletal muscle cells.

The mechanism by which the DHPR signals the RyR1 is poorly understood [12,13]. Domains in the cytoplasmic linker between repeats II and III have been clearly implicated [14–19], and some regions such as Thr671-Lue690 were suggested to trigger RyR1 opening by binding to RyR1 [15]. However, extensive deletions within the II-III linker that eliminate the RyR1 binding region, and other suggested signaling regions in the II-III loop [16], do not entirely eliminate EC coupling [20,21]. Hence additional domains of α1S and/or other DHPR subunits appear to be engaged by the voltage sensor and contribute to an EC coupling signal. In this respect, the contribution of the β1a subunit of the DHPR to EC coupling in skeletal muscle cells has been extensively documented [4,22–24].

In the present report, we characterized a frame-shift mutant of α1S that expresses two complementary fragments of α1S. Complementation between the two α1S fragments produced recovery of EC coupling in dysgenic muscle cells lacking α1S. The results suggest the EC coupling voltage sensor of skeletal muscle is modular in function and can be assembled from separate hemi-Ca2+ channel fragments.

**Results and Discussion**

**Expression of a frame-shift mutation of α1S in dysgenic myotubes**

Primers for the frame-shift mutant, fs-α1S, were originally designed to delete the 20-mer Thr671-Leu690 in the cytosolic loop between repeats II and III of α1S and to generate a full-length α1S carrying this internal deletion. A proofreading error during a PCR reaction resulted in an amplified DNA with the desired deletion but also containing an additional thymidine following the TTG codon for Leu670 (Fig. 1A). The one-base shift in reading frame introduced a serine at position 671 followed immediately by a stop codon (Fig. 1B). This frame-shift mutation was re-ligated into an otherwise full-length α1S, subcloned into the mammalian expression vector pSG5, and transfected into dysgenic (α1S null) myotubes. Fs-α1S was abundantly expressed in primary dysgenic myotubes in culture (Fig. 1C) and produced the expected truncated α1S protein (Fig 1D). Western blots using N-terminus T7-tagged fs-α1S and T7-tagged full-length α1S showed that the expressed full-length α1S protein migrated with an apparent molecular weight of approximately 185 KDa under reducing conditions. This result is consistent with the mobility of the native purified skeletal muscle α1S subunit [25]. The fs-α1S migrated with a molecular weight of approximately 90 KDa which is entirely consistent with the theoretical molecular weight of the expressed fragment which was 85.6 KDa. Furthermore, 5-fold overloading of the SDS-PAGE gel failed to detect any fragment of a size comparable to full-length α1S (not shown).

**Recovery of EC coupling by the frame-shift α1S cDNA**

EC coupling was investigated in voltage-clamped myotubes with simultaneous monitoring of intracellular Ca2+ using confocal fluorescence of fluo-4 [26]. Controls shown in Fig. 2A indicated that the overwhelming majority of non-transfected dysgenic myotubes (13 of 15 cells) did not produce detectable Ca2+ transients (<0.1 ΔF/Fo) or Ca2+ currents (<20 pA/cell) in response to depolarization under voltage-clamp. This is shown in the line-scan images of fluo-4 fluorescence in Fig. 2A and the corresponding traces of ICa2+ during a 50-ms depolarization to +30 mV and +90 mV delivered at the start of the line scan in the same cell. However, in two cells (2 of 15 cells) we observed Idys, the low-density endogenous Ca2+ current previously described in dysgenic myotubes [27,28]. The reason for the low abundance of this current in these cultured myotubes is unknown. Ca2+ currents and stimulated fluorescence for one of the cells expressing Idys is shown in Fig. 2B. We observed a peak ICa2+ density of approximately 0.8 pA/pF and a barely detectable fluorescence signal which in Fig. 2B is indicated by the arrow in the trace of integrated fluorescence at +30 mV. This small fluorescence signal disappeared entirely at +90 mV, suggesting it might be contributed directly by Idys or might be due to SR Ca2+ release induced by Idys. The voltage dependence of the fluorescence signal and ICa2+ are compared in Fig. 2C for the two cells expressing Idys and for the vast majority of cells which altogether did not express intracellular Ca2+ transients or ICa2+. The maximum fluorescence signal contributed by Idys, when Idys was present, was <0.2 ΔF/Fo units. Furthermore, the shape of the fluorescence vs. voltage relationship was bell-shaped and a mirror image of the ICa2+ vs. voltage curve. These
Figure 1
Nucleotide sequence and protein expression of fs-α1S. A) Nucleotide sequence of wt-α1S and fs-α1S in the region of the frame-shift. B) The three reading frames of fs-α1S in the region of the frame-shift are shown. Translation of the C-terminal half of α1S is explained by a restart of translation at the indicated ATG codon which is located 25 bases downstream from the termination codon indicated by the asterisk. C) Confocal images (calibration bar is 10 microns) show details of the intracellular distribution of the expressed proteins. Cells were transfected with the CD8 cDNA plus wt-α1S or fs-α1S. Cells were incubated with CD8 antibody beads, fixed, and stained with T7 primary/fluorescein-conjugated secondary antibodies. Pixel intensity was converted to a 16-level inverted gray scale with high-intensity pixels in black color. Asterisks show on-focus CD8 antibody beads (diameter 4.5 microns) bound to cells. NT indicates a non-transfected myotube in the same focal plane of the transfected cell. D) Immunoblots using anti T7 antibody of cultures of dysgenic myotubes expressing wt-α1S and fs-α1S. Indicated are 3 of 7 molecular weight markers run in the same gel.
Figure 2
Absence of EC coupling in non-transfected dysgenic myotubes. The confocal line-scan images in color show fluo-4 fluorescence across myotubes in response to a 50-ms depolarization from a holding potential of -40 mV. Line scan images have a constant temporal dimension of 2.05 s (horizontal) and a variable spatial dimension (vertical) depending on the cell size. Traces immediately above each line scan show the time course of the fluorescence change in resting units (ΔF/Fo). The amplitude and the timing of the depolarization are indicated under each line-scan. Arrow indicates a small Ca²⁺ transient elicited in 1 of 2 cells found to express Idys. Traces next to lines-cans show ICa²⁺ during the 50 ms depolarization used to stimulate the Ca²⁺ transient. Current calibration bars are 10 ms and 1 pA/pF. A) Absence of Ca²⁺ transients and ICa²⁺ in a typical dysgenic cell. B) Minor Ca²⁺ transient and ICa²⁺ in a cell expressing Idys. Note that fluorescence calibration bar is 0.5 ΔF/Fo. A 16-color calibration bar in ΔF/Fo units is included in Fig. 3 for visual reference. C) Voltage dependence of the mean (± SEM) ICa²⁺ and mean peak Ca²⁺ transient (± SEM) in 13 cells not expressing ICa²⁺ and 2 cells expressing Idys (mean only).
controls indicated that non-transfected dysgenic myotubes are low-background cells that do not express voltage-activated Ca\textsuperscript{2+} signals of major consequence for the present studies.

Fig. 3 shows that fs-\(\alpha\)1\(\text{S}\) recovered a significant fraction of the voltage-activated Ca\textsuperscript{2+} transient compared to that expressed by full-length wt-\(\alpha\)1\(\text{S}\). The magnitude of the fluorescence signal expressed by fs-\(\alpha\)1\(\text{S}\) was approximately 5-fold larger than the largest Ca\textsuperscript{2+} transient detected in non-transfected myotubes expressing Idys, >20-fold larger than the average Ca\textsuperscript{2+} transient detectable in non-transfected cells, and about 1/3 of the maximum SR Ca\textsuperscript{2+} release expressed by the control wt-\(\alpha\)1\(\text{S}\) construct. Thus, we are confident that the voltage-evoked Ca\textsuperscript{2+} transient in cells transfected by fs-\(\alpha\)1\(\text{S}\) cells was a direct consequence of the expressed protein. Also shown in Fig. 3 is ICa\textsuperscript{2+} during the 50 ms depolarization used to stimulate the Ca\textsuperscript{2+} transient. The amplitude and the timing of the depolarization are indicated under each line scan. Note that fluorescence calibration bar is 1 \(\Delta F/F_o\). A 16-color calibration bar in \(\Delta F/F_o\) units is included for visual reference.

The confocal line-scan images in color show fluo-4 fluorescence across myotubes in response to a 50-ms depolarization from a holding potential of -40 mV to +30 mV (top) and +90 mV (bottom). Line scan images have a constant temporal dimension of 2.05 s (horizontal) and a variable spatial dimension (vertical) depending on the cell size. Traces immediately above each line scan show the time course of the fluorescence change in resting units (\(\Delta F/F_o\)). Traces under lines cans show ICa\textsuperscript{2+} during the 50 ms depolarization used to stimulate the Ca\textsuperscript{2+} transient. The amplitude and the timing of the depolarization are indicated under each line scan. Note that fluorescence calibration bar is 1 \(\Delta F/F_o\). A 16-color calibration bar in \(\Delta F/F_o\) units is included for visual reference.

**Figure 3**
Ca\textsuperscript{2+} transients in dysgenic myotubes transfected with fs-\(\alpha\)1\(\text{S}\).
The confocal line-scan images in color show fluo-4 fluorescence across myotubes in response to a 50-ms depolarization from a holding potential of -40 mV to +30 mV (top) and +90 mV (bottom). Line scan images have a constant temporal dimension of 2.05 s (horizontal) and a variable spatial dimension (vertical) depending on the cell size. Traces immediately above each line scan show the time course of the fluorescence change in resting units (\(\Delta F/F_o\)). Traces under lines cans show ICa\textsuperscript{2+} during the 50 ms depolarization used to stimulate the Ca\textsuperscript{2+} transient. The amplitude and the timing of the depolarization are indicated under each line scan. Note that fluorescence calibration bar is 1 \(\Delta F/F_o\). A 16-color calibration bar in \(\Delta F/F_o\) units is included for visual reference.

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**fs-\(\alpha\)1\(\text{S}\) expresses two complementary protein fragments**

The EC coupling recovered by fs-\(\alpha\)1\(\text{S}\) could be due either to the activity of the N-terminal half of \(\alpha\)1\(\text{S}\) alone or to protein-protein complementation between the N-terminal half and a fragment expressing the C-terminal half of \(\alpha\)1\(\text{S}\). The C-terminal half of \(\alpha\)1\(\text{S}\) could have been translated by the fs-\(\alpha\)1\(\text{S}\) expression vector if the ATG codon (Met701), which is downstream from the TGA termination codon and in-frame with the wild-type message (Fig 1B) served as open reading frame for translation of the second half of the wt message. Although this would be unusual, the fact that the codon for Met701 is only 25 bases downstream from the termination codon could have substantially increased the possibility of a re-start of the translation of the second half of the message at Met701. This phenomenon has been described in eukaryotic cells and in viral-infected mammalian cells and is known as translation by leaky ribosomal scanning [32,33]. To test this explanation, the presumptive restart codon, Met701, was mutated to Ile701 in the fs-\(\alpha\)1\(\text{S}\) template. If fs-\(\alpha\)1\(\text{S}\) recovered EC coupling by virtue of expressing a single protein fragment, then fs-\(\alpha\)1\(\text{S}\)M701I should also recover EC coupling since the mutation was introduced downstream from the stop codon. Fig. 5 shows that this was not the case. Fs-\(\alpha\)1\(\text{S}\)M701I did not recover Ca\textsuperscript{2+} transients in 9 of 9 tested cells, consistent with leaky ribosomal scanning. As a positive control, we coexpressed fs-\(\alpha\)1\(\text{S}\)M701I and the C-terminus half of \(\alpha\)1\(\text{S}\), namely \(\alpha\)1\(\text{S}\)Δ1–700, cloned into a separate pSG5 vector. The results in Fig. 5 indicated that \(\alpha\)1\(\text{S}\)M701I alone was inactive. However, when myotubes were cotransfected with fs-\(\alpha\)1\(\text{S}\)M701I and \(\alpha\)1\(\text{S}\)Δ1–700, each in a separate pSG5 vector, there was a robust recovery of Ca\textsuperscript{2+} transients in 5 of 5 cells. Fig. 6A shows fluorescence vs. voltage relationships for the fs-\(\alpha\)1\(\text{S}\)M701I mutant and for this mutant coexpressed with \(\alpha\)1\(\text{S}\)Δ1–700. The combined expression of the two complementary frag-
ments of $\alpha_{1S}$ resulted in a robust recovery of EC coupling with sigmoidal Ca$^{2+}$ release vs. voltage characteristics. A summary of the maximum fluorescence during the Ca$^{2+}$ transient in response to a depolarization to +90 mV is shown in Fig. 6B. The magnitude of the Ca$^{2+}$ transient expressed by $\alpha_{1S}$M701I + $\alpha_{1S}$A1–700 was indistinguishable from that of wt-$\alpha_{1S}$ (t-test significance $p = 0.671$, see figure legend). To confirm expression of the C-terminus half of $\alpha_{1S}$ in cells transfected with $\alpha_{1S}$M701I, we used the II-III loop polyclonal antibody SKI [34] directed against epitope Ala739-Ile752 which is downstream from the II-III linker or III-IV linker was cut and the two fragments were coexpressed each in a separate vector [37]. We would thus conclude that in the case of the Ca$^{2+}$ channel, an in-

Figure 4
Skeletal-type EC coupling expressed by $\alpha_{1S}$. A) Voltage-dependence of peak Ca$^{2+}$ for 5 control myotubes expressing wt-$\alpha_{1S}$ and 5 myotubes expressing $\alpha_{1S}$. Ca$^{2+}$transients for 15 non-transfected myotubes (NT) are included for reference. The sigmoidal lines are a Boltzmann fit with parameters $\Delta F/Fo$ max = 2.9, 1.4 $\Delta F/Fo$; $V1/2 = 11.7, 20$ mV; $k = 8.7, 13.2$ mV, for wt-$\alpha_{1S}$ and fs-$\alpha_{1S}$ respectively. B) Line-scans (horizontal dimension is 2.05 seconds) and traces of integrated fluorescence in $\Delta F/Fo$ units for depolarizations to +30 mV. Top line-scans are for the same fs-$\alpha_{1S}$ transfected myotube in standard external solution (10 mM CaCl$_2$) and the same solution without added CaCl$_2$ (0 Ca$^{2+}$). Bottom line-scans show fs-$\alpha_{1S}$ transfected KO myotubes lacking DHPR $\beta_{1a}$ or lacking RyR1 in standard external solution.

Implications for EC coupling in skeletal myotubes
Except for the magnitude, the SR Ca$^{2+}$ release signal expressed by $\alpha_{1S}$ was entirely typical of skeletal myotubes with sigmoidal voltage-dependence, proceeding in the absence of external Ca$^{2+}$ and requiring RyR1. A comparison of the maximum fluorescence ($\Delta F/Fo$ max) at +90 mV (Fig. 6B) shows that the signal generated by $\alpha_{1S}$ was significantly smaller than that generated by the control construct (wt-$\alpha_{1S}$ vs. fs-$\alpha_{1S}$ t-test significance $p = 0.014$) and smaller than that generated by the two coexpressed fragments (fs-$\alpha_{1S}$ vs. $\alpha_{1S}$M701I + $\alpha_{1S}$A1–700 t-test significance $p = 0.013$). These observations suggest that the magnitude of the Ca$^{2+}$ release appears to be limited by the low yield of expression of the C-terminal fragment achieved by leaky ribosomal scanning of the second half of the $\alpha_{1S}$ message. To test this explanation further, we coexpressed $\alpha_{1S}$ and the C-terminal half of $\alpha_{1S}$ each in a separate pSG5 vector. We found that $\alpha_{1S}$ and $\alpha_{1S}$A1–700 together expressed Ca$^{2+}$ transients with a $\Delta F/Fo$ max similar to wt-$\alpha_{1S}$ control (not shown). Thus we are certain that the EC coupling expressed by $\alpha_{1S}$ is mechanistically similar to control skeletal-type EC coupling but limited in magnitude by a comparatively lower density of functional DHPRs that are assembled in cells expressing $\alpha_{1S}$. It is conceivable that the functional integrity of the fragmented $\alpha_{1S}$ protein is maintained in part by the $\beta$ subunit of the DHPR which spans both halves of the $\alpha_{1}$ pore subunit by binding to the I-II loop and the C-terminus [35,36]. Consistent with this explanation, we failed to detect EC coupling recovery when fs-$\alpha_{1S}$ was expressed in $\beta_{1a}$ null myotubes.

Earlier studies in the voltage-gated Na$^{+}$ channel had shown that pore function was not compromised when the II-III linker or III-IV linker was cut and the two fragments were coexpressed each in a separate vector [37]. We would thus conclude that in the case of the Ca$^{2+}$ channel, an in-
tact II-III loop is essential for this function since neither fs-α1S nor the combined expression of the two truncated fragments (not shown) was able to rescue Ca2+ current. This result is entirely consistent with the identification of the II-III loop as critical for enhancement of L-type Ca2+ current expression by the RyR1 [38]. However, EC coupling, per se, can clearly proceed with a cut in the II-III loop. This was shown here by the behavior of the fs-α1S construct and elsewhere by expressing the α15 construct(s) indicated at the top of each column. A 16-color calibration bar in ΔF/F0 units is included in Fig. 3 for visual reference.

Figure 5
EC coupling generated by two complementary fragments of α15. Line scans (horizontal dimension is 2.05 seconds) of fluo-4 fluorescence show Ca2+ transients in response to the indicated 50-ms depolarization from a holding potential of -40 mV. Trace of integrated fluorescence in ΔF/F0 units is shown for each line scan. Each set of depolarizations is from a separate dysgenic myotube expressing the α15 construct(s) indicated at the top of each column. A 16-color calibration bar in ΔF/F0 units is included in Fig. 3 for visual reference.

The present studies show EC coupling recovery by a frame-shift mutant of α15 due to protein-protein complementation of the N-terminal and C-terminal halves of α15. The N-terminal half houses repeats I and II with the adjoining cytosolic loop and the C-terminal half houses most of the II-III loop, along with repeats III and IV with the adjoining loop. Protein-protein complementation between the N-terminal and C-terminal fragments produced a DHPR capable of functioning as EC coupling voltage sensor, thus suggesting the presence of at least two functional modules within α15. Recent evidence suggests that the four internal repeats of the voltage-gated Na+ channel, which is closely related to the L-type Ca2+ channel encod-
Figure 6
Expression of C-terminal fragment of α1S is essential for EC coupling. A) Voltage dependence of peak Ca$^{2+}$ during the Ca$^{2+}$ transient for dysgenic myotubes transfected with the indicated constructs. The sigmoidal curve is a Boltzmann fit with parameters $\Delta F/ F_0 \text{max} = 2.45$; $V_1/2 = 15.4 \text{ mV}$; $k = 9.3 \text{ mV}$ for 5 cells coexpressing fs-$\alpha_{1S}$M701I + $\alpha_{1S}\Delta 1-700$. Absence of response is shown for 9 cells expressing fs-$\alpha_{1S}$M701I alone. B) $\Delta F/ F_0 \text{max} \text{ (mean ± SEM)}$ obtained from a depolarization to $+90 \text{ mV}$ is shown for the indicated number of cells. NT denotes non-transfected dysgenic myotubes. Compared to wt-$\alpha_{1S}$ (control), the statistical significance in unpaired t-Student test was $p = 1.6 \times 10^{-6}$ (non-transfected, NT); 0.014 (fs-$\alpha_{1S}$); 0.0002 (fs-$\alpha_{1S}$M701I); 0.0008 ($\alpha_{1S}\Delta 1-700$); 0.671 (fs-$\alpha_{1S}$M701I + $\alpha_{1S}\Delta 1-700$). Compared to fs-$\alpha_{1S}$, the statistical significance was $p = 1 \times 10^{-6}$ (non-transfected, NT); 0.014 (wt-$\alpha_{1S}$); 0.00019 (fs-$\alpha_{1S}$M701I); 0.0008 ($\alpha_{1S}\Delta 1-700$); 0.013 (fs-$\alpha_{1S}$M701I + $\alpha_{1S}\Delta 1-700$). C) Immunoblots using a polyclonal antibody directed to the II-III loop epitope Ala739-Ile752 [34] in cultures of dysgenic myotubes expressing fs-$\alpha_{1S}$ and fs-$\alpha_{1S}$M701I. Indicated are 3 of 7 molecular weight markers run in the same gel. D) Confocal images of cells transfected with the CD8 cDNA plus T7-tagged fs-$\alpha_{1S}$M701I or T7-tagged fs-$\alpha_{1S}$M701I + untagged $\alpha_{1S}\Delta 1-700$. Cells were incubated with CD8 antibody beads, fixed, and stained with T7 primary/fluorescein-conjugated secondary antibodies. Pixel intensity was converted to a 16-level inverted gray scale with high-intensity pixels in black color. NT indicates a non-transfected myotube. CD8 antibody beads have a diameter of 4.5 microns. Calibration bar is 10 microns.
ed by the DHPR, have non-equivalent functional roles because the S4 segments of repeats I and II move much faster than those of repeats III and IV [40]. By analogy, the “fast-moving” module of the DHPR would be represented by the N-terminal fragment and the “slower-moving” module by the C-terminal fragment. Interactions between these two modules are likely to be critical for intramembrane charge movements in the assembled four-repeat channel and for coupling the movement of the S4 gating charges to the opening of the RyR1 channel. Future studies of gating currents in each hemi-Ca\(^{2+}\) channel fragment should provide valuable information on how the “fast” and “slow” gating modules interact during EC coupling in skeletal muscle.

The C-terminal fragment was generated by an unusual re-start of translation of the fs-α\(_{1S}\) message at M701, presumably by leaky ribosomal scanning, and was eliminated by a M701I mutation. Hence, a premature stop codon in the II-III loop upstream of M701 may not necessarily cause a loss of DHPR function because in these cases, function would be recovered by complementation between protein fragments expressed by the same cDNA. From a methodological perspective, leaky scanning could be further used as a means to control protein expression to desired levels, since restart of translation after a premature stop codon is sensitive to the number of nucleotides separating the stop and restart codons [39]. By changing the position of the restart methionine relative to the premature stop codon, it might be possible to significantly change the level of expression of the distal protein fragment and hence functional protein as a whole. Thus, leaky scanning remains as an attractive possibility for boosting or depressing protein levels in a transfected cell.

Materials and Methods

**Primary cultures of mouse myotubes**

Primary cultures were prepared from hind limbs of day 18 embryos (E18) as described previously [23]. cDNAs of interest and a separate expression vector encoding the T-cell membrane antigen CD8 were subcloned into the mammalian expression vector pSG5 (Stratagene, CA) and were mixed and cotransfected with the polyamine LT-1 (Panvera, WI). Whole-cell recordings and immunostaining were done 3–5 days after transfection. Cotransfected cells were recognized by incubation with CD8 antibody beads (Dynal, Norway). The coincidence of expression of CD8 and a cDNA of interest was >85%.

**α\(_{1S}\) cDNA constructs**

All cDNA constructs were sequenced twice or more using BigDye technology (Perkin Elmer, CA) at a campus facility. For epitope tagging and expression in mammalian cells, the unmodified full-length rabbit α\(_{1S}\) cDNA encoding residues 1–1873 (Genebank #M23919 nucleotide coordinates nt 226 to nt 5847) was fused in frame to the first 11 amino acids of the phage T7 gene 10 protein in pSG5 using AgeI and NotI cloning sites. All constructs were made using the T7 tagged α\(_{1S}\) as template in PCR-based strategies, some previously described [20, 21]. All primers were HPLC-purified (Operon, CA) and a phosphate was tagged to the 5’-end of the sense primer. Genebank #M23919 nucleotide coordinates are used below to describe primers.

**pSG5 wt-α\(_{1S}\)**

A unique silent HindIII site was introduced by PCR at nt 2228 in the full-length α\(_{1S}\) template and cloned into the T7-α\(_{1S}\) pSG5 vector using AgeI and XhoI sites. The HindIII-XhoI fragment (nt 2228 to nt 2878) encompassing the II-III loop was subcloned into pcRII TOPO TA (Invitrogen, CA) and this plasmid was further used for PCR reactions.

**pSG5 fs-α\(_{1S}\)**

PCR reactions for deletion of residues 671–690, consisted of 10 nanograms pCR 2.1 TOPO/HindIII-XhoI insert, 15 pmoles of each primer, 0.5 mM dNTPs, 1X cloned Pfu buffer (Stratagene) and 2.5 U cloned Pfu DNA polymerase (Stratagene). The antisense primer was complementary to nt 2202 to nt 2235 and the sense primer was nt 2236 to nt 2237. Amplification was carried out for 30 cycles at 95°C for 45 seconds, 60°C for 2 minutes and 72°C for 2 minutes/kb of plasmid. The PCR reaction was treated with 10 U of DpnI (Stratagene) and recircularized with T4 DNA ligase (Stratagene). Once amplified by PCR, the HindIII-XhoI digest was ligated into the T7-α\(_{1S}\) pSG5 vector using the same restriction sites.

**pSG5 fs-α\(_{1S}\)M701I**

The construct was produced by a two-step PCR reaction using fs α\(_{1S}\) as template. Using conditions as above, the sense primer (nt 1932 to nt 1959) was paired with antisense primer 5’TGACAGTTTCTGGCGATCAAGTCTCGTCC3’ carrying the point mutation. In a separate reaction, sense primer 5’GGAGAAGTCTGTGATCGCCAAGAAGCTGGA3’ was paired with antisense primer (nt 3100 to nt 3081). The two PCR products were diluted 1:500 in ddH\(_2\)O and then hybridized to each other for 2 minutes at 95°C, 1 minute at 60°C, 1 minute at 72°C for 4 cycles. 15 pmol of nt 1932 to nt 1959 primer and 15 pmol of nt 3100 to 3081 primer were added and further cycled for 4 minutes at 95°C, then 30X of 2 minutes at 95°C, 1 minute at 60°C, 1 minute at 72°C and finally 10 minutes at 72°C. The PCR product was then cloned into the fs α\(_{1S}\) construct using HindIII and XhoI sites.

**pSG5 α\(_{1S}\)Δ1–700**

The construct was produced by cutting pSG5 fs α\(_{1S}\) with AgeI and HindIII enzymes and filling-in the overhangs.
with klenow fragments. The plasmid was religated using DNA T4 ligase.

**Whole-cell voltage-clamp**

Whole-cell recordings were performed as described previously [22] using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). All experiments were performed at room temperature. Patch pipettes had a resistance of 1–2 MΩ. The external solution was (in mM) 130 TEA-Methanesulfonate, 10 CaCl₂, 1 MgCl₂, 10 HEPES-TEA(OH), pH 7.4. The pipette solution was (in mM) 140 Cs-aspartate, 5 MgCl₂, 0.1 EGTA (for Ca²⁺ transients) or 5 EGTA (for Ca²⁺ current). 10 MOPS-CsOH, pH 7.2. The voltage dependence of peak intracellular Ca²⁺ loading buffer composed 100 mM Tris-Cl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue and 20% glycerol. Samples were incubated at 100°C for 20 minutes. Approximately 10 mg of total protein was applied to a 5–15% SDS polyacrylamide gel and electrophoresed for 2 hours at 40 mA. Proteins were transferred to PVDF membranes and analyzed with either anti-T7 or SKI antibodies and the appropriate secondary antibodies. The subunits were visualized using SuperSignal ECL reagent (Pierce, Rockford, IL). The images were captured on a Chemi-Imager (Alpha Innotech, San Leandro, CA) set to a level just below saturation.

**Confocal fluorescence microscopy**

Line-scans were performed as described [26] in cells loaded with 4 mM fluo-3 AM (fluo-3 acetoxymethyl ester, Molecular Probes, OR) for ~30 minutes at room temperature. Cells were viewed with an inverted Olympus microscope with a 20X objective (N.A. 0.4) and a Fluoview confocal attachment (Olympus, NY). Excitation light was provided by a 5 mW Argon laser attenuated to 20% with neutral density filters. For immunofluorescence, confocal images had a dimension of 1024 by 1024 pixels (0.35 microns/ pixel) and were obtained with a 40X oil-immersion objective (N.A. 1.3).

**Immunostaining**

Cells were fixed and processed for immunofluorescence as described [4,20]. The N-terminal fragment expressed by fs-α₁S or wt-α₁S was identified with a mouse monoclonal antibody against a T7 epitope fused to the N-terminus of α₁S. The anti-T7 antibody (Novagen, WI) was used at a dilution of 1:1000. Secondary antibodies were a fluorescein-conjugated goat anti mouse IgG (Boehringer Mannheim, IN) used at a dilution of 1:1000 and a fluorescein-conjugated donkey anti-rabbit IgG (Chemicon, CA) used at a dilution of 1:1000.

**Western blots**

The C-terminal fragment was identified with SKI, a rabbit polyclonal antibody against the II-III loop of α₁S (Ala739-Ile752) previously characterized [34]. Cells were scrapped from tissue cultures dishes with cold PBS plus protease inhibitors and spun in a cold table-top centrifuge. Cells were homogenized in a glass-teflon homogenizer in a minimal volume of PBS and diluted 1:1 (vol:vol) with SDS-gel loading buffer composed 100 mM Tris-Cl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue and 20% glycerol. Samples were incubated at 100°C for 20 minutes. Approximately 10 mg of total protein was applied to a 5–15% SDS polyacrylamide gel and electrophoresed for 2 hours at 40 mA. Proteins were transferred to PVDF membranes and analyzed with either anti-T7 or SKI antibodies and the appropriate secondary antibodies. The subunits were visualized using SuperSignal ECL reagent (Pierce, Rockford, IL). The images were captured on a Chemi-Imager (Alpha Innotech, San Leandro, CA) set to a level just below saturation.

**Abbreviations**

DSMO (dimethyl sulfoxide); EGTA (ethylene glycol bis-α-monoethylether tetraacetic acid); HEPES (2-hydroxyethyl piperazine 2-ethane sulfonic acid); MOPS (3N-Morpholino-propane sulfonic acid); PVDF (polyvinylidene difluoride); TTX (tetrodotoxin); TEA (tetraethylammonium); ORF (origin of replication).

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