Insertion of the full-length calcium channel α_{1S} subunit into triads of skeletal muscle in vitro

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Abstract   A full-length and a C-terminally truncated form of the calcium channel α_{1S} subunit can be isolated from skeletal muscle. Here we studied whether full-length α_{1S} is functionally incorporated into the skeletal muscle excitation-contraction coupling apparatus. A fusion protein of α_{1S} with the green fluorescent protein attached to its C-terminus (α_{1S}-GFP) or α_{1S} and GFP separately (α_{1S}+GFP) were expressed in dysgenic myotubes, which lack endogenous α_{1S}. Full-length α_{1S}-GFP was targeted into triad junctions and restored calcium currents and excitation-contraction coupling. GFP remained colocalized with α_{1S}, indicating that intact α_{1S}-GFP was inserted into triads and that the C-terminus remained associated with the excitation-contraction coupling apparatus.
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Key words: Calcium channel; Excitation-contraction coupling; Immunofluorescence; Skeletal muscle

1. Introduction

Voltage-gated calcium channels are a family of heteromeric membrane proteins composed of the pore forming α_1 subunit and the accessory α_2/δ, β, and – at least in skeletal muscle – a γ subunit [1]. Several classes of α_1 subunits have been characterized, some of which exist as multiple variants. Alternative mRNA splicing most likely yields multiple isoforms of the neuronal P-type and N-type channels and of the class D channels, resulting in α_1 subunits which differ in the length of the C-terminus [2–6]. Splice variants of the class C calcium channel differ in internal regions of the protein [7] and in the C-terminus [8]. Furthermore, both the cardiac class C (α_{1C}) and the skeletal class S (α_{1S}) channels exist as a long and a short isoform [9–11]. For the short isoforms no mRNAs have been characterized, suggesting that these isoforms arise from proteolytic truncation at the C-terminus.

The full-length skeletal muscle α_{1S} subunit has a molecular mass of 214 kDa, which is consistent with the molecular mass predicted from the primary structure. The short form has a molecular mass of 193 kDa, both as determined by Ferguson analysis [11]. However, both forms migrate abnormally with respect to molecular weight markers on SDS gels, giving rise to bands at apparent lower molecular weights. The two size forms of α_{1S} are differentially phosphorylated by cAMP-dependent protein kinase [12,13], with the C-terminus of the long form carrying two to three specific, potentially important phosphorylation sites for receptor-mediated modulation via protein kinase A [14]. Expression of the two size forms in skeletal muscle in vivo and in vitro is strongly unbalanced, in that the truncated α_{1S} isoform is predominant constituting over 90% of the total [10,11]. The skeletal muscle α_{1S} subunit is localized in the junctional transverse tubule (t-tubule) membrane [15,16], but it is not known if one or both length forms are expressed in the junction or whether the two length forms exist in distinct subcellular compartments.

In skeletal muscle α_{1S} serves two functions (see [17] for review): first, it is the voltage sensor for depolarization-induced release of calcium from the sarcoplasmic reticulum (SR) calcium stores. This process activates rapidly and is independent of the conductance of calcium through the channel. Second, α_{1S} functions as a slowly activating, dihydropyridine-sensitive calcium channel. Only a small fraction of the total dihydropyridine binding sites in skeletal muscle can account for the measured calcium conductance [18]. Thus, it has been suggested that the minor full-length channel isoform functions as the calcium channel, whereas the predominant truncated isoform constitutes the voltage sensor for excitation-contraction coupling [19]. This hypothesis was partially refuted by an experiment in which expression of a short isoform from cDNA restored excitation-contraction coupling as well as L-type calcium conductance in dysgenic myotubes, which lack the endogenous α_{1S} [20]. This showed that the truncated isoform alone is capable of performing both functions. However, the question about the localization and biological function of the long α_{1S} in skeletal muscle remained unresolved.

Recent findings about the cardiac α_{1C} provided new fuel to the controversy about the biologically relevant α_1 subunit length form in muscle [21]. Immunolabeling and quantitative biochemistry showed that despite the fact that after purification the bulk of α_{1C} was truncated, in the cardiac myocyte α_{1C} existed in the non-truncated form. This observation prompted us to address this problem in skeletal muscle. Here we asked the question of whether in skeletal myotubes full-length α_{1S} can be correctly incorporated into the triad junctions without prior truncation at the C-terminus and whether it can function as both calcium channel and voltage sensor in excitation-contraction coupling. Expression of a fusion protein of α_{1S} and a C-terminally located green fluorescent protein (GFP) in dysgenic myotubes revealed that cleavage of the C-terminus of α_{1S} is not required for normal targeting and dual function of the channel.

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2. Materials and methods

2.1. Cell culture and transfections

Myotubes of the homozygous dysgenic (*mdg/mdg*) cell line GLT were cultured as described by Powell et al. [22]. At the height of myoblast fusion (2–4 days after addition of differentiation medium) GLT cultures were transfected using a liposomal transfection reagent (DOTAP, Boehringer Mannheim). A total DNA concentration of 5–10 μg was applied per 35-mm culture dish. In cotransfections two expression plasmids were combined at equimolar concentrations, yielding coexpression in approximately 80% of transfected cells. One day after transfection the medium was changed and the cultures were incubated for 2–4 days at 37°C or at 30°C to achieve optimal folding of GFP.

2.2. Expression plasmids

For coexpression experiments of α_{1S} and of GFP (α_{1S}+GFP) rabbit skeletal muscle α_{1S} cDNA cloned into pcDNA3 [23] and the S65T version of GFP inserted into pRK5 [24] were used. α_{1S}-GFP cDNA encoding a full-length α_{1S} subunit with GFP S65T fused to its C-terminus was constructed as follows. The coding region of GFP S65T was excised from plasmid pRK5 by digestion with *Bam*HI (immediately preceding the 5′-end of GFP) and with *Eco*RI (downstream of the stop codon). This GFP cDNA was ligated into the multiple cloning site (a *Hin*dIII–*Eco*RI polylinker fragment of plasmid pSP72 (Promega)) of a proprietary mammalian expression plasmid under the control of a cytomegalovirus promoter. To yield α_{1S}-GFP this intermediary construct was opened by *Sal*I/*Xba*I restriction cuts and coligated with the *Sal*I*–*Bgl*II fragment (nt 5–4488) and the PCR-generated *Bgl*II–*Xba*I* fragment (nt 4488–5620) of α_{1S} (accession number X05921). Asterisks indicate restriction enzyme sites introduced by PCR. The artificial *Xba*I* site (nt 5620) erases the native TGA stop codon of α_{1S} (nt 5620–5622) and allows an in-frame fusion with GFP. The PCR-modified cDNA portion was confirmed by sequence analysis.

2.3. GFP and immunofluorescence labeling

Differentiated GLT cultures were fixed in 4% paraformaldehyde and immunostained as previously described [25], using the monoclonal antibody 1A against the DHP receptor α_{1S} subunit at a final concentration of 1:1000 [26], the affinity-purified antibody #5 against the type 1 ryanodine receptor (RyR) at a dilution of 1:5000 [25], and the affinity-purified anti-GFP antibody at a dilution of 1:2000 (Molecular Probes, Eugene, OR, USA). The antibodies were carefully characterized for their use in immunofluorescence experiments in previous studies [25,27,28]. The following antibody and fluorochrome combinations were used: in the double-labeling experiments with anti-GFP and anti-α_{1S}, anti-GFP was used in combination with a fluorescein-conjugated secondary antibody so that both the intrinsic GFP signal and the GFP antibody label were seen in the green channel. The intrinsic GFP fluorescence and the GFP immunolabel gave identical labeling patterns, however, the immunofluorescence signal was stronger and more stable than the intrinsic GFP fluorescence. Therefore it was used for the quantitative analysis of the labeling patterns. Controls, for example the omission of primary antibodies and incubation with inappropriate antibodies, were routinely performed.

Quantitative analysis of the labeling patterns was performed by systematically screening the coverslips for transfected myotubes using a 63×, 1.4 NA planapochromat objective on an epifluorescence microscope (Carl Zeiss, Oberkochen, Germany). The immunofluorescence labeling patterns of positive myotubes with more than two nuclei were classified as either 'clustered', 'endoplasmic/sarcoplasmic reticulum' (ER/SR), 'diffuse/cytoplasmic', or 'other' in cases where the labeled compartment was not clearly identifiable. The counts were obtained from several samples of at least three different experiments for each condition.

2.4. Combined patch-clamp and calcium recording

Whole-cell patch-clamp recordings were performed with an Axopatch 200A amplifier controlled by pClamp 6.0 software (Axon Instruments, Foster City, CA, USA). The bath solution contained (in mM): 10 $CaCl_2$, 145 tetraethylammonium chloride, and 10 HEPES (adjusted to pH 7.4 using TEA-OH). Patch pipettes had resistances of 2–4 MΩ when filled with 145 Cs-aspartate, 10 HEPES, 2 Mg-ATP, 2 Cs-EGTA, 0.5 $MgCl_2$ and 0.2 Fluo-3-K_5 (adjusted to pH 7.4 with CsOH). Capacitative currents were compensated using the built-in analog circuits. Leak currents were digitally subtracted by a P/4 prepulse protocol. Recordings were low-pass Bessel filtered at 1 kHz and sampled at 2 kHz. *I–V* curves were determined with 500 ms depolarizing steps from a holding potential of −80 mV to test potentials of −60, −50 ... to +80 mV. Calcium current densities were normalized by linear cell capacitance (expressed in p*A*/p*F*). The recording chamber was mounted on a Zeiss Axiovert epifluorescence microscope equipped with a Photon Technology International (PTI, S. Brunswick, NJ, USA) photometer system allowing the simultaneous recording of the Ca^{2+} fluorescence signal. Transient changes in fluorescence intensity were normalized by the resting fluorescence ($\Delta F/F$).

Action potential-induced Ca^{2+} transients were recorded in cultures incubated with 5 μM Fluo-3-AM plus 0.1% Pluronic F-127 (Molecular Probes, Eugene, OR, USA) in HEPES and bicarbonate-buffered DME for 45 min at room temperature as described by Flucher et al. [25]. Action potentials were elicited by passing 1-ms pulses of 20–30 V across the 19-mm incubation chamber. The fluorescence signals from single myotubes were recorded at a sampling rate of 200 Hz using PTI Oscar software. Traces were normalized by calculating the $\Delta F/F$ ratio.

3. Results

3.1. Restoration of I_{Ca} and excitation-contraction coupling in dysgenic myotubes by α_{1S}-GFP

A mammalian expression plasmid encoding a fusion protein of the full-length α_{1S} subunit of the skeletal muscle L-type calcium channel with GFP attached to the C-terminus (α_{1S}-GFP) was generated. Transient transfection of the dysgenic cell line GLT, which lacks the endogenous α_{1S}, with the plasmid encoding the α_{1S}-GFP fusion proteins restored calcium currents and excitation-contraction coupling.

Stimulation of α_{1S}-GFP-transfected cultures with extracellular voltage pulses gave rise to calcium transients (Fig. 1A) with properties similar to those previously observed in GLT myotubes reconstituted with α_{1S} [27] or in myotubes of normal muscle cultures [28]. Stimulation with increasing voltage pulses gave an all-or-none response (not shown), indicating that the calcium transients were induced by action potentials. Myotubes expressing α_{1S}-GFP were also selected for combined whole-cell patch-clamp recordings and calcium measurements with the fluorescent calcium indicator Fluo-3. Depolarizing voltage steps gave rise to slowly activating voltage-dependent calcium currents which peaked between +30 and +40 mV and to increases of cytoplasmic free calcium concentrations (Fig. 1B). The current properties were characteristic of skeletal muscle L-type calcium currents and were equal to those recorded in GLT myotubes reconstituted with the wild type α_{1S} subunit [27]. The calcium transients showed a voltage dependence different from that of the currents. Activation of depolarization-induced calcium transients started at 20 mV more negative test potentials than current activation (Fig. 1C). Moreover, calcium transients did not decline in parallel with calcium currents at test pulse potentials above +30/+40 mV and had still maximal magnitude at test potentials to near the reversal potential (+80 mV), where no calcium influx occurred as a result of reduced driving force. This indicates that calcium transients were not dependent on the influx of calcium ions but represent skeletal muscle-specific depolarization-induced calcium release from the SR. Comparable calcium transients were recorded in myotubes of the skeletal muscle cell line C_2C_{12} (not shown). Together these experiments show that transfection of dysgenic myotubes with α_{1S}-GFP restores
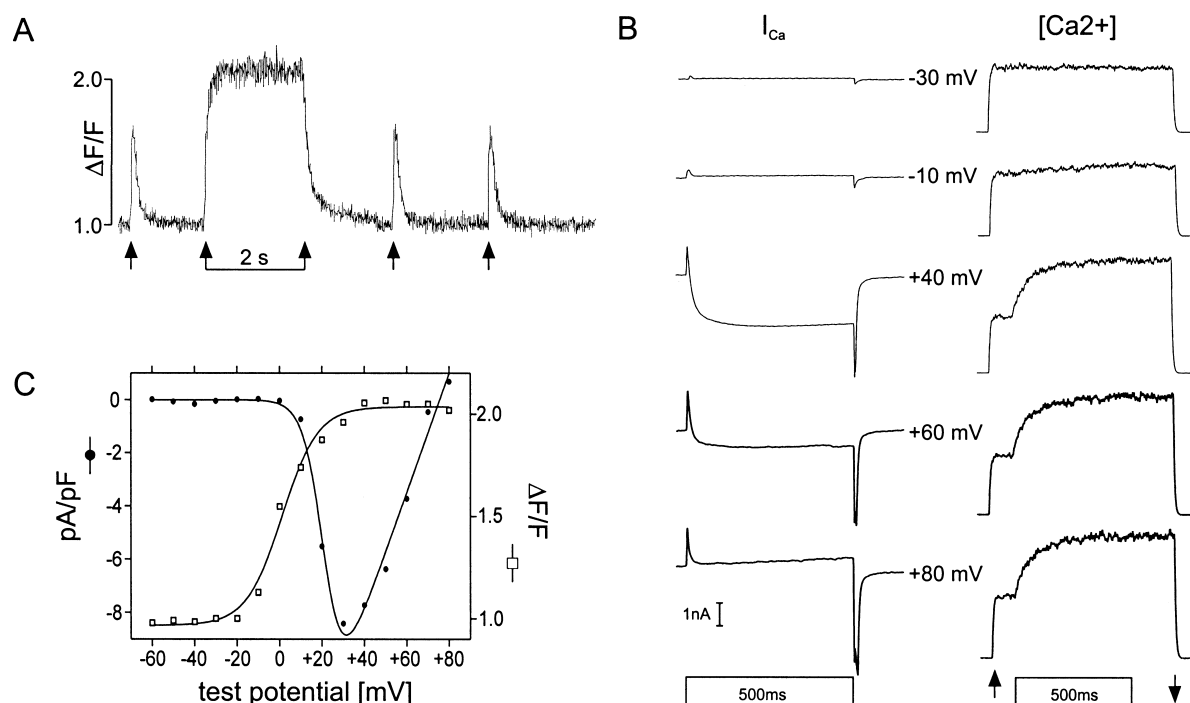
Fig. 1. Full-length α_{1S}-GFP restores skeletal muscle excitation-contraction coupling in transfected dysgenic myotubes. A: Action potential-induced calcium transients recorded from an α_{1S}-GFP-transfected myotube loaded with Fluo-3 AM. Individual stimuli are indicated by arrows, a 2-s 20-Hz stimulation train by the bracketed arrows. B: Simultaneous recordings of calcium currents (I_{Ca}) and depolarization-induced calcium transients ([Ca^{2+}]) under whole-cell voltage-clamp conditions. Step depolarizations were applied from a holding potential of -80 mV to the indicated membrane potentials. The duration of the test pulse (500 ms) and the opening and closing of the excitation light shutter (arrows, right) are indicated at the bottom. Linear cell capacitance was 182 pF. C: Voltage dependence of peak current densities (pA/pF, filled symbols) and of peak fluorescence ($\Delta F/F$; open symbols) of the recordings shown in B. The fact that calcium transients activate at more negative test potentials than the L-type calcium currents and are equally high at $+30$ mV (where I_{Ca} is maximal) and at $+80$ mV (where I_{Ca} is small as a result of reduced driving force) indicates that α_{1S}-GFP elicits skeletal-type excitation-contraction coupling.

skeletal muscle L-type calcium currents and skeletal-type excitation-contraction coupling.

3.2. α_{1S}-GFP is incorporated into SR/plasma membrane and SR/t-tubule junctions

The intrinsic GFP fluorescence or immunolabeling with an antibody against α_{1S} identified myotubes expressing the fusion protein. Approximately half of these myotubes showed a clustered distribution of the fluorescent label (Table 1), reminiscent of the distribution pattern of triad proteins in normal myotubes [28]. The number of anti-α_{1S}-labeled myotubes per coverslip and the distribution patterns were similar in wild type α_{1S} (α_{1S}+GFP)- and α_{1S}-GFP-transfected myotubes, indicating that the C-terminally attached GFP did not affect expression of the α_{1S} subunit. Double immunofluorescence labeling of cultures transfected with the fusion proteins using antibodies against α_{1S} and against the RyR revealed the co-

localization of both proteins in the clusters (Fig. 2a,b). The clusters were found in linear arrays in the cell periphery and randomly arranged in deeper regions of the myotubes. Based on the discrete colocalization of α_{1S}-GFP with the RyR, the calcium release channel of the SR, these clusters are identified as junctions of the SR with either the plasma membrane or the t-tubules, called peripheral couplings and triads, respectively. Thus, heterologous expression of full-length α_{1S}-GFP in dysgenic myotubes results in the normal incorporation of the α_{1S} subunit into the junctions responsible for excitation-contraction coupling.

In those myotubes where no clusters could be identified, anti-α_{1S} labeled a reticular cytoplasmic membrane compartment. This network was very dense in the perinuclear region but the tubular nature of this compartment was best resolved in peripheral regions of flat myotubes (see Fig. 3c,g). This network represents the ER/SR system of developing myo-

Table 1
Subcellular distribution of the α_{1S} subunit and of GFP when expressed as a fusion protein (α_{1S}-GFP) or as separate proteins (α_{1S}+GFP)

cDNA construct	Antibody	Subcellular distribution (%)			n
		Cluster	ER/SR	Cytoplasmic	
α_{1S}-GFP	anti-α_{1S}	53.0	45.0	0.0	728
	anti-GFP	47.0	52.0	0.0	953
α_{1S}+GFP	anti-α_{1S}	54.4	41.6	0.0	769
	anti-GFP	0.0	0.0	100.0	696

Percentages give the fraction of total number of counted myotubes (n) in each category. The balance to 100% represents myotubes that could not unambiguously be classified.
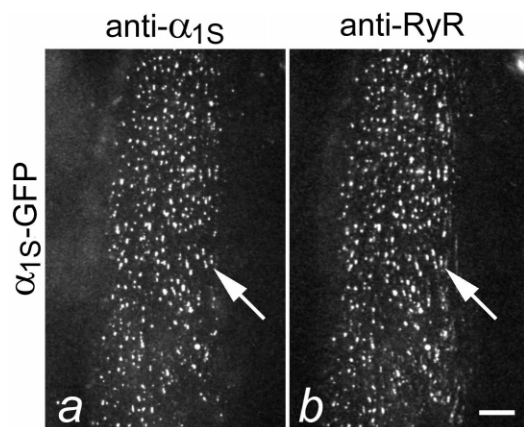
Fig. 2. Expression and subcellular localization of α_{1S}-GFP in a transiently transfected dysgenic myotube. Double immunofluorescence labeling with antibodies against α_{1S} (a) and against the ryanodine receptor (b) shows the colocalization of both channels in clusters (examples indicated by arrows), representing junctions of the SR with t-tubules or the plasma membrane. Bar, 10 μm.

tubes, suggesting that α_{1S} is expressed at high concentrations in the biosynthetic apparatus. The two labeling patterns – clustered and reticular – were not mutually exclusive. Myotubes with clearly identifiable clusters in one region could show ER/SR distribution near the nuclei of another region. However, the ER/SR labeling pattern was always strongly reduced in myotubes that also expressed clusters. The clustered distribution was more often found in cultures with an

overall higher degree of differentiation. This suggests that expression of α_{1S} or α_{1S}-GFP initially results in an accumulation of the gene products in the ER/SR and that this is reduced when the channels are efficiently exported to the outer membrane upon differentiation of the excitation-contraction coupling apparatus.

3.3. The C-terminus of α_{1S}-GFP is present in SR/plasma membrane and SR/t-tubule junctions

If the C-terminus of the α_{1S} subunit needed to be cleaved prior to insertion into peripheral couplings and triads, the C-terminally attached GFP should not be found in the junctions. Comparison of α_{1S} immunolabel with the distribution pattern of GFP fluorescence in α_{1S}-GFP-transfected myotubes indicated the colocalization of both proteins in the clusters. However, after immunolabeling the intrinsic GFP fluorescence was much weaker than the immunofluorescence signal obtained with the α_{1S} antibody. Therefore we used a specific antibody against GFP to analyze the two distribution patterns under comparable conditions. Intrinsic GFP fluorescence and anti-GFP immunofluorescence resulted in the same labeling patterns, but the immunolabel was stronger and more resistant to photobleaching.

Double immunolabeling of myotubes transfected with α_{1S}-GFP with antibodies specific for α_{1S} and for GFP showed a colocalization of both proteins in the clusters (Fig. 3a,b). This was clearly distinct from the distribution patterns obtained when the myotubes were transfected with wild type α_{1S} and GFP encoded on separate plasmids (Fig. 3e,f). In this case α_{1S} was localized in the clusters, whereas GFP was absent from
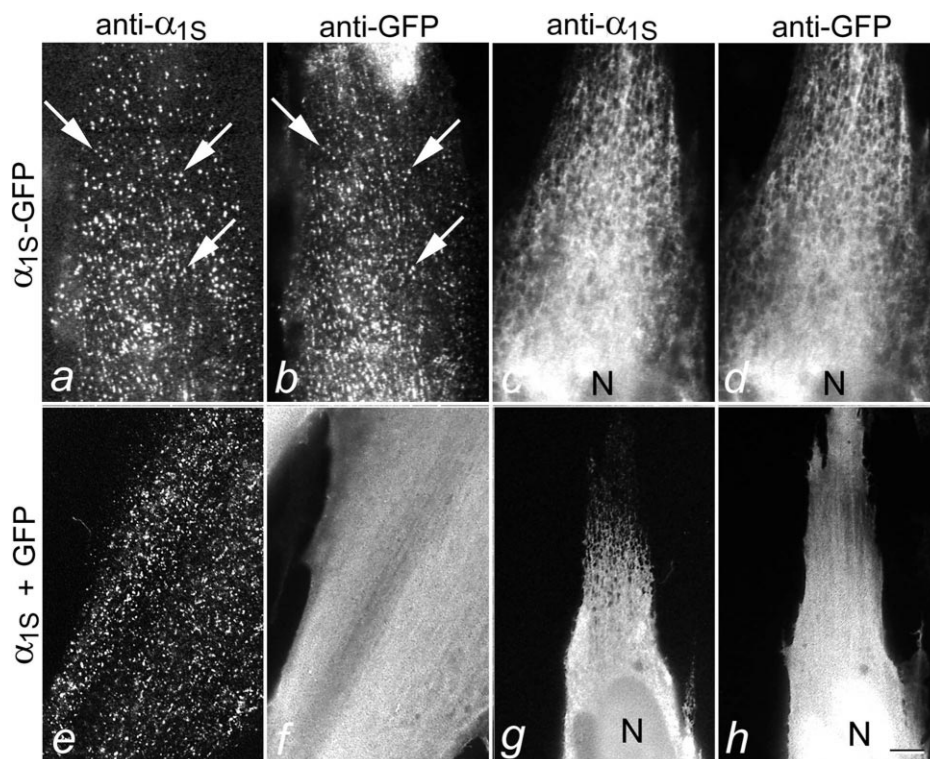

Fig. 3. Comparison of the distribution of α_{1S} and GFP in myotubes transfected either with the α_{1S}-GFP fusion protein or with separate α_{1S} and GFP (α_{1S}+GFP). In α_{1S}-GFP transfected myotubes (upper row) anti-α_{1S} and anti-GFP label is always colocalized. Either both antibodies detect the fusion protein in clusters representing junctions (a,b; examples indicated with arrows), or in the ER (c,d). In myotubes transfected with α_{1S} plus GFP from separate plasmids (lower row), anti-α_{1S} shows the same clustered distribution as the fusion protein (e), whereas GFP is diffusely distributed throughout the cytoplasm (f). N, nuclei; bar, 10 μm.

clusters but showed a diffuse distribution, indicative of a cytoplasmic localization. A similar diffuse distribution would have been expected in α_{1S}-GFP-transfected myotubes if the C-terminus with the attached GFP had been truncated before incorporation of the channel into the junction. However, there was no indication of cytoplasmic localization of GFP in myotubes transfected with α_{1S}-GFP and fixed 72 h later. The distribution patterns observed in the myotubes which had not formed clusters but showed the ER/SR localization of the channels gave corresponding results. In myotubes transfected with the fusion protein anti-α_{1S} and anti-GFP, labeled the tubular cytoplasmic network (Fig. 3c,d), whereas in myotubes transfected with the separate plasmids (α_{1S}+GFP), α_{1S} was localized in the tubular network (Fig. 3g) and GFP was diffusely localized throughout the cytoplasm (Fig. 3h). Quantitative analysis of the labeling patterns in 696–953 myotubes per condition from multiple cultures and experiments (Table 1) confirmed that in α_{1S}-GFP-transfected myotubes GFP was always colocalized with α_{1S}. In contrast, separately expressed GFP was never found concentrated in clusters or in the ER/SR network. Thus, the α_{1S}-GFP fusion protein is synthesized in the ER of developing myotubes, targeted and inserted into peripheral couplings and triads without prior truncation at the C-terminus and the C-terminus is still associated with the junctions at the time when they commence their function in excitation-contraction coupling.

4. Discussion

The results of the present study demonstrate that the C-terminus of the fusion protein α_{1S}-GFP is not truncated prior to insertion into the junctional domains of the excitation-contraction coupling apparatus. Thus, truncation of the C-terminus is not a prerequisite for the normal targeting and incorporation of α_{1S}. The C-terminally attached GFP remains associated with the α_{1S} in the junction for several days and myotubes containing the α_{1S}-GFP in their junctions show normal skeletal-type calcium currents and depolarization-induced calcium release. Beam et al. [20] obtained the restoration of calcium currents and excitation-contraction coupling in dysgenic myotubes using an expression plasmid encoding a truncated α_{1S}. This result disproved the notion that only the long form of the skeletal muscle α_{1S} subunit is capable of current conduction [19]. But if the short form is able to function both as a voltage sensor in excitation-contraction coupling and in conducting L-type calcium currents, what is the function of the long form and where in the muscle cells is it expressed? Our present data do not prove that the full-length α_{1S} is the isoform expressed in mature skeletal muscles in vivo, but they clearly show that non-truncated α_{1S}-GFP can be inserted into triads and peripheral couplings and that it remains there intact at least for a period of several days. Furthermore, the results suggest that the long isoform can support excitation-contraction coupling and calcium conductance, because at a stage where there was no indication of C-terminal cleavage, many myotubes showed depolarization-induced calcium release. It cannot be excluded that a minor fraction of truncated α_{1S} existed besides the intact α_{1S}-GFP in the junctions and that only these truncated α_{1S} were responsible for the observed depolarization-induced calcium transients. However, if truncation was a slowly progressive process, one would expect to find some myotubes at least in the better differentiated cultures that showed reduced colocalization of anti-α_{1S} and anti-GFP. But the opposite was true, the better the overall differentiation of the myotubes was, the clearer the colocalization appeared to be.

The finding that α_{1S}-GFP exists in peripheral couplings and triads in its non-truncated form is in stark contrast to the consistent observations that α_{1S} isolated from skeletal muscle tissue and from muscle cultures [10–13] predominantly exists in its short form. However, α_{1S} expressed in heterologous non-muscle expression systems is isolated in the long form [29,30]. Thus, either there is no truncation but two splice variants give rise to the two size forms in native tissue, or muscle contains a tissue-specific mechanism for the truncation of α_{1S} that is missing in the heterologous expression systems. So far all efforts to isolate α_{1S} mRNA from muscle have failed to detect a specific message for the short form of α_{1S}, or for α_{1C}, which also exists in two length forms. The possibility that non-specific proteolytic cleavage during the purification procedure is the cause of the truncation of α_{1S} in native tissue is unlikely, not only because great care has been taken in many laboratories to minimize proteolysis [9,31] but also because of the observation that a similar proteolytic cleavage did not occur when recombinant α_{1S} was isolated from heterologous expression systems [29,30]. If proteolytic processing of the C-terminus was a muscle-specific property, expression of recombinant α_{1S} in dysgenic muscle cells should have resulted in the truncation of the channel. Unfortunately, the low expression efficiency in differentiated myotubes ($<5\%$) of transiently transfected dysgenic cultures does not allow biochemical analysis. However, our immunocytochemical analysis showed no evidence of α_{1S}-GFP truncation within a 72-h expression period.

Support for the notion that the excitation-contraction coupling apparatus of native muscle cells contains the full-length α_1 subunit comes from a recent study in cardiac muscle [21]. Immunolocalization of α_{1C} with an antibody directed against the C-terminus indicated a t-tubule localization of the full-length protein. Furthermore, an estimate of the ratio of truncated to full-length α_{1C} provided the first experimental evidence that the α_{1C} subunits expressed in cardiac myocytes contain an intact C-terminus. This is consistent with our present finding that recombinant α_{1S} is inserted in skeletal muscle peripheral couplings and triads without prior truncation at the C-terminus. The authors of the cardiac study [21] offer an interesting explanation for the different proportions of long to short form in situ and in isolated preparations. If posttranslational cleavage occurs in the junction but the C-terminus remains associated with the channel complex, antibodies against the C-terminus would label the junctions in situ, whereas the non-covalently associated C-terminus would get lost during the isolation procedure. Although at this point still speculative, such a mechanism would also be consistent with our present results and could be true for skeletal muscle as well.

What are the functional implications, if full-length α_{1S} is at least partially responsible for skeletal muscle excitation-contraction coupling? The part of the C-terminus that is considered to be missing in the truncated form of α_{1S} contains several phosphorylation sites specific for cAMP-dependent protein kinase [12–14]. If, and only if, the C-terminus remains located in the junctions and constitutes part of the calcium channel complex, phosphorylation of these C-terminal sites

could represent the molecular basis for the modulation of skeletal muscle excitation-contraction coupling.

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