Endogenous cardiac Ca\textsuperscript{2+} channels do not overcome the E–C coupling defect in immortalized dysgenic muscle cells: evidence for a missing link

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Abstract The expression of subunit genes of the Ca\textsuperscript{2+} channel complex was studied in differentiating, immortalized mouse mdg cells. These cells expressed \(\alpha_{\text{s}}\) and \(\alpha_{\beta}\) subunit transcripts and several known transcript variants of skeletal, cardiac and brain \(\beta\) genes. The \(\alpha_{\text{s}}\) mutation is retained in the 129DA\textsubscript{3} cell line and occurs exclusively at nucleotide position 4010 in the skeletal \(\alpha_{\text{s}}\) transcript in which a cytosine residue is deleted. In early stages of differentiation and fusion, \(\text{Ba}^{2+}\) currents were detected in dysgenic myotubes the same as the cardiac L-type Ca\textsuperscript{2+} channel. These data provide specific structural evidence for a missing link coupling.

Key words: Muscular dysgenesis; Calcium channel; \(\alpha_{\text{s}}\) Subunit; Skeletal; Cardiac; Point mutation; Mouse

1. Introduction

Muscular dysgenisis (mdg), a spontaneous, lethal mutation in mice [1], was found to be transmitted as a single autosomal, recessive gene [2,3]. Phenotypically, the mutation appears in homozygous embryos as a severe general deficiency of the skeletal musculature while cardiac and smooth muscles show normal development and function [2,3].

Extensive functional and molecular studies [4–8] showed that the probable gene defect that is the critical locus for the development of the dysgenic phenotype is located in or in close proximity to the skeletal muscle L-type voltage-gated calcium channel \(\alpha_{\text{s}}\) subunit gene (CACHL1A3). Apparently, normal level expression was found for the \(\alpha_{\text{s}}\) subunit (CACHL2A) [7]. Thus far, no studies have been conducted for detection of \(\beta\) subunit transcripts or proteins. In recent molecular genetic analyses, we assigned the CACHL1A3 gene as the \(\text{mdg}\) mutation in the 129DA\textsubscript{3} mouse disclosed a consistently appearing point mutation, a cytosine deletion at nt 4010 that causes a frameshift and premature translation stop in the reading frame of the skeletal muscle L-VGCC \(\alpha_{\text{s}}\) transcript [10]. This observation, however, is contradictory to a previous analysis [8] with genomic DNA for the region of the CACHL1A3 gene, in which alterations were found in the restriction fragment length of the 5'-end and 5'-flanking region for the CACHL1A3 gene, providing evidence that the lack of expression or low level expression of the \(\alpha_{\text{s}}\) transcript is likely a consequence of a mutation in the promoter region of the gene.

Permanent, immortalized cell line was recently established from the \(\text{mdg}\) mouse [11] that retains the differentiation capability and all functional and phenotypical characteristics of the dysgenic mutation. Here, we present molecular evidence that the dysgenic mutation is fully retained in these cells as a point mutation in the CACHL1A3 gene at nt position 4010 of the open reading frame. We observed considerable expression of the CACHL1A3 gene transcripts as well as transcription of the class C-type (cardiac) CACHL1A1 calcium channel gene. The transcription of the latter gene can be either due to aberrant differentiation [12] in mdg cells or to a ‘turn-on’ of a compensatory mechanism that controls the level of cardiac channel transcripts. Consistent with this finding, we detected class C-type calcium channel activity in these mdg cells.

2. Materials and methods

2.1. Cell cultures

The normal mouse muscle cell line (129CB1) and the dysgenic muscle line (129DA\textsubscript{3}) were grown at 34°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum and 1.25% chicken embryo extract. Differentiation was induced by deprivation of growth factors and shifting the temperature of growth to 39°C [11]. The differentiation medium contained 10% horse serum and 1.25% chicken embryo extract in DMEM. For routine growth of the cells, tissue culture dishes were coated with 1% gelatine.

2.2. RNA extraction, Northern blotting and hybridization

Total RNA was extracted from \(10^7–10^8\) cells by the acidic guanidinium thiocyanate method [13] and polyA\textsuperscript{+} RNA was purified on oligo dT cellulose columns [14]. PolyA\textsuperscript{+} RNA (2–5 \(\mu\)g) was denatured and size separated on 1.2% denaturing formaldehyde-agarose gels and transferred onto Hybond C filters (Amersham). Filters were hybridized with \(^{32}\)P-labeled [15] DNA probes in 50% formamide, 0.5 M NaCl, 10 mM Na\textsubscript{PO}\textsubscript{4}, pH 7.4, 5 mM EDTA, 0.1% SDS and 10% dextrane sulphate at 45°C. The highest stringency washing of filters was done in 150 mM NaCl, 10 mM Na\textsubscript{PO}\textsubscript{4}, pH 7.4, 1 mM EDTA and 0.1% SDS at 55°C. Blots were exposed to Kodak X-OMAT films at −70°C for various times (from 2 to 24 h).

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Abbreviations: mdg, muscular dysgenesis; L-VGCC, L-type voltage-gated Ca\textsuperscript{2+} channel; nt, nucleotide.
2.3. PCR amplification of segments of skeletal and cardiac α gene products

Multiplex PCR was performed on 2 µg polyA* RNA isolated from non-differentiated and differentiated cultured mouse muscle cells (129CB3, 129DA3) and from murine muscle tissues. The first strand synthesis was done with 1 µg polyA* RNA using 200 U of Superscript reverse transcriptase (GIBCO-BRL) and 6-mer random primers at 50°C for 1 h. For the second strand synthesis and amplification, multiplex PCR was performed using 2 U of Vent polymerase and three primers. The reverse primer was designed for a region in motif I that is highly homologous between skeletal [10] and cardiac α subunits [16]. This sequence lies betweennts 1942-1962 and 675-695 for cardiac and skeletal muscle clones, respectively. The two forward primers were designed for a more 5'-upstream sequence that is non-homologous between the cardiac (1249-1268) and skeletal (206-226) messages. PCR (Roche Molecular Systems) was done in 32 cycles including denaturation at 95°C for 2 min, annealing at 68°C for 30 sec and extension for 2 min at 72°C. The PCR products were separated on 2% sieving agarose gels. RT-PCR amplification for the motif IVS-S segment of both skeletal and cardiac α was done utilizing the same conditions as described for motif I regions. The primers were designed for nts 3935-3954 (forward) and 4108-4087 (reverse) corresponding to the skeletal α sequence. The PCR products were isolated from preparative gels, subcloned into pBluescriptSK(+) at the EcoRV site and sequenced using the dideoxy chain termination method [17].

2.4. Cell cultures for electrophysiology and electrophysiological recordings

Cells were plated at very low density (100–500 cells/60 mm dish) and grown on glass coverslips coated with 1% gelatin. 24 h after plating, differentiation was induced as described above and electrophysiological recordings were done at a very early stage of differentiation (usually on the second and third day of differentiation) when most of the cells were in the non-fused stage or myotubes did not contain more than 3 nuclei/cell. Barium currents (Ib) were recorded in the whole-cell configuration [18] at room temperature (20-22°C). The bathing solution was produced using the pCLAMP software package (version 5.5; Axon Instruments, CA). The hybridizing RNA band was of identical size with that from that seen in normal skeletal muscle. The immortalized normal and dysgenic (129DA3) mouse muscle cells. PolyA* RNAs from 129CB3 and 129DA3 cells were hybridized with various Ca²⁺ channel subunit cDNA probes. ND, non-differentiated; D, differentiated. The probes are: skeletal α1; 6.1-kb A-val fragment of the full-length rabbit cDNA [19], cardiac α1; 5.2-kb EcoRI fragment of the rabbit cDNA [20], skeletal α2; 3.5-kb EcoRI fragment of the rabbit cDNA [19], skeletal β1; 1.6-kb EcoRI fragment of the rabbit cDNA [21], Rβ; 1.6-kb PstI fragment of the cross-hybridizing rat β-actin probe [23], GAPDH; 0.7-kb PstI/XbaI fragment of the glyceraldehyde-3-phosphate dehydrogenase cDNA [22].

3. Results

In Northern hybridizations, a skeletal muscle L-VGCC α1 (α1S) subunit probe provided clear signals with a 6.5-kb mRNA species for both of the differentiated 129CB3, and 129DA3 cells. The hybridizing RNA band was of identical size with that detectable in a mouse skeletal muscle preparation. When the same blot was hybridized with a cardiac L-VGCC α1 (α1C) probe, cross-hybridization was noted with the 6.5-kb band, however, an 8.6-kb band was clearly detectable in differentiated 129DA3 cells, the size of which was identical with cardiac α1 mRNA from mouse heart (Fig. 1). A skeletal muscle α2 probe detected hybridization with a 8.5-kb message for 129CB3, and 129DA3 cells as well. The skeletal muscle β1 probe cross-hybridized with four bands corresponding to RNA sizes of 1.9, 3.5, 5.3 and 6.8 kb. These correspond to products of β1, β2, β3, and β4 genes. The pattern of expression of β messages in immortalized normal and dysgenic muscle cells is somewhat different from that seen in normal skeletal muscle. The immortalized cells express commensurable amounts of all four β messages while the β1 gene product is prevalent in normal skeletal muscle and the expression of β2 and β3 genes is significantly lower. The β4 gene expression was not detectable in mouse skeletal muscle.

A cross-hybridizing β-actin gene cDNA was used to monitor the progress of differentiation. Hybridization with this probe shows that during differentiation the expression of α1-actin is turned on, however, β4-actin expression is not completely eliminated. This phenomenon is typical for the immortalized muscle cells [23]. The expression of skeletal and cardiac α1 transcripts were assessed relative to the level of the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene. We estimated that skeletal α1 expression was 10–30-fold lower in differentiated 129DA3 cells compared to that of 129CB3 normal mouse muscle cells.

RT-PCR amplification of α1S- and α1C-specific cDNA se-
The DNA sequences of the PCR products showed 100% identity with the mouse skeletal and cardiac \( \alpha_{1} \) sequences. The amplifications have not been optimized on quantitative scale, thus no conclusions can be reached for the relative abundance of \( \alpha_{1S} \) and \( \alpha_{1C} \) transcripts in these cells.

PCR amplification on a segment (IVS2-IVS6) between nts 3935-4087 resulted in the appearance of a product with 152 bp in length both for 129CB3 and 129DA3 mRNA preparations (data not shown). Sequencing of the 129DA3 mRNA related PCR product on both strands revealed a DNA sequence that was 100% identical with that of the mouse \( \alpha_{1C} \) except at nt position 4010. At this position, a deletion mutation, the lack of a cytosine nucleoside residue, was noted compared to the sequence obtained from 129CB3 cells and matched that published previously [10] (Fig. 3A). The existence and nature of this mutation was confirmed by using several different mRNA preparations from 129CB3 and 129DA3 cells and also by independent amplifications of the same RNA preparation. We have found that the deletion mutation consistently appears in 129DA3 cells and never appears in the normal counterpart, in 129CB3 cells. The mouse \( \alpha_{1C} \) sequence for this region did not show this mutation and was found to be fully identical with the published mouse cardiac \( \alpha_{1} \) sequence (data not shown) [16].

The cytosine deletion at nt 4010 in the 129DA3 \( \alpha_{1} \) sequence generates a frameshift mutation in the conceptual translation. This frameshift generates a stop codon at nt 4173 that prematurely terminates the translation of the \( \alpha_{1} \) message. Consequently, part of the topological arrangement of the \( \alpha_{1} \) in motif IV is altered. The translation stops in the mutant \( \alpha_{1D} \) after the motif IV S1 region and the critical SS2 segment that carries glutamic acid residues important for Ca\(^{2+}\) selectivity are missing [24–26]. Further, the IVS6 and the C-terminal tail are deleted (Fig. 3B).

Depolarization of voltage-clamped 129DA3 cells from a holding potential of −80 mV to various test potentials from −40 to +50 mV induced inward barium currents (IBa) in a voltage-dependent manner (Fig. 4A). The currents activated with fast kinetics and decayed following inactivation kinetics, in a manner typical for cardiac L-type IBa's. The current-voltage relationship of these current recordings is depicted in Fig. 4B. The currents activated at −30 mV and reached peak current values at +10 mV potential. This high-voltage activated IBa was found to be sensitive to dihydropyridine agonists. Application of 1 μM Bay K 8644 increased the peak current −4-fold, shifted the voltage-dependence of activation and inactivation to hyperpolarizing potentials and accelerated inactivation (Fig. 4C). The IBa also exhibited sensitivity to β-adrenergic modulation. Isoproterenol at a concentration of 10 μM increased IBa −2-fold (Fig. 4D). In our experiments, we never detected slow L-type IBa that would be related to skeletal L-type Ca\(^{2+}\) channel activity.

4. Discussion

We have shown that the \( mdg \) mutation is molecularly retained in an immortalized cell line (129DA3) and is in the \( \alpha_{1} \) sequences was carried out on mRNA preparations from non-differentiated and differentiated forms of 129CB3 and 129DA3 cells. Multiplex PCR resulted in the appearance of 470 and 694 bp products in both cell lines that are characteristic cDNA segments for skeletal and cardiac \( \alpha_{1} \), respectively (Fig. 2A). The DNA sequences of the PCR products showed 100% identity with the mouse skeletal and cardiac \( \alpha_{1} \) sequences (Fig. 2B). The amplifications have not been optimized on quantitative scale, thus no conclusions can be reached for the relative abundance of \( \alpha_{1S} \) and \( \alpha_{1C} \) transcripts in these cells.
the nature and function of the skeletal muscle L-VGCC α₁ subunit. First, due to the presence of the premature stop codon, the mutant transcript codes for a protein with a length of 1352 amino acids and a molecular weight of 153,681. This is a considerable truncation compared to that of the full-length sequence of the α₁ from normal mouse muscle (1873 amino acids, molecular weight: 212,018). Second, in the mutant protein part of the IVS1, the IVS2, the extracellular connecting loop to IVS6, the IVS6 and the entire C-terminal cytoplasmic tail is missing. Some of these segments, in particular the IVS1, IVS2, and the putative E-F hand [27] on the C-terminal tail may also be involved in Ca²⁺ binding. Based on hydropathy analysis and prediction of secondary structure, we suggest that the C-terminus of the mutant protein will be largely globular and localized intracellularly. Therefore, it is unlikely that the mutant α₁ protein will and can exert calcium channel function. Our electrophysiological data are consistent with this since no skeletal L-type I₅Na was recorded in dysgenic 129DA₃ cells [11].

Immortalized dysgenic mouse skeletal muscle cells express detectable amounts of skeletal and cardiac L-VGCC α₁ subunit mRNAs. The expression level for the skeletal α₁ transcript was reported to be dramatically diminished compared to that obtained in normal mouse skeletal muscle cells [10]. We cannot understand, however, how the single point mutation around the middle of the mRNA could be responsible for the reduced mRNA levels [10]. Most likely, the decreased expression is due to some compensatory mechanisms activated by the altered Ca²⁺ homeostasis of these cells. Alternatively, decreased mRNA stability is also possible [10,12] although it is difficult to understand the underlying molecular mechanisms. The present and previous data [10,12] are contradictory to an earlier description that localized the mutation of muscular dysgenesis in the 5'-end and flanking sequences of the skeletal muscle L-VGCC α₁ subunit gene [8]. Two other skeletal muscle L-VGCC subunit mRNAs; two transcripts for α₂/β and numerous β subunit transcripts are expressed at a level similar to that observed in control cells. Further, this provides an explanation as to why expression of skeletal [8], cardiac [28] and brain [29] α₁ cDNAs in primary cultures of mdg muscle cells always elicited Ca²⁺ channel currents that were very close to the native behavior.

It is tempting to speculate about possible phenotypic characteristic of dysgenic skeletal muscle in view of the causative point mutation and the present discovery of cardiac Ca²⁺ channels.
It is conceivable that the lack of excitation–contraction (E–C) coupling is due not only to the low level of aberrant α1S subunits but also to a process that excludes the endogenous α1C from interacting with the coupling machinery. Interestingly, overexpression of cardiac Ca\(^{2+}\) channels restores a modified, cardiac-like E–C coupling [28] while overexpression of α1A [29] or α1B [30] cDNAs, which deliver sizable Ca\(^{2+}\) currents, fail to restore E–C coupling.

Our data provide evidence that α1C is overexpressed in the 129DA\(_3\) dysgenic cell line, compared to the normal 129CB\(_3\) counterpart. This result is reminiscent to that observed in developing skeletal muscle [12]. Consistent with the presence of the α1C transcript, we routinely recorded cardiac L-type current in differentiating myotubes of 129DA\(_3\) cells. Here, we have demonstrated that the I\(_{\text{Ca}}\) recorded in dysgenic myotubes reflect the activity of α1C-directed Ca\(^{2+}\) channels whose electrophysiological, pharmacological and β-adrenergic regulation properties are the same as those of the class C-type Ca\(^{2+}\) channels [31]. This is the first experimental evidence showing that the appearance of α1C transcript in mdg cells gives rise to functional, C-type Ca\(^{2+}\) channel activity. While it is possible that the level of expression of the endogenous α1C, as well as the corresponding Ca\(^{2+}\) channel current may be unable to provide enough ‘Ca\(^{2+}\)’ entry to restore E–C coupling in the dysgenic cell line [11,32], this is unlikely since, the amount of ‘trigger Ca\(^{2+}\)’ required to elicit a contraction is extremely small [33,34]. We therefore suspect that some component required for E–C coupling is missing. On the other hand Tanabe et al. [28] demonstrated that overexpression of α1C in dysgenic myotubes can restore C-type E–C coupling. The presence of cardiac L-type Ca\(^{2+}\) channel in mdg myotubes is probably related to the etiology of the disorder [35] and is compensatory in nature. Thus, the expression of cardiac α1C in 129DA\(_3\) cells would be reminiscent of the early stages of skeletal muscle development.

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**References**