

Expression of chloride channel 1 mRNA in cultured myogenic cells: a marker of myotube maturation

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Received 9 September 1996

Abstract The chloride channel CIC-1 is required to maintain a normal excitability of mature muscle fibers; its blockade leads to hyperexcitability, the hallmark of the disease myotonia. In mouse and rat myotubes, representing the embryonic stage of muscle, CIC-1 mRNA is not detectable by Northern blotting. From neonatal to adult, CIC-1 expression increases at least fourfold. Using RT-PCR and hybridization on cultured myotubes we found CIC-1 mRNA at a level of 0.4–1.1% of that in mature mouse muscle, and $\leq 0.01\%$ in myoblasts, at stages when desmin mRNA levels are already high. The level of CIC-1 mRNA is thus a sensitive and specific indicator of the maturation of skeletal muscle cells.

Key words: Skeletal muscle development; Cell culture; Chloride channel 1; Myotonia; Quantitative RT-PCR

1. Introduction

Myogenesis is a striking example of differentiation that can be achieved under well controlled cell culture conditions. Cloned secondary myoblasts and certain myogenic cell lines are able to fuse in the absence of other cell types to yield contractile myotubes, indicating that the components of the excitable membrane, excitation-contraction coupling and the contractile apparatus achieve a functional status in the absence of other cell types [1]. Yet, these myotubes are still very different from neonatal muscle fibers and even more so from adult muscle fibers in situ, as exemplified by the conductance properties of the plasma membrane.

In rodent development, the chloride conductance of the muscular plasma membrane increases by a factor of 200 from the myotube stage to the adult fiber [2,3]. In adult muscle, the chloride channel CIC-1 contributes to about 70% of the sarcolemmal resting conductance and is responsible for the muscle fiber to produce action potentials only when stimulated by the motor nerve. Consequently, pharmacological blockade and hereditary defects of CIC-1 lead to hyperexcitability of the sarcolemma, and a cramp-like muscle stiffness known as myotonia [4,5]. Hereditary myotonias are known in man, goat and mouse. The first case of the genetic basis of a myotonia to be molecularly unravelled was the ADR mouse (ADR stands for 'arrested development of righting response' [6]), in which the myotonia [7] is caused by an insertion of a

retropon into *adr/Cic1*, the gene coding for CIC-1 [8,9]. The low chloride conductance of cultured myotubes from wild-type or myotonic ADR mice is not dependent on the function of the *Cic1* gene. By Northern blotting, the mRNA for muscular chloride channel CIC-1 is just detectable in neonatal muscle, but has not been observed in cultured myotubes [2].

Here we applied reverse transcription in combination with PCR (RT-PCR) and hybridization with a CIC-1 cDNA probe to study low levels of CIC-1 mRNA in cultured myogenic cells and to compare several myogenic cell strains and successive stages of muscle fiber differentiation and maturation.

A preliminary report of this work has been given [10].

2. Material and methods

2.1. Cells and tissues

The following myogenic cells were used as sources for RNA: the murine myogenic cell line C2C12 (the original C2 line had been derived from a wild-type CBA mouse [11]); the secondary cell strains M12 (derived from a wild-type C57BL10 mouse; Heinrich and Rode mann, unpublished; [12]) and *adrladr*-26.5 (derived from a myotonic A2G ADR mouse [2,13]) primary myogenic cells from limb muscles of neonatal Sprague-Dawley rats. For control experiments: organs from 1- and 80-day-old wild-type mice of strain C57BL/6.

2.2. Cell culture

The myogenic cell line C2C12 was cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal calf serum (FCS), and fusion was induced in subconfluent cultures by switching to 'fusion medium', DMEM supplemented with 2% FCS [14]. M12 and *adrladr*-26.5 secondary and rat primary cells were cultured on collagenized tissue culture dishes in F12 medium with 20% FCS, 1% chicken embryo extract. Fusion was induced by changing the medium to DMEM containing 5% horse serum, 5% FCS.

2.3. RNA extraction and RNA blots

Myoblast cultures were harvested at 70–80% confluence, myotubes 3 days after induction of fusion. Total RNA was extracted from cell culture and tissue samples according to Chomczynski and Sacchi [15]. RNA blots were done according to standard protocols. 3 µg per lane of total RNA in denaturing sample buffer [16] was separated in 1% agarose minigels (70 mm×100 mm×3 mm) and blotted onto nylon membranes (Biodyne B, Pall Filtrationstechnik, Dreieich, Germany). The blots were subsequently hybridized with [α -³²P]dATP random prime labelled cDNA probes [17] in 1% bovine serum albumin, 7% SDS, 1 mM EDTA, 0.5 M NaHPO₄, pH 7.2 [18] at 65°C overnight and washed for 30 min (Desmin) or 1 h (18S rRNA) in 0.1×SSC, 0.1% SDS at 65°C.

2.4. Molecular probes and primers

Hybridization was performed with the following probes: mouse desmin cDNA (1.5 kB *Eco*RI fragment [19]; rat CIC-1 cDNA (1.4 kB *Eco*RI/*Hind*III fragment; nt -83 to nt +1324 [20]); 18S rRNA (1.5 kB *Eco*RI fragment; Cat. No. 77242, American Type Culture Collection, Rockville, MD, USA). PCR primers to amplify CIC-1 transcripts were R/M II-1 (nt 503, 5'-CAC TTA TCC TCA TCC TCT TC-3') as sense and R/M II-2 (nt 1078, 5'-ACC CGC AGC AAA TCC CAA T-3') as antisense primer [21].

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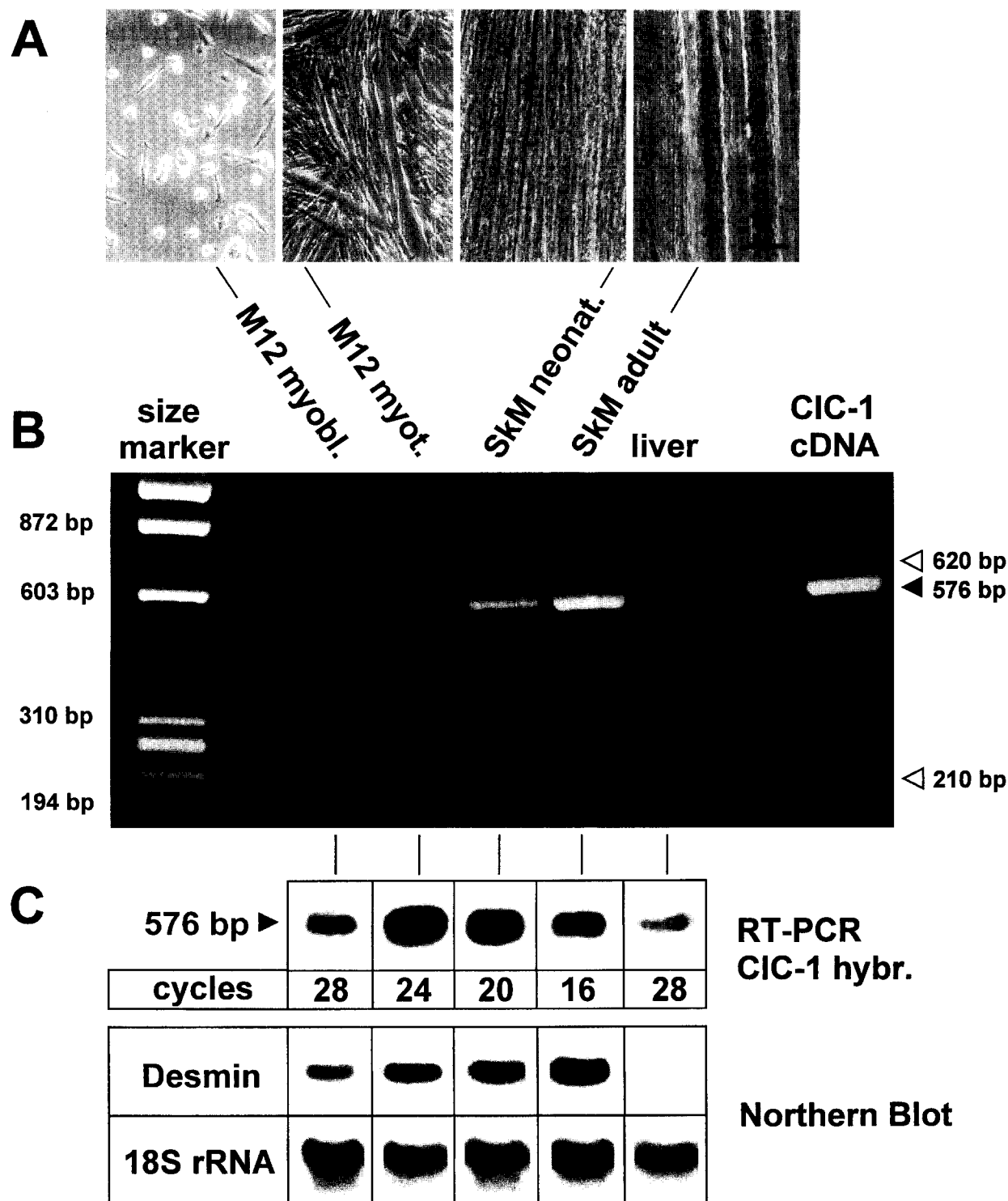


Fig. 1. Dependence of CIC-1 expression on development and maturation of myogenic cells and tissues. Abbreviations: myobl., myoblasts; myot., myotubes; SkM, skeletal muscle; neonat., neonatal. A: Myogenic cells and tissues as used for this study. Phase contrast micrographs. Skeletal muscle: 100 μ M vibratome sections of formaldehyde fixed tissues. Bar = 100 μ M. B: Ethidium bromide stained RT-PCR amplification products obtained with CIC-1 primers from total RNAs. Size marker: bacteriophage ϕ X174 DNA, *Hae*III digest. Filled arrowhead, standard CIC-1 amplification product; open arrowheads, non-standard amplification products. C: CIC-1 expression as analyzed by variable PCR cycle numbers and hybridization with the CIC-1 probe. Signals shown result from cycle number given below. Controls on identical RNA samples: Northern hybridization with desmin and 18S rRNA probes.

2.5. Reverse transcriptase polymerase chain reaction for CIC-1 mRNA

One microgram of total RNA in 20 μ l reaction volume was subjected to reverse transcription according to Kawasaki [22] using SuperScript reverse transcriptase (Life Technologies GmbH, Eggen-

stein, Germany). 5 μ l of this solution was amplified by PCR in a volume of 50 μ l containing 10 pmol of each primer with AmpliTaq (Perkin-Elmer GmbH, Überlingen, Germany) under the following conditions: 4 min 94°C, varying number of cycles with 1 min 58°C,

1.5 min 72°C, 1 min 90°C (Trio-Thermoblock from Biometra, Göttingen, Germany). The resulting PCR products were separated in TRIS-borate/EDTA (TBE) buffered 1% agarose gels and visualized by ethidium bromide staining or blotted onto nylon membranes for subsequent hybridization.

2.6. Quantification of RT-PCR products

After hybridization with a CIC-1 specific probe and stringent washing ($0.1 \times \text{SSC}$, 0.1% SDS for 45 min at 65°C), areas on the membrane showing signals after autoradiography were cut out and quantitated by liquid scintillation counting in a TriCarb 300 (Packard-Canberra, Frankfurt/M., Germany). The RT-PCR data were normalized to 18S rRNA Northern blot signals, which were obtained by densitometric scanning using a Hewlett Packard Scan Jet IIcx/T and Quantiscan program (Biosoft). Variation of 18S rRNA Northern blot signals was < 2 .

3. Results

Differentiation and maturation of myogenic cells in culture and in situ were followed by morphological, physiological and biochemical criteria (Fig. 1A,C). Three days after the induction of fusion, myotubes in culture had highly variable diameters (5–50 μm) and lengths; on average, C2C12 myotubes were thinner and shorter than those formed by secondary mouse and primary rat myogenic cells. Neonatal muscle fibers were about 10 μm , adult ones (of the anterior tibial muscle) about 50 μm in diameter (Fig. 1A). M12, *adrladr*-26.5, and rat myotubes started twitching at the time of harvest, but this was not seen in C2C12 myotubes. Cross-striations were observed in some of the rat myotubes. From the myoblast stage onward, myogenic cells had high levels of desmin mRNA, whereas liver tissue used as control gave no desmin signal in the Northern blot (Fig. 1C).

In the study of CIC-1 mRNA, cultured myogenic cells and tissues with extremely divergent CIC-1 expression levels were compared. As the CIC-1 transcript is not detectable in myotubes by Northern blot analysis, a more sensitive RT-PCR protocol had to be established. With the primers used, an amplification product of 576 bp is obtained from mouse muscle RNA [21]. A minimum of 36 cycles of PCR was required to clearly visualize the CIC-1 amplification product of myotubes by ethidium bromide staining of agarose gels (Fig. 1B; filled arrowhead). Under these conditions, saturation problems led to non-exponential amplification kinetics and hence to difficulties in quantification. Furthermore, in some samples slightly larger (~ 620 bp; myotubes, muscle) or smaller (~ 210 bp; liver) than standard amplification products were seen (Fig. 1B; open arrowheads). In control experiments with the CIC-1 cDNA as a template, these bands were missing (Fig. 1B). Therefore we performed PCR with low and variable numbers of cycles and subsequent blotting and hybridization of the PCR product with a CIC-1 probe to gain additional sensitivity and specificity (Fig. 1C). Using a combination of low cycle numbers and hybridization, the increment of the amount of CIC-1 amplification product was exponential within the optimal range of cycles (Fig. 2). Furthermore, the bands of non-standard size did not yield a CIC-1 hybridization signal (data not shown).

CIC-1 expression levels in myotube cultures were two orders of magnitude lower than in adult muscle, with the values of primary and secondary mouse myotubes (M12 and *adrladr*-26.5) slightly higher (0.9–1.1% relative to adult tissue) than those of C2C12 derived myotubes (0.4%) (Fig. 3). This ten-

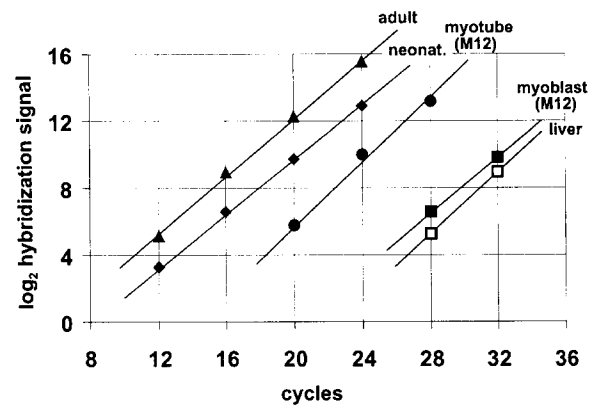


Fig. 2. Increment of CIC-1 PCR product with cycle number. Quantification was performed by liquid scintillation counting of hybridized CIC-1 ^{32}P probe. Binary logarithms of counts per minute are given on the ordinate.

gency, which was evident in several independent experiments, fitted to the maturation stage of myotubes (see above). In *adrladr*-26.5 myotubes, CIC-1 expression was identical to that of wild-type secondary M12 myotubes (cf. [2]).

Using RT-PCR combined with concomitant hybridization to a CIC-1 specific probe, CIC-1 mRNA was detectable even in liver and in myoblasts, at levels of 0.01% or less of that of the adult skeletal muscle. The slightly higher signal in myoblasts could be due to a small proportion of cells which had started to differentiate in the absence of fusion medium.

4. Discussion

Chloride channel 1 is not required for the basic functions of vertebrate skeletal muscle, excitability, excitation-contraction coupling, and contractility, but rather modulates excitability in adult muscle. Using sensitive RT-PCR methods we now show significant levels of CIC-1 mRNA in cultured myotubes, and low levels even in myoblasts. The low chloride conductance detectable in myotubes, however, is not dependent on a functional *Cle1* gene [2,3], although it is of the same order as the CIC-1 mRNA level (1/200 to 1/100 of the adult value). It is conceivable that the low levels of CIC-1 mRNA in myotubes do not translate into corresponding levels of functional CIC-1 channels, i.e. that, in addition to the control of mRNA levels post-transcriptional control is involved in Cl^- conductance during muscle differentiation and maturation. CIC-1 mRNA levels of $< 10^{-4}$ of the adult concentrations, as found in myoblasts and liver, are almost certainly physiologically irrelevant.

In rodents, the expression pattern of CIC-1 is similar to that of parvalbumin: extremely low levels in myotubes and embryonic muscle are followed by a steep upregulation in the first postnatal weeks [23]. In mature muscle, CIC-1 expression [24], like that of parvalbumin [23], is highly dependent on fiber types, innervation, and pattern of electrical activity [25]. On the other hand, desmin mRNA and protein are classical markers of early muscle differentiation; its expression is not nerve-dependent [26,27]. Furthermore, desmin is present in all three muscle types, smooth, cardiac and skeletal. Myosin heavy chains and their isoforms mark the subsequent steps of functional muscle differentiation [1]. In comparison to these, CIC-1 appears as a marker strictly confined to skeletal muscle and with high levels indicating the late steps of maturation. As

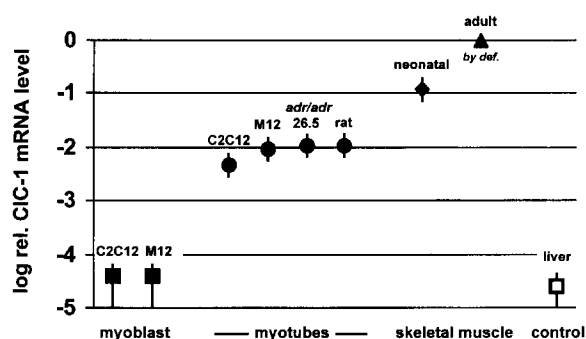


Fig. 3. Relative CIC-1 mRNA levels of cultured cells and tissues. Adult skeletal muscle CIC-1 expression level was defined as 1 (log=0). The error bars indicate an estimated maximum variability (on a linear scale) of a factor of 3, except for the lower limits of the lowest values, which cannot be estimated.

such it should be ideal to study factors, i.e. substrata, medium components, electrical activity, and co-cultured neuronal cells that may promote myotube maturation in culture.

Acknowledgements: This work was supported by Deutsche Forschungsgemeinschaft, Graduiertenkolleg 'Zelluläre Grundlagen biotechnischer Prozesse' and SFB 223 'Pathomechanismen zellulärer Wechselwirkungen'. We thank Drs. Steinmeyer and Jentsch for providing the rat CIC-1 cDNA, C. Schütze for RNA samples from *adr/adr-26.5* cells, and Dr. J.-W. Bartsch for advice.

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