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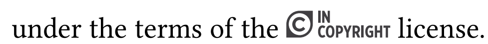
under the terms of the  license.

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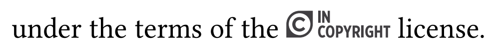
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CLCN1 MYOTONIA CONGENITA MUTATION WITH A VARIABLE PATTERN OF INHERITANCE SUGGESTS A NOVEL MECHANISM OF DOMINANT MYOTONIA

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Abstract

Introduction: Mutations in CLCN1 cause recessive or dominant forms of myotonia congenita (MC). Some mutations have been found to exhibit both patterns of inheritance but the mechanism explaining this behavior is unknown.
Methods: A known recessive missense mutation, A493E, was identified in a family with dominant MC. The mutant p.A493E alone or in co- expression with wild-type (WT) ClC-1 was expressed in Xenopus oocytes. Currents were measured and biochemical assays were performed.
Results: The mutant showed no significant activity and reduced total and plasma membrane (PM) protein levels. Co-expression with the mutant reduced the activity and PM levels of an engineered lower expression variant of ClC-1, whereas no effect was observed on a higher expression variant.
Discussion: Our results suggest that the dominant effect of some CLCN1 mutations showing recessive or dominant inheritance patterns may be due to a dose-dependent defect in PM delivery of the WT channel.

Abbreviations

cRNA, complementary RNA; ER, endoplasmic reticulum; MC, myotonia congenita; PCR, polymerase chain reaction; PM, plasma membrane; WT, wild-type.

Key words

ClC-1; dominant-negative; myotonia; surface expression; trafficking
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Introduction

Myotonia congenita (MC) is a hereditary chloride channel neuromuscular disorder.[1] It is caused by mutations of the CLCN1 gene, which encodes ClC- 1, a chloride channel of the CLC channel/trans- porter family.[2] ClC-1 activity in skeletal muscle is important for maintaining resting potential.[3] CLCN1 mutations may be associated with a recessive (Becker phenotype) or dominant (Thomsen phenotype) pattern of inheritance.[4] However,

some mutations have been associated with both recessive and dominant MC by unknown mechanisms.[1] It has been suggested that this could be explained by reduced penetrance of these dominant-negative mutations[5] or by variations in allelic expression of the mutant mRNA allele.[6]

Materials and Methods

Direct sequencing of CLCN1 (primers and polymerase chain reaction [PCR] conditions are shown in Supplementary methods) revealed the heterozygous variant c.1478C>A, leading to the p.Arg483Glu missense mutation, in two of the affected siblings (Fig. 1). The daughter of one of the affected subjects (3.5) was an asymptomatic (or, alternatively, presymptomatic at age 7 years) carrier of the same variant. Another mildly affected individual (3.2) was not genotyped and neither was his aunt (2,3) who displayed mild calf and trapezius muscle hypertrophy.

DNA constructs were made using recombinant PCR. Other constructs were designed using the Multisite Gateway system (Invitrogen, Carlsbad, CA). For expression in Xenopus oocytes, after linearization with NotI, complementary RNA (cRNA) was transcribed using the mMessage mMachine SP6 kit (Ambion, Waltham, MA). Oocytes were harvested from *Xenopus* laevis frogs that had been anesthetized with ethyl 3-aminobenzoate methanesulfonate salt (Sigma Aldrich, St. Louis, MO) at a concentration of 1 g/L. The animal protocol was approved by the Ethics Committee for Animal Experimentation of the University of Barcelona. The oocytes were enzymatically defolliculated by a 2–3 h treatment with collagenase type I A in ORII solution with gentle shaking at 17 ◦C. Defolliculated stage IV oocytes were injected with 50 nl of cRNA.

Currents were measured using the two-electrode voltage clamp technique.[7] Surface expression was examined using a chemiluminescence technique.[8] Western blot and co-immunoprecipitation experiments were performed using phosphate buffered saline/1% Triton X-100-solubilized extracts from oocytes.[8] To detect ClC-1, we developed a rabbit polyclonal antibody against the peptide C- RSTDEEDEDELIL, using the services provided by Eurogentec. In co-immunoprecipitation experiments, extracts were immunoprecipitated with the mouse monoclonal anti-flag M2 (Sigma-Aldrich) and detected with the rabbit polyclonal anti-HA antibody (Invitrogen).

Values depicted are mean ± SEM. For the determination of the statistical significance between groups, Student’s unpaired t-test was used.

Results

We identified a multigenerational family diagnosed with MC (Fig. 1A). The onset of myotonia occurred in all affected family members around puberty. All the patients had clear symptoms of myotonia, such as generalized muscle stiffness, especially in the lower limbs. Furthermore, they showed moderate muscle hypertrophy, mainly in the lower limbs, biceps brachii, and neck. Sanger sequencing of CLCN1 uncovered a heterozygous p.A493E variant which, in several of the affected individuals, segregated with the phenotype and followed a dominant pattern of inheritance. No other mutations were identified in CLCN1 in the family presented here by the methods used.

To understand the functional consequences of the p.A493E CLCN1 mutation, we introduced it into ClC-1HAL (Fig. S1) and studied it using voltage-clamp and through biochemical analysis after heterologous expression in Xenopus oocytes. Mutant p.A493E

showed no currents (Fig. 1B), in parallel with a reduced surface and total expression levels (Fig. 1C). Next, we aimed to explain what the molecular basis of its dominant behavior is. We first addressed the issue of whether the mutant was still capable of interacting with wild-type (WT) ClC-1. To this end, we co-expressed ClC-1flag (Supplementary Fig. S1, which is available online) with ClC-1HA or p.A493EHA (Supplementary Fig. S1) and performed co-immunoprecipitation experiments. No ClC-1HA was present in the eluate of the immunoprecipitation when no antibody was used (Fig. 2A). In contrast, we detected ClC-1HA and p.A493EHA when the flag antibody was used to immunoprecipitate (Fig. 2A). These results suggested that the p.A493E mutant was still capable of interacting with WT ClC-1, in agreement with the fact that the residue is not found in interacting domains of CLC channels.[9]

We hypothesized that the expression of the WT allele may influence the mode of inheritance. To address this issue, we took advantage of the fact that ClC-1HAL (Supplementary Fig. S1) has weaker currents than HAClC-1HAL (Fig. 2B), probably due to a reduction in surface and total protein expression levels (Fig. 2C). Both proteins show similar gating properties (not shown), suggesting that the tags do not interfere with the function of the protein. We then determined by means of co-expression experiments of the p.A493E mutant with ClC-1HAL or HAClC-1HAL whether their expression levels influence the mode of inheritance of the p.A493E mutation. To mimic the heterozygous state of the patients, we co- injected both RNAs in a 1:1 ratio.

Co-expression of p.A493E with HAClC-1HAL did not decrease the HAClC-1HAL currents (Fig. 2D). Similarly, p.A493E did not reduce the surface expression of HAClC-1HAL (Fig. 2E). The experi- ments were done in linear condition of expression, as co-expression of HAClC-1HAL with ClC-1flag potentiated the currents (Fig. 2D), but did not increase surface expression. Thus, p.A493E was act- ing as a typical recessive mutation when co-expressed with HAClC-1HAL.

The situation was completely different when p.A493E was co-expressed with ClC-1HAL. p.A493E reduced the ClC-1 mediated current (Fig. 2F) and ClC-1 surface expression (Fig. 2G). No significant effect was observed on the voltage dependence of ClC-1HAL in co-expression with p.A493E (not shown). To verify that this effect was specific for this mutation, we performed additional controls (Supplementary Fig. S2). Thus, we tested whether this reduction was observed when co-expressing ClC-1flag with an unrelated membrane protein (LRRC8A) or co-expressing ClC-1HAL with a clearly defined recessive mutation, p.Q412P, identified in Costa Rican patients.10 LRRC8A and p.Q412P did not reduce the ClC-1 mediated currents or surface expression, even when injected at 1:3 ratio (Supplementary Fig. S2). The expression of all proteins was not in saturation, as ClC-1 co-expression increased current amplitudes and slightly potentiated the sur- face expression of ClC-1HAL (Fig. 2F,G).

Discussion

We have identified the p.A493E mutation in a family with dominant myotonia. Of interest, this mutation was found previously in a patient with recessive myotonia.[11] We conclude that the p.A493E mutation can be found in both dominant and recessive pedigrees, as can the previously reported missense mutations G230E and A531V, and the deletion R894X.5 Here, we demonstrate that the mutant A493E showed reduced total and plasma membrane (PM) expression, suggesting a folding defect. Previous biochemical characterization of the mutant R894X also showed reduced total and PM expression.[7] As the mutant A493E is still capable of forming a heterodimer with WT ClC-1, it seems likely

that the dominant character of this mutation would be explained by the heterodimer mutant/WT being targeted by endoplasmic reticulum (ER)- and endolysosomal-associated protein degradation processes, as found in many other membrane proteins with folding mutations.[12]

Thus, based on these studies, we suggest that the unusual phenomenon of some pathogenic mutations for MC occurring in both dominant and recessive pedigrees could be explained by a difference in the ratio of protein expression of the two alleles of the CLCN1 gene. Possibly, all these pathogenic mutations maintain the ability to oligomerize with WT ClC-1 and are able to retain the WT protein in the ER. However, previous experiments expressing 1:1 WT:R894X in oocytes yielded only slightly reduced currents,[4] similar to our findings in A493E mutant:WT co-expression experiments with HAClC-1HAL. Possibly, experiments should be performed with lower doses of this mutant or lower expression variants of ClC-1 such as the one reported here (ClC-1HAL).

In other studies with the mutation R894X, the mRNA allelic expression ratio found in heterozygous members of a dominant pedigree was identical to that found in members of recessive pedigrees.[6] We speculate that it is possible that mRNA levels do not correlate with the levels of the expressed protein, which may be controlled by different regulatory mechanisms, such as the recently identified ubiquitin ligases or molecular chaperones.[13,14]

Thus, taking into account these results, we suggest that strategies aimed at increasing expression of the WT allele or reducing expression of the mutant allele may be beneficial for these patients.

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Conflicts of Interest

None of the authors has any conflict of interest to disclose.

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Figure legends

Figure 1. Identification and functional characterization of a dominant CLCN1 mutation (p.A493E).

(A) Pedigree of the family, segregating the p.A493E mutation (mut) in an autosomal dominant manner. Filled symbols indicate classical myotonia (Thomsen). Individual 1.2 died from an acute event consistent with malignant hyperthermia and is considered putatively affected (gray symbol) and so are 2.3 and 3.2, both displaying generalized and mild muscle hypertrophy. Individual 3.5 (age 7) is an asymptomatic/presymptomatic carrier. Individual 3.6 is affected with neurofibromatosis type 1, not with myotonia. (B,C) Mean currents, surface and total expression analysis of ClC-1 and p.A493E. Data indicate the mean 6 SEM of a representative experiment ***P < 0.001 (Student's t-test comparing the mutant with the WT). Uninj. means un-injected oocytes. Inset in C: Western-blot analysis of total ClC-1 protein levels, detected using an antibody against the C-terminus of ClC-1. Tubulin detection was used as a loading control.

Figure 2. Interaction analysis of mutant p.A493E with ClC-1.

(A) Co-immunoprecipitation using C-terminally epitope-tagged (flag or HA) ClC-1 and A493E mutant. In IP-, no flag antibody is coupled to the beads. SN, supernatant fraction; P, pellet fraction. (B,C) Mean currents and PM and total protein levels mediated by HAClC-1HAL or ClC-1HAL. Data indicate the mean 6 SEM of a representative experiment. ***P < 0.001 (Student's t-test comparing the ClC-1HAL with the HAClC-1HAL). Another independent experiment yielded similar results. Uninj. means un-injected oocytes. Inset: Western-blot analysis with a ClC-1 C-terminal antibody shows that the steady- state levels of HAClC-1HAL are higher than those of ClC-1HAL. Tubulin detection was used as a loading control. (D-G) Oocytes were injected with 20 ng of HAClC-1HAL (D,E) or ClC-1HAL (F,G), alone or together with 20 ng of A493Eflag or ClC-1flag. Currents and PM were measured and normalized (dotted line) to the value of oocytes injected with HAClC-1HAL or ClC-1HAL. Data indicate the mean- 6 SEM of 3 or 4 independent experiments for co-expressions with HAClC-1HAL or ClC-1HAL, respectively. *P < 0.05 ***P < 0.001. ns: not significant (Student's t-test comparing the co-expressed groups with the groups expressed individually).