Spectrum of Mutations in the Major Human Skeletal Muscle Chloride Channel Gene (CLCN1) Leading to Myotonia

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Summary

Autosomal dominant myotonia congenita and autosomal recessive generalized myotonia (GM) are genetic disorders characterized by the symptom of myotonia, which is based on an electrical instability of the muscle fiber membrane. Recently, these two phenotypes have been associated with mutations in the major muscle chloride channel gene CLCN1 on human chromosome 7q35. We have systematically screened the open reading frame of the CLCN1 gene for mutations by SSC analysis (SSCA) in a panel of 24 families and 17 single unrelated patients with human myotonia. By direct sequencing of aberrant SSCA conformers we revealed 15 different mutations in a total of 18 unrelated families and 13 single patients. Of these, 10 were novel (7 missense mutations, 2 mutations leading to frameshift, and 1 mutation predicted to affect normal splicing). In our overall sample of 94 GM chromosomes we were able to detect 48 (51%) mutant GM alleles. Three mutations (F413C, R894X, and a 14-bp deletion in exon 13) account for 32% of the GM chromosomes in the German population. Our finding that A437T is probably a polymorphism is in contrast to a recent report that the recessive phenotype GM is associated with this amino acid change. We also demonstrate that the R894X mutation may act as a recessive or a dominant mutation in the CLCN1 gene, probably depending on the genetic background. Functional expression of the R894X mutant in Xenopus oocytes revealed a large reduction, but not complete abolition, of chloride currents. Further, it had a weak dominant negative effect on wild-type currents in coexpression studies. Reduction of currents predicted for heterozygous carriers are close to the borderline value, which is sufficient to elicit myotonia.

Introduction

The primary skeletal muscle diseases autosomal dominant myotonia congenita Thomsen (MC) and autosomal recessive generalized myotonia Becker (GM) are rare nondystrophic disorders with a reported prevalence of ~1:20,000–50,000 in the German population (Becker 1977). On clinical examination, both disorders share the phenomenon of myotonic stiffness, which is due to repetitive action potentials of the muscle membranes. This can be seen as characteristic myotonic discharges on electromyogram (EMG). The two disorders differ clinically by age at onset, by spreading of the symptom myotonia, by a typical transient muscle weakness only present in GM, and genetically by dominant and recessive transmission (Ricker et al. 1978; Koch et al. 1992, 1993). Electrophysiological in vitro studies on muscle biopsies from myotonic goats, from a recessive myotonic mouse strain ADR, and from patients with recessive myotonia revealed a diminished sarcolemmal chloride conductance (Bryant and Morales-Aguilera 1971; Lipicky et al. 1971; Mehrke et al. 1988; Rüdel et al. 1988). The high resting chloride conductance of skeletal muscle contributes significantly to the repolarization of action potentials in that tissue, and its reduction will lead to an electrical instability. Thus, a skeletal muscle chloride channel appeared to be a good candidate gene for these disorders. Immediately after cloning of the major skeletal muscle chloride channel CLC-1, it was indeed shown that its coding potential was destroyed by a transposon in the myotonic ADR mouse strain (Steinmeyer et al. 1991a; 1991b). Subsequently, a partial human CLC-1 cDNA was cloned, and its gene (CLCN1) was mapped to human chromosome 7q35 (Koch et al. 1992). Both dominant and recessive myotonia were linked to this CLCN1 gene in German families (Koch et al. 1992), and in North American families MC was mapped to the same region (Abdalla et al. 1992).

Several mutations in the chloride channel gene have now been identified in MC and GM patients (Koch et al. 1992; George et al. 1993, 1994; Heine et al. 1994; Koty et al. 1994; Lorenz et al. 1994; Meyer-Kleine et al. 1994b; Steinmeyer et al. 1994; Lehmann-Horn et al. 1995; Pusch et al., in press). This demonstrates that

both disorders are due to mutational inactivations of the chloride channel. At present, it is not generally possible to predict from the sequence alone whether a certain point mutation will cause a dominant or a recessive phenotype. However, functional expression in Xenopus oocytes is able to predict the recessive or dominant character of the CLCN1 mutations (Lorenz et al. 1994; Steinmeyer et al. 1994; Pusch et al., in press). Total loss of function of one allele results in recessive myotonia (GM), while a dominant mutation must reduce the chloride conductance further by a dominant negative mechanism. This implies a multimeric structure of the chloride channel. Analysis of two MC mutations (P480L and G230E) provides strong evidence for a homomultimeric channel with at least three and probably four identical subunits (Steinmeyer et al. 1994).

Detailed analyses of CLCN1 mutations are necessary to gain more insight into the relationship of phenotype, genotype, and genetic transmission. The large number of rare mutations in the MC and GM phenotype suggests that many additional mutations remain to be discovered in this gene. The availability of the complete sequence with intron-exon structures permits a systematic study of all 23 exons in respective families (Lorenz et al. 1994). In an effort to determine the spectrum of mutations in the CLCN1 gene, we have undertaken an analysis of all 23 exons in our well-characterized MC and GM patients. We have found 10 novel mutations and 5 mutations that have been reported elsewhere. The R894X mutation, elsewhere reported to cause dominant myotonia (MC) is clearly recessive in our families. By in vitro analysis in Xenopus oocytes we demonstrate that it displays a weak dominant negative effect, explaining that it may act dominantly in some families and recessively in others.

Families, Material, and Methods

Family Studies

A total of 24 families (23 GM and 1 MC) recruited throughout Germany were included in the present study. The majority of the families (*n* = 20) were already part of our previous linkage studies and our mutational analyses to investigate the frequency of the F413C mutation in the German population (Koch et al. 1992, 1993). In addition, 17 single unrelated GM probands were investigated. For all families and single patients the same diagnostic criteria as outlined in Koch et al. (1993) were used assigning clinical status. The total number of investigated GM chromosomes was 80.

DNA Extraction and SSC Analysis (SSCA)

All venous blood samples from the family members and the German control probands used were obtained with the approval of the ethics committee of the Univer-

Table I

Primer Pairs for 10 Novel Mutations in the CLCN1 Gene for MC and GM

Exon	Primer Pairs[a]
3	F 5′ TTTTCCCTCATCTCTTCCTA 3′ R 5′ CCATAACACACCCTGCTTAC 3′
4	F 5′ CGGTGGACACGGCTGCTCAG 3′ R 5′ GCCGAGTCTGGTGGCAAGTT 3′
8	F 5′ TCCTTAGGTCCAAGCAGT 3′ R 5′ AGAGTTTTCCTCTGCACC 3′
9	F 5′ ATTAATCCTGAAAACTGC 3′ R 5′ GTCCCTTCCAATCTACAG 3′
10	F 5′ CCTGCAGTAGTTATGTCC 3′ R 5′ AAGGGAGGAACTCTTGGA 3′
11	F 5′ CTTCAGCTTGCCATCGTT 3′ R 5′ ACTACTTATGCTCTGAAG 3′
12	F 5′ GGGGAATAAGTTCTCTAA 3′ R 5′ GGTTCATAGATTGAAAACAGATGG 3′
13	F 5′ GGAATTGTGTGTGCATGTCTATTG 3′ R 5′ GGCCTTTCCTTATGTTTCCTGTAT 3′
14	F 5′ ATGCCCAAGGAGAGATTGGTTCTG 3′ R 5′ ATCCAATGGGAGAGTTTAAGTGTG 3′
23	F 5′ TCTGTGTCTCTCACTGCCCCCGTC 3′ R 5′ GGAGATGGCACAGGGGTC 3′

[a] F = forward; and R = reverse.

sity of Marburg. Genomic DNA was prepared from patients peripheral or cultured lymphocytes by a standard phenol/chloroform extraction method or by a modification of the salting-out procedure. Oligonucleotide primers were designed to amplify the entire coding sequence of the CLCN1 gene as well as the adjacent splice junctions (Lorenz et al. 1994). In addition to the 23 exons, the primer set amplifies 208 bp of the 5′ UTR and 90 bp of the 3′ UTR. The primer pairs used for the 10 novel mutations (George et al. 1993; Lorenz et al. 1994) are shown in table 1. SSCA was performed according to Orita et al. (1989) with minor modifications employing a radioisotope method (Meyer-Kleine et al. 1994a, 1994b).

Approximately 100 ng of genomic DNA of one affected proband of each family was amplified using the respective intronic primer pairs. Each reaction was cycled as described elsewhere and separated on 5%–10% polyacrylamid gels at 4°C and/or room temperature (Lorenz et al. 1994; Meyer-Kleine et al. 1994b). All samples were run in duplicate under different conditions. Electrophoresis was carried out at 5–30 W constant power for 6–20 h. Gels were dried and exposed to Kodak X-OMAT AR film or to Kodak BioMax MR film. All those

patient DNA samples that yielded aberrant SSCA patterns were checked for segregation in the family and for absence in 200 control chromosomes.

DNA Sequencing

Samples showing an altered electrophoretic migration in SSCA were amplified and analyzed by direct sequencing of both strands of the PCR product using the dideoxy chain termination method (γ-ATP) or the fluorescence-based dideoxy chain termination method (Prism® Ready reaction, Applied Biosystems) and analyzed on the ABI 373A automatic DNA sequencer. The 1262insC mutation was additionally cloned into the pGEM-T vector, and 10 clones from both directions were sequenced. Nucleotides affected by mutation were numbered from the cDNA as published in Lorenz et al. (1994). All sequence variants were confirmed in PCR products derived from genomic DNA of affected family members. In any case each sequence result was obtained on more than one sample.

Site-Directed Mutagenesis and Electrophysiology

Mutations were introduced into the functional human CLC-1 cDNA by recombinant PCR (Steinmeyer et al. 1994). The stretch modified by PCR was fully verified on both strands by sequencing. All CLC-1 cDNAs were subcloned into a vector (PTLN) that uses the Xenopus β-globin 5' and 3' UTRs to boost expression in the oocyte system (Krieg and Melton 1984). Capped cRNA was transcribed in vitro using Sp6 RNA polymerase (Sp6 mMessage mMachine kit, Ambion) and checked for integrity by agarose gel electrophoresis. As an additional control, we performed in vitro translation using a rabbit reticulocyte lysate system (Promega) in the presence of ^{35}S-methionine (Amersham-Buchler). The translation products were separated by SDS-PAGE, the gel dried, and autoradiography performed. The R894X mutant protein showed the expected reduction in size, and identical amounts of wild-type (WT) and mutant proteins were made, which is important for the coinjection experiments. The cRNA was injected in a volume of 50 nl into Xenopus oocytes prepared and handled as described elsewhere (Steinmeyer et al. 1991b). RNA concentration was quantified by UV absorption and gel electrophoresis. Experiments were performed with three different batches of RNA (to control for differences in translatability) and oocytes, with similar results. Currents were measured after 2 d by using the Turbotec (Npi Instruments) two-electrode voltage-clamp amplifier and pCLAMP (Axona) software. The bath solution was ND96.

Results

We have screened one MC family, 23 GM families, and 17 single unrelated GM patients for the 23 CLCN1

exons, which include parts of the 5' and 3' UTR. SSCA screening and subsequent DNA sequence analyses resulted in identifying 15 different mutations in 1 MC family and 17 GM families. In six GM families both mutations were detected, but in six families we were unable to find any mutation, although they fulfilled the diagnostic criteria for the disease and showed positive linkage results. In the group of single unrelated GM patients, we were able to detect in 11 patients one mutation; in 2 patients both mutations were identified; and in 4 none. The locations of all identified mutations and their amino acid positions are listed in table 2 and are shown in the preliminary model of the skeletal muscle chloride channel protein (fig. 1).

Of the 15 mutations we have identified in the project, 10 are missense mutations; 3 are frameshift mutations due to deletion and insertion of nucleotides; 1 is a nonsense mutation; and 1 affects a 5' splice site. Ten of these mutations are novel, while five others have already been reported by us and other investigators (Koch et al. 1992, 1993 [F413C]; George et al. 1994 [F167L and R894X]; Heine et al. 1994 [D136G]; Meyer-Kleine et al. 1994 [Del 14 bp]).

In our sample the R105C, D136G, F167L, and I329T exchanges were observed in one single unrelated GM patient each. The G482R exchange was found in two single unrelated patients. In one GM family a V165G and in another a M485V exchange segregated on one chromosome. In two families and one single GM patient, the E291K exchange was found. In our sample none of the amino acid exchanges were detected in a homozygous state. In the MC family, the R317Q exchange cosegregated with the disease phenotype in all affected members (figs. 2 and 3, pedigree 3004).

One-third of the identified mutations are predicted to produce a shortened protein product. In three cases the resulting frameshift leads to a premature stop codon within 24–60 bp. The nonsense mutation R894X was observed in seven GM families and four single GM patients on one chromosome. The 14-bp deletion in exon 13 was found in one family and one single patient. A 2-bp deletion in exon 10 within an overlapping direct repeat of 3 bp (TGTGT) segregated in one GM family. The 1262insC mutation, within a run of originally four existing cytosine bases, was identified in one family, and a 5' splice-donor mutation (1471+1 g-to-a in figs. 2 and 4) altering the highly conserved consensus sequence (Ggt) in three families.

In six GM families we were able to identify both mutations (table 3). The F413C exchange in family 4026 (fig. 3) was reported elsewhere (Koch et al. 1993). Each listed compound heterozygous and homozygous status was found only once, with the exception of 1437–1450 del14bp/R894X. We found this status in two families, as well as in two single GM patients. While the mutations

Table 2

Mutations in the CLCN1 Gene for MC and GM

Phenotype	Exon	Sequence Change	Codon Change	Reference
GM	3	313C to T	R105C	Present study
GM	3	407A to G	D136G	Present study; Heine et al. 1994
GM	4	494T to G	V165G	Present study
GM	4	501C to G	F167L	Present study; George et al. 1994
MC	5	689G to A	G230E	George et al. 1993
MC	8	870C to G	I290M	Koty et al. 1994; Lehmann-Horn et al. 1995; Pusch et al., in press
GM	8	871G to A	E291K	Present study
GM	8	898C to T	R300X	George et al. 1994
MC	8	950G to A	R317Q	Present study
GM	8	979-1G to A	Splice mutation	Lorenz et al. 1994
GM	9	986T to C	I329T	Present study
GM	9	1013G to A	R338Q	George et al. 1994
GM	10	del 2 bp (1095+1096 or 1096+1097 or 1098+1099)	fs387X	Present study
GM	11	1238T to G	F413C	Present study; Koch et al. 1992
GM	12	1262insC	fs429X	Present study
GM	12	1278-1281 del 4bp	fs to 433X	Heine et al. 1994
GM	13	1437-1450 del 14bp	fs to 503X	Present study; Meyer-Kleine et al. 1994
MC	13	1439C to T	P480L	Steinmeyer et al. 1994
GM	13	1444G to A	G482R	Present study
GM	13	1453A to G	M485V	Present study
GM	13	1471+1 g to a	Splice mutation	Present study
GM	14	1488G to T	R496S	Lorenz et al. 1994
MC	15	1655A to G	Q552R	Lehmann-Horn et al. 1995
GM/MC	23	2680C to T	R894X	Present study; George et al. 1994

segregated on different chromosomes in the families, we could not prove the independent segregation of the mutant alleles in the single patients, as the parents were not available.

Polymorphisms

In addition to the R300Q polymorphism described elsewhere (Steinmeyer et al. 1994), we identified one
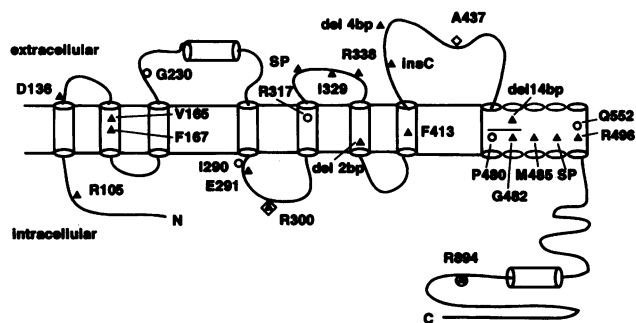

Figure I Location of mutations in the CLC-1 chloride channel protein. A preliminary transmembrane model is shown that is based on Jentsch et al. (1990) as modified by Pusch et al. (1995). The broad hydrophobic region D10-D12 is not further subdivided, as predictions are particularly difficult in this stretch. A circle (O) represents dominant mutations; a triangle (▲) represents recessive mutations; and a diamond (◇) represents polymorphisms.

further sequence change suspicious of being a polymorphism, an alanin-to-threonine substitution at codon position 437 (A437T). This substitution was observed in three of our GM families and in 5 of 200 control chromosomes. The A437T exchange segregated with the disease in our families and was reported in an American GM family by Koty et al. (1994) as a disease-causing mutation. Therefore, a mutant A437T cRNA was functionally expressed in Xenopus oocytes. It induced currents that were indistinguishable from WT currents (data not shown). While this supports that it is a polymorphism, we cannot exclude the possibility that it cooperates with recessive myotonic mutations to decrease chloride conductance.

Functional Expression of R894X in Xenopus Oocytes

The R894X mutation is clearly associated with GM in our families, whereas it was described elsewhere as a dominant mutation (George et al. 1994). We therefore focused on this mutant for our functional analysis. Xenopus oocytes were injected with mutant R894X cRNA and examined by two-electrode voltage clamping after 2 d. This yielded currents which were much smaller than WT currents, but displayed the same typical voltage- and time-dependence (fig. 5). Current amplitudes were ~10%–15% of WT currents, and could reach even 4
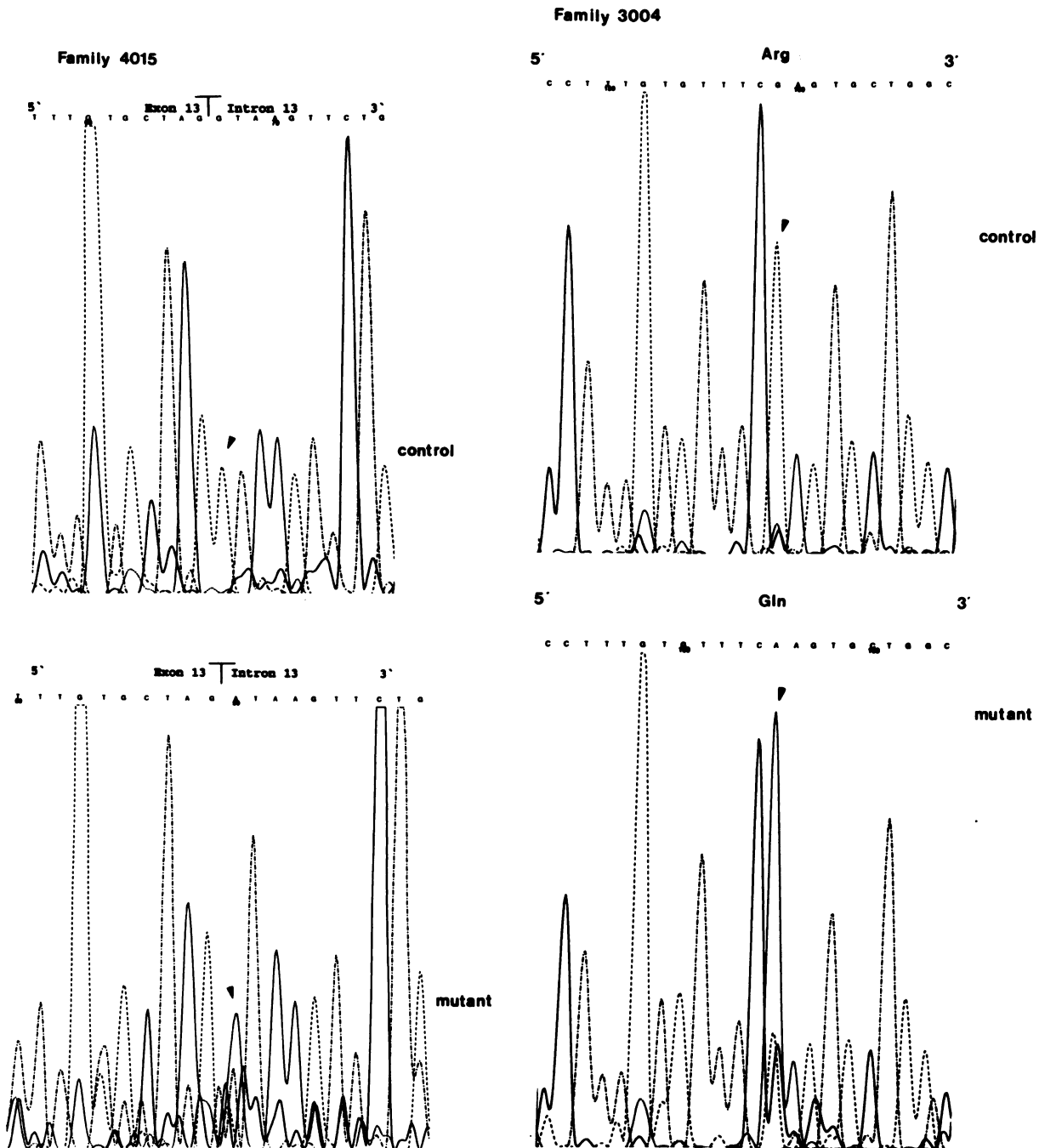
Family 3004

Family 4015

Figure 2   Sequence detection of the 950 G-to-A transition in exon 8 (R317Q) in MC family 3004 (pedigree in fig. 3) and the splice-site mutation 1471 + 1 g to a in exon 13 in GM family 4015 (pedigree and SSCA in fig. 4).

μA in well-expressing batches of oocytes (fig. 6). As shown by our previous functional analysis of the recessive R496S and the dominant P480L and G230E mutations, coexpression with WT in Xenopus oocytes is able to predict the mode of inheritance of myotonic mutations (Lorenz et al. 1994; Steinmeyer et al. 1994). We therefore coexpressed R894X and WT CLC-1 RNA at a 1:1 concentration ratio and compared channel expres-

sion to that of WT CLC-1 injected at the same total RNA concentration (fig. 6). For comparison we included R496S/WT and P480L/WT coexpressions representing recessive and strongly dominant mutations, respectively (Lorenz et al. 1994; Steinmeyer et al. 1994). Confirming our previous studies, R496S/WT currents were ~50% of WT currents, whereas coexpression with P480L reduced currents to ~10% of WT. Even though R894X
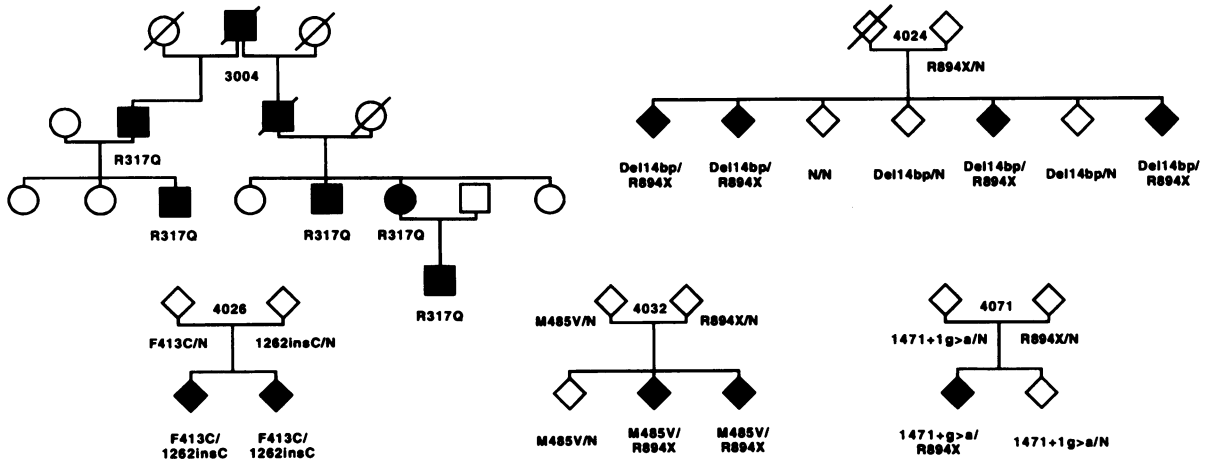
Figure 3 Selected pedigrees with mutation results from the present study. Pedigree 3004 shows autosomal dominantly inherited MC; all other pedigrees show autosomal recessively inherited GM.

channels yielded typical CLC-1 currents, coexpression with WT RNA gave currents that were intermediate between a fully recessive (R496S) and a fully dominant (P480L) mutant. Thus, in vitro analysis suggests that the R894X mutation is close to the border between a recessive and a dominant mutation.

Discussion

The present article describes our ongoing efforts to identify and describe alterations in the CLCN1 gene in autosomal dominant and autosomal recessive human myotonia. Including our previous reports (Koch et al. 1992, 1993; Lorenz et al. 1994; Meyer-Kleine et al. 1994*b*) we have characterized 16 different mutations in 94 GM chromosomes (26 families and 21 single pa-

Figure 4 SSCA analysis of Exon 10 (GM family 4017, sequence change del 2 bp) and Exon 13 (GM family 4015, sequence change 1471 + 1 g to a). The single-stranded alleles corresponding to the respective mutation are indicated by arrowheads, the polymorphism in exon 13 with asterisks (*) (Meyer-Kleine et al. 1994*b*). ds = double-stranded control; ss = single-stranded control.

tients) and two mutations in two of three MC families. We were unable to find mutations in six GM families and four single GM patients. The nonsense mutation R894X (11 of 94 chromosomes), the missense mutation F413C (10 of 94 chromosomes) and the 14-bp deletion in exon 13 (9 of 94 chromosomes) were the most frequent alterations and account for 32% of our 94 investigated GM chromosomes. In contrast to the two other more frequent mutations the R894X nonsense mutation was never observed in a homozygous state. The F413C mutation is probably the most common alteration on GM chromosomes. It was not only observed in our family material but in addition on five GM alleles reported by two other research groups (Heine et al. 1994; Koty et al. 1994). As there are no highly informative polymorphisms in and around the CLCN1 gene, it is currently not possible to investigate the genetic background against which the more frequent mutations might have arisen, i.e., from one or several founder chromosomes. In 9 of our 26 GM families, we were able to identify mutations on both chromosomes (table 3). Only the compound heterozygous status 14-bp deletion/R894X was observed more than once, in two nonrelated families and two nonrelated single patients (fig. 3). When all mutations identified so far are taken into account, the majority of events involved missense mutations (*n* = 16), whereas the number of nonsense, frameshift, and splice-site mutations (*n* = 8) is considerably lower.

The seven new amino acid substitutions described will presumably result in direct alteration of function of the channel protein, as in each case an amino acid in a conserved stretch is exchanged. The amino acids glutamic acid 291, arginine 317, and glycine 482 are conserved between different members of the voltage-gated chloride channel family, namely in Clc-0, Clc-1 to Clc-4, and also in the human kidney–specific channels
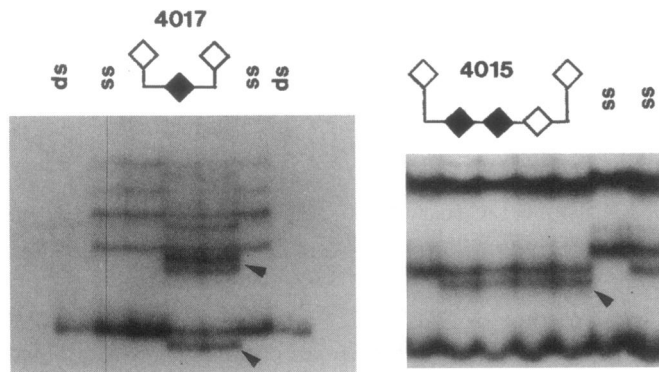
Table 3

GM Families in Which Both Mutations Are Identified

Phenotype	Exon	Codon Change or Sequence Change	Reference
GM	3/3	D136G/D136G	Heine et al. 1994
GM	8/9	R300X/R338Q	George et al. 1994
GM	8/14	Splice mutation/R496S	Lorenz et al. 1994
GM	8/23	E291K/R894X	Present study
GM	11/11	F413C/F413C	Koch et al. 1992
GM	11/12	F413C/1262insC	Present study
GM	12/12	1278-1281del 4bp/1278-1281del 4bp	Heine et al. 1994
GM	13/13	1437-1450del 14bp/1437-1450del 14bp	Meyer-Kleine et al. 1994 Lehmann-Horn et al. 1995
GM	13/23	1437-1450del 14bp/R894X	Present study
GM	13/23	M485V/R894X	Present study
GM	13/23	1471+1g to a/R894X	Present study

(Jentsch et al. 1990; Steinmeyer et al. 1991*b*; Thiemann et al. 1992; Kawasaki et al. 1994; Kieferle et al. 1994; van Slegtenhorst et al. 1994). Arginine 105, valine 165, isoleucine 329, and methionine 485 are all conserved within the rat skeletal muscle chloride channel (rClc-1) and the ubiquitous swelling-activated chloride channel (rClc-2), but not in the more distantly related Clc-0, Clc-3, Clc-4, and Clc-k channels. It is unlikely that the described alterations are merely polymorphisms and not the disease-causing mutations, since they were not found on 200 alleles from the general population that were analyzed as controls. A strict proof of this point, however, will require a functional analysis of the mutant proteins. In contrast to the findings of Koty et al. (1994), we found the A437T exchange not only in GM families but also in 5 of 200 normal control chromosomes. The exchange is located in the extracellular loop between the presumptive transmembrane domains D8 and D9 in a stretch of amino acids that are poorly conserved in the CLC-family. In addition, the chloride currents func-
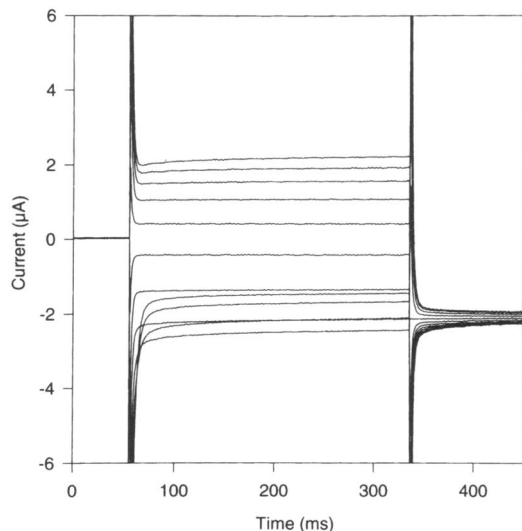

Figure 5 Voltage-clamp traces of mutant R894X CLC-1 expressed in a Xenopus oocyte and investigated by two-electrode voltage-clamp technique. Time- and voltage-dependence of currents are very similar to WT CLC-1 currents (Steinmeyer et al. 1991*a*, 1994). The two-oocyte membrane voltage was clamped sequentially to values between +60 and −180 mV in 20-mV steps from a holding potential of −30 mV, followed by a pulse to −80 mV. Currents injected into the oocyte are shown as superimposed on the time scale.
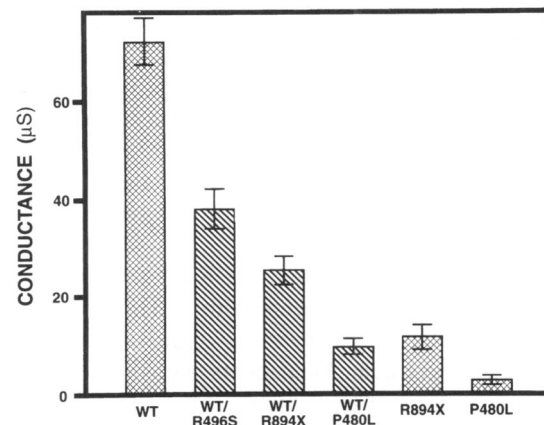

Figure 6 Quantitative analysis of WT and mutant CLC-1 currents. Oocytes were injected with identical amounts of RNA (≈10 ng), either with pure WT or mutant RNA or with a 1:1 mixture of WT and mutant RNA. This mixture mimics the situation in a heterozygous patient and allows determination of the recessive or dominant nature of the mutant. Injections of identical total amounts of RNA ensures that saturation of translation in the oocyte system can be neglected (Steinmeyer et al. 1994). The means of measurements of at least seven oocytes are shown. Error bars indicate SEM. Background conductance was not subtracted. Injections of R894X cRNA gave ~13%, WT/R496S 51%, WT/R894X 33%, and WT/P480L 10% of WT currents. Thus, R894X is intermediate between a fully recessive (R496S) and a fully dominant (P480L) mutant. Similar results were seen with three different batches of RNA and three batches of oocytes.

tionally expressed in Xenopus oocytes were comparable to WT. Therefore we conclude that this A437T exchange is more likely a silent sequence alteration.

In one-third (*n* = 8) of all identified mutations the described mutation event will result in a truncation of the predicted protein product. With the exception of R894X, which truncates the protein close to the carboxy-terminus and eliminates a poorly conserved stretch, this will probably lead to a total loss of function. This fits with our observation that these mutations are associated with the autosomal recessive GM phenotype. We do not know the amounts of truncated proteins being made in muscle but would anticipate from our in vitro studies that these would be unable to associate with normal CLC-1 subunits encoded by the other allele. Thus, they should be unable to exert a dominant effect. The locations of all the identified mutations along the preliminary channel protein model are shown in figure 1. There is no cluster for dominantly or recessively inherited mutations in the channel gene; both are rather evenly spread in the protein. Considerable phenotypic variation was not found within affected members of GM families and between families. Therefore we could not establish a phenotype-genotype correlation in our sample population. There is a slight accumulation of mutations (*n* = 5) in exon 13 in direct vicinity of the transposon insertion mutation of the ADR mouse. The region includes three missense mutations, two of them (P480L/dominant and G482R/recessive) within the recessively inherited 14-bp deletion, one (M485V/recessive) very close to it, and finally a 5' splice donor mutation at the end of the exon. It might be worth it to evaluate this region with more sensitive mutation-detection methods than SSCA in our remaining GM alleles, in order to verify a propensity of this site for mutation (Mashal et al. 1995; Youil et al. 1995).

Our data demonstrate that the R894X mutation is inherited as an autosomal recessive trait. This nonsense mutation was detected in probands with the phenotype of generalized myotonia. All patients had the characteristic sign of myotonic stiffness followed by a transient weakness that is especially pronounced in the arm and hand muscles. In none of the heterozygous R894X parents and sibs were we able to demonstrate unambiguous clinical signs of myotonia or a pathologic EMG. In contrast, we were able to detect discrete myotonic discharges in F413C heterozygotes in two families (Koch et al. 1993). In 7 of the 11 probands with the R894X mutation we found the mutation on the second GM chromosome (4 × 14-bp deletion/R894X, 1 × E291K/R894X, 1 × M485V/R894X, 1 × 1471 + 1 g-to-a/R894X). This strongly suggests that R894X behaves as a recessive mutation in these families. As this mutation was described in two autosomal dominant families by George et al. (1994), we investigated the functional effect of this mutation.

We have demonstrated elsewhere that dominant myotonia is due to dominant negative effects of mutated CLC-1 subunits (Steinmeyer et al. 1994). Detailed analysis of coexpression experiments suggested that CLC-1 functions as an oligomer of at last three, but probably four, identical subunits. It is interesting that we found that the P480L mutation exerted a more pronounced dominant negative effect than the G230E mutation described in North American MC families. This was explained by a model in which a single P480L subunit suffices to destroy channel activity when inserted into the tetrameric channel complex. In contrast, the channel functionally tolerates one G230E subunit, which leads to a less pronounced dominant negative effect.

It is important that this in vitro result was confirmed by clinical data recently. The G230E mutation was identified in a North American pedigree which at first seemed to have a recessive myotonia (P. P. Koty, G. Hobson, E. Pegoraro, H. G. Marks, A. Turel, D. Flagler, M. Cadaldini, C. Angelini, and E. P. Hoffmann, personal communication). One of the parents also had the mutated allele, though he was clinically normal. EMG, however, revealed subclinical myotonia, indicating that the G230E mutant has a reduced penetrance. This nicely confirmed our previous in vitro studies and suggests that coexpression in oocytes faithfully predicts the dominant or recessive mode of inheritance.

Our coexpression studies with the R894X mutant give similar results. One-to-one coexpression with WT yields ~30%–40% of WT current, which is in the same range as found with a 1:1 coexpression of G230E with WT RNA (Steinmeyer et al. 1994). This again places the dominant effect at the border where reduction in chloride currents is just beginning to be pathogenic. Again, this mutation has been described to be dominant in some families and recessive in ours (George et al. 1994). The factors determining the inheritance of such an intermediate mutation are unclear at present. They might depend on genetic background and may be due to the expression level of other channels.

The fact that certain CLCN1 mutations are recessive in some families but dominant in others may also explain the fact that in some cases no second mutation can be found in patients assumed to have GM. It could be that the R894X mutation belongs to a "borderline" type of mutation, which is dominant in the genetic background of a particular patient.

R894X is located after the conserved domain D13 (fig. 1). The introduction of a stop codon at this position deletes 94 amino acids of the C-terminus of the protein, a portion that is not conserved between different members of this gene family. Therefore this should not necessarily change the qualitative properties of CLC-1 currents. However, R894X strongly reduces current amplitudes in two different expression systems, Xeno-

pus oocytes and human skeletal muscle. The mechanism leading to this reduction in current is not apparent. It may be due to a reduced trafficking of the protein to the cell surface or to a reduction of protein levels. The partial dominant negative effect suggests that the mutant subunit can still associate with WT subunits. Thus, our study also demonstrates that the domains mediating subunit assembly are not located at the extreme carboxy-terminus of the protein.

Disorders that are sometimes dominantly and sometimes recessively inherited, even though the mutations are in the same gene, are increasingly described. The experience with CLCN1 mutations in autosomal dominant and recessive myotonias shows that it is impossible to predict from mutation data alone the difference in the MC and GM phenotype and inheritance. An exception might be truncations in the first part of the protein, which will probably always result in recessive inheritance. For missense mutations, however, a detailed functional analysis is necessary. Our experience shows that the Xenopus oocyte expression system is a valuable tool for predicting the mode of inheritance of CLCN1 mutations leading to myotonia.

Acknowledgments

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