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Absence of the Skeletal Muscle Sarcolemma Chloride Channel ClC-1 in Myotonic Mice*

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The voltage-dependent chloride channel ClC-1 stabilizes resting membrane potential in skeletal muscle. Mutations in the ClC-1 gene are responsible for both human autosomal recessive generalized myotonia and autosomal dominant myotonia congenita. To understand the tissue distribution and subcellular localization of ClC-1 and to evaluate its role in an animal model of myotonia, antibodies were raised against the carboxyl terminus of this protein. Expression of the 130-kDa ClC-1 protein is unique to skeletal muscle, consistent with its mRNA tissue distribution. Immunolocalization shows prominent ClC-1 antigen in the sarcolemma of both type I and II muscle fibers. Sarcolemma localization is confirmed by Western analysis of skeletal muscle subcellular fractions. The ADR myotonic mouse (phenotype ADR, genotype *adr/adr*), in which defective ClC-1 mRNA has been identified, is shown here to be absent in ClC-1 protein expression, whereas other skeletal muscle sarcolemma protein expression appears normal. Immunohistochemistry of skeletal muscle from ADR and other mouse models of human muscle disease demonstrate that the absence of ClC-1 chloride channel is a defect specific to ADR mice.

The voltage-dependent Cl^- channel ClC-1[1] is responsible for maintaining resting membrane potential in skeletal muscle (1). Hyperexcitability of muscle membranes following voluntary contractions is a hallmark of myotonia, suggesting either an increase in sodium conductance and/or a decrease in chloride conductance. Blockage of Cl^- conductance elicits myotonia in experimental animals (2, 3). Moreover, mutations in the ClC-1 gene have been shown to be responsible for hereditary myotonia in mouse (4, 5) and human (6–9).

The first member of the ClC family of voltage-gated chloride channels, ClC-0, was identified by expression cloning of Torpedo electric organ cDNAs (10). Rat skeletal muscle ClC-1, which is 55% identical to ClC-0, is a 994-amino acid protein with a predicted molecular mass of 110 kDa (11). Northern blot analysis of ClC-1 detected prominent mRNA expression in skeletal muscle, with weak expression in kidney, liver, heart, and smooth muscle (11). The apparent muscle-specific expression of the ClC-1 mRNA lies in stark contrast to the ubiquitous epithelial and non-epithelial expression of ClC-2 (12). ClC-1 and ClC-2 sequences are 55% homologous, with greatest divergence in the amino and carboxyl termini and between domains D12 and D13. Both proteins are proposed to have 12 transmembrane domains, although a 13th potential transmembrane domain is presumed to be entirely cytoplasmic, based on the inability of carboxyl-terminal deletions to affect channel inactivation (13).

The ADR ("arrested development of righting response") mouse has frequently been used as a model of human recessive autosomal myotonia. Human myotonias are similar to this mouse model on the basis of hyperexcitability of the plasma membrane and a physiological defect in sarcolemmal Cl^- conductance (14). The insertion of a transposon of the *Etn* family after the D9 coding sequence of the ClC-1 gene leads to aberrant small mRNA fragments which appear insufficient to encode a functional Cl^- channel (4). However, no study has examined ClC-1 protein expression in the ADR mouse or in any human with this genetic disease.

Although the voltage-dependent chloride channel ClC-1 has been well studied electrophysiologically and genetically due to the involvement of patients with defects in this gene, there has been very little biochemical characterization of this protein. In this study, the 130-kDa voltage-dependent chloride channel ClC-1 is shown to be localized to the sarcolemma by immunofluorescence and subcellular fractionation of skeletal muscle membranes. Protein expression appears to be limited to skeletal muscle. By immunocytochemistry, we demonstrate the specific absence of the chloride channel ClC-1 protein in ADR mice.

EXPERIMENTAL PROCEDURES

Antibody Preparation—A peptide representing the 15 carboxyl-terminal amino acids (amino acids 980–994) of the cloned rat skeletal muscle chloride channel ClC-1 was prepared by the HHMI Peptide Facility (Washington University, St. Louis, MO) as an NH_2-terminal *p*-benzoylbenzoic acid photoprobe. Peptide was conjugated to keyhole limpet hemocyanin and injected into rabbits as described (15). Peptide was also conjugated to bovine serum albumin (BSA) for immunoblot analysis. Antibodies to the α_2 subunit of the voltage-dependent Ca^{2+} channel were prepared against an 18-amino acid peptide corresponding to residues 839–856 of rabbit skeletal muscle according to De Jongh *et al.* (16). Peptide was coupled to keyhole limpet hemocyanin and BSA using the bifunctional coupling agent *m*-maleimidobenzoic acid-*N*-hydroxysuccinimide ester through an NH_2-terminal cysteine residue on the peptide. Rabbit polyclonal antibodies were affinity-purified against immobilized strips of COOH-terminal peptide coupled to BSA as described previously (17). Na,K-ATPase monoclonal antibody was provided by Dr. Kathleen Sweadner (Harvard University).

Membrane Preparation—KCl-washed membranes were prepared from three age-matched control (?/+) and *adr/adr* mice as described previously (18). Sarcolemma membranes were separated from KCl-washed microsomes by the wheat germ agglutination method described by Ohlendieck and Campbell (18).

SDS-PAGE and Immunoblotting—SDS-PAGE (19) was carried out on 3–12% gradient gels in the presence of 1% 2-mercaptoethanol (unless otherwise indicated) and stained with Coomassie Blue or transferred to nitrocellulose (20). Molecular weight standards were purchased from

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[1] The abbreviations used are: ClC-1, skeletal muscle chloride channel gene product; ADR, *adr*, arrested development of righting response (phenotype, gene); PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; WGA, wheat germ agglutinin; dfw, recessive deaf waddler; dy, dystrophia muscularis; mdx, X-linked muscular dystrophy; mAb, monoclonal antibody.

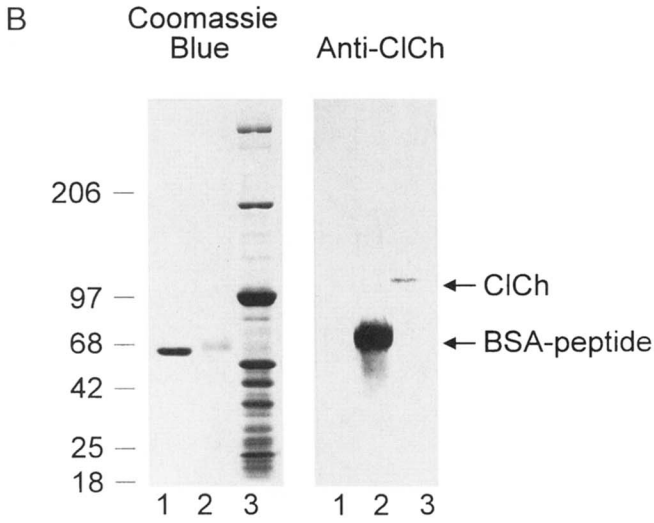

FIG. 1. **Characterization of rabbit antibody to the carboxyl terminus of a rat skeletal muscle chloride channel ClC-1.** *A*, the 15 carboxyl-terminal amino acids of the cloned rat skeletal muscle chloride channel (10). *B*, shown are a Coomassie Blue-stained gel (*left panel*) and a nitrocellulose transfer (*right panel*) stained with affinity-purified rabbit anti-ClCh antibodies. *Lane 1*, BSA (3 μg); *lane 2*, BSA-conjugated to carboxyl-terminal peptide (3 μg); *lane 3*, rabbit KCl-washed skeletal muscle microsomes (150 μg). *Arrows* indicate the positions of the chloride channel (*ClCh*) and the BSA-conjugated COOH-terminal peptide (*BSA-peptide*). Molecular weight markers are indicated on the *left*.

Life Technologies, Inc. Nitrocellulose transfers were stained with polyclonal antibodies, affinity-purified antibodies, or monoclonal antibodies as described previously (21). Affinity-purified antibodies were used at a 1:50 dilution for immunoblotting.

Immunofluorescence—Cryosections (7 μm) of quadriceps muscle age-matched mice were incubated with nondiluted affinity purified COOH-terminal ClC-1 antibody or 1:1,000 dilution of mAb XIXC2 (specific for dystrophin). Sections had been initially blocked for 1 h in 5% BSA in phosphate-buffered saline. Incubation in 1:200 biotinylated-labeled goat anti-rabbit IgG secondary for 1 h was followed by incubation for 30 min with 1:1,000 diluted fluorescein-conjugated streptavidin (Jackson ImmunoResearch Laboratories). Conventional fluorescence microscopy was carried out with an Axioplan photomicroscope. Samples were prepared uniformly, and photographs were taken under identical conditions with same exposure times.

RESULTS AND DISCUSSION

A single immunoreactive protein of approximately 130 kDa is recognized in skeletal muscle microsomes with antibodies prepared against the deduced 15 carboxyl-terminal amino acids of ClC-1 chloride channel (Fig. 1). This antibody also recognizes the BSA-conjugated peptide, but not unconjugated BSA. The skeletal muscle tissue specificity of ClC-1 is demonstrated by the inability of the ClC-1 carboxyl-terminal antibody to recognize protein in membranes of any other tissue (data not shown). The absence of ClC-1 protein in tissues other than skeletal muscle is in accordance with its mRNA expression (11), although small amounts of mRNA were detected in liver, kidney, and heart, but were not detected by Western blot analysis of crude membrane preparations. The carboxyl-terminal 15 amino acids of rat skeletal muscle are unique to ClC-1 and are not found in any other chloride channels identified to date, including the Torpedo electroplax ClC-0 (10), the ubiquitously expressed ClC-2 (12), or the K-1 of the inner medulla of the kidney (22).

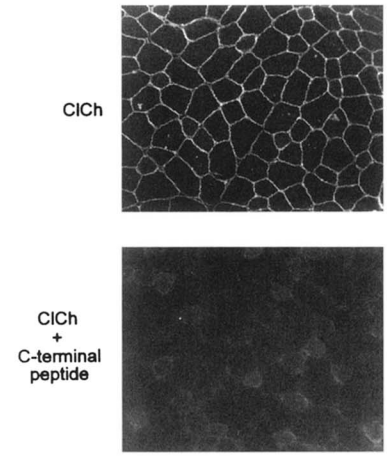

FIG. 2. **Sarcolemma distribution of ClC-1 in mouse skeletal muscle.** Immunofluorescence microscopy of mouse skeletal muscle transverse cryosections stained with affinity-purified chloride channel carboxyl-terminal antibody in the absence and presence of competing carboxyl-terminal peptide. Antibody specifically labeled the cell periphery, whereas specific staining was not observed in the internal regions of muscle fibers.

Cross-reactivity of the carboxyl-terminal antibody with rabbit, dog, mouse, hamster, guinea pig, and human skeletal muscle ClC-1 suggests conservation of at least a portion of this carboxyl-terminal sequence in mammals (data not shown). Although this antibody did not appear to react with nonmammalian tissue, an exhaustive test of various species was not performed.

Establishing the distribution of chloride-conducting channels in the sarcolemma and transverse tubule membrane system is useful in understanding the action potential repolarization phase and action potential propagation, especially as it relates to disease states such as myotonia (23–25). Labeling of transverse skeletal muscle cryosections with carboxyl-terminal ClC-1 antibody reveals exclusive staining of the cell periphery, suggestive of a sarcolemmal localization (Fig. 2). Staining was uniformly distributed along the sarcolemmal surface of all fiber types, supporting the presence of ClC-1 in both type I and II muscle fibers. Specific staining was able to be competed by preincubation of the antibody with the 15-amino acid peptide against which this antibody was raised.

Confirmation of ClC-1 distribution by fractionation of skeletal muscle membranes into purified sarcolemma and WGA-void fraction, which contains both transverse tubules and sarcoplasmic reticulum, demonstrates the presence of ClC-1 in the sarcolemma and its absence in the WGA-void fraction (Fig. 3). This is in contrast to the voltage-dependent Ca^{2+} channel α_2 subunit, which is characteristically localized to the transverse tubules and is thus enriched in the WGA-void (18, 26). These results differ dramatically from previous physiological localization of skeletal muscle chloride conducting channels. Chloride conductance in mammalian muscle was previously shown to be similar on surface and tubular membranes (24, 27), which when corrected for surface area suggested that up to 80% of chloride conductance may be associated with transverse tubules. Although ClC-1 does not appear to be present in transverse tubules by both immunofluorescence and cell fractionation, it is likely that either a distinctly different chloride conducting channel or a ClC-1 splice variant which differs at the carboxyl terminus may be present in transverse tubules. The defects in human and animal models of myotonia identified to date, however, appear to be specific for the sarcolemma protein ClC-1. The sarcolemma localization of ClC-1 has strik-

ing implications for understanding the mechanisms of myotonic properties such as depolarizing afterpotentials which allow repetitive firing of action potentials and tubular depolarization.

The single channel properties of ClC-0 and the dominant negative effect of Thomsen-type myotonia congenita ClC-1 mutations predict a homodimeric or homotetrameric structure for this chloride channel gene family (8, 28). To investigate whether this oligomeric structure may be due to disulfide linked monomers, crude surface membranes were run under reduced and nonreduced conditions. Although the characteristic 25-kDa mobility shift was seen in the voltage-dependent Ca^{2+} channel α_2 subunit on dissociation of the disulfide linked δ subunit (29), disulfide reduction appeared to have no effect on ClC-1 mobility (data not shown). The absence of a mobility shift on disulfide bond reduction suggests that, if this channel is indeed a homooligomeric structure, it is not comprised of disulfide-linked ClC-1 monomers. However, voltage-dependent K^+ channels form a well established homotetramer without intersubunit disulfide bonds, and the chloride channel oligomeric structure may be similar (30).

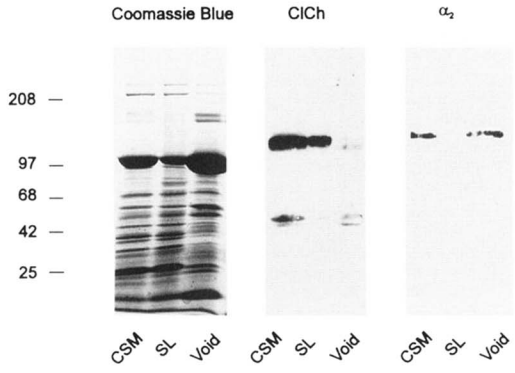

FIG. 3. **Immunoblot analysis of purified sarcolemma preparation.** Coomassie Blue-stained gel (*left panel*) and nitrocellulose transfer (*middle* and *right panel*) stained with affinity-purified rabbit chloride channel (*ClCh*) antibodies and affinity-purified voltage-dependent Ca^{2+} channel α_2 (α_2) subunit antibodies and detected by ECL (Amersham Corp.). *First lane*, 150 μg of mouse crude surface membranes; *second lane*, 150 μg of sarcolemma; *third lane*, 150 μg of WGA-void fraction. Molecular weight standards are indicated on the *left*. Low molecular weight fragments identified by affinity-purified chloride channel antibodies are degradation products.

Recent evidence has suggested that a highly conserved consensus site for *N*-linked glycosylation between domains D8 and D9 is glycosylated *in vitro* (31). Biochemical deglycosylation with glycanase F resulted in a small, but difficult to detect and reproduce, mobility shift of 2–3 kDa, which may represent deglycosylation of a single *N*-glycosylation site (data not shown). Under identical conditions, complete deglycosylation was observed in the heavily glycosylated voltage-dependent Ca^{2+} channel α_2 subunit. However, the majority of 1% digitonin solubilized ClC-1 is not retained by WGA affinity chromatography (data not shown) and no differences are seen on concanavalin A peroxidase staining of control and ADR mouse skeletal muscle (Fig. 4*A*). Experiments on purified chloride channel complex may further elucidate the presence and location of glycosylated residues. Glycosylation also does not account for the difference in the 110 kDa predicted molecular mass based on sequence analysis (11) and the 130 kDa calculated for the native protein. Anomalous SDS-PAGE mobility of ClC-1 may be due to the multiple membrane-spanning regions or the highly negatively charged domain between D12 and D13.

Although ClC-1 protein expression is predicted to be absent in ADR mice based on phenotypic and genetic analysis (11), here we confirm its absence in skeletal muscle membranes. ClC-1 protein is specifically absent in *adr/adr* mouse skeletal

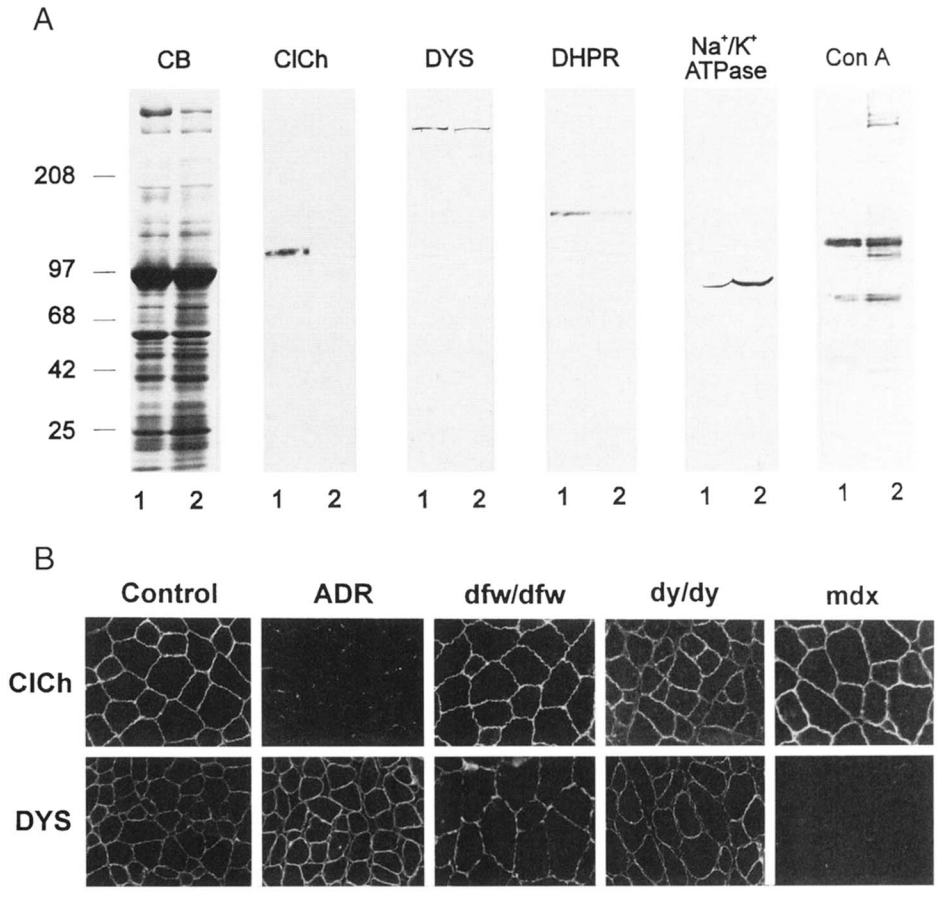

FIG. 4. **Specific deficiency of ClC-1 chloride channel protein in ADR mice.** *A*, immunoblot analysis of control and *adr/adr* mouse muscle membranes. Shown are a Coomassie Blue-stained gel (*CB*) and identical immunoblots stained with affinity-purified anti-chloride channel antibodies (*ClCh*), affinity-purified rabbit antibody to the COOH terminus of dystrophin (*DYS*), mAb IIF7 to the α_1 subunit of the skeletal muscle dihydropyridine receptor (*DHPR*), mAb McB2 to the Na^+/K^+-ATPase, and concanavalin A-peroxidase (*ConA*). *Lanes 1* represent (?/+) control membranes and *lanes 2* represent *adr/adr* membranes. Protein amounts (from *left* to *right*) are 100, 200, 100, 100, 50, and 100 μg. *B*, immunofluorescence labeling of mouse skeletal muscle with chloride channel (ClC-1) and dystrophin antibodies. Transverse cryosections were labeled for indirect immunofluorescence with affinity-purified anti-chloride channel antibodies (*ClCh*) and mAb XIXC2 against dystrophin (*DYS*). Cryosections are from skeletal muscle of age-matched control (?/+), *adr/adr*, dfw/dfw, dy/dy, and mdx mice.

muscle as compared with age-matched controls (Fig. 4*A*). The defect is specific for ClC-1, as expression of dystrophin, dihydropyridine receptor, and Na^+/K^+-ATPase all appear to be normal in ADR mice. Immunofluorescence microscopy also demonstrates the specific absence of ClC-1 sarcolemma staining in ADR mice (Fig. 4*B*). Dystrophin, which is also localized to the sarcolemma, is expressed normally in the myofibers of ADR mice. Loss of ClC-1 immunoreactivity does not appear to be a nonspecific marker of muscle disease, as this protein is expressed at normal levels in dy/dy, dfw/dfw, and mdx mice.

This is the first biochemical characterization of the skeletal muscle voltage-dependent chloride channel ClC-1. Although an earlier study identified a 110–120-kDa protein in purified sarcolemma fractions of rabbit skeletal muscle that correlated with chloride channel activity and indanyloxyacetic acid binding, this protein could not be unambiguously identified as ClC-1 without the use of specific antibodies (32). Interestingly, a 60-kDa protein also copurified in the fractions with chloride channel activity, suggesting the presence of an auxiliary subunit such as has been found to associate with most other voltage-gated channels. Further purification of the chloride channel complex should clarify its multimeric structure.

While several investigators have identified and examined the electrophysiological properties of mutant ClC-1 proteins expressed in *Xenopus* oocytes, a biochemical approach will allow us to determine whether ClC-1 may be regulated by the serine/threonine protein kinase implicated in the pathogenesis of myotonic dystrophy. Also, since we have identified the absence of ClC-1 protein in ADR mice, it is probable that some forms of human autosomal recessive myotonia congenita may also be associated with a deficiency of this protein. Determination of ClC-1 expression by immunofluorescence microscopy of muscle biopsy may aid genetic analysis in the diagnosis of patients with myotonia congenita.

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