Adhalin mRNA and cDNA sequence are normal in the cardiomyopathic hamster

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Abstract Adhalin is deficient in two forms of human muscular dystrophy, one due to mutations in the adhalin gene and one linked to an unidentified gene on chromosome 13. Because adhalin is deficient in skeletal and cardiac muscles of BIO 14.6 hamsters, which experience both myopathy and cardiomyopathy, cDNA encoding adhalin from BIO 14.6 hamster skeletal muscle was cloned and sequenced. Adhalin mRNA was expressed at normal levels in BIO 14.6 hamster cardiac muscle, and no mutation in adhalin coding sequence was found, indicating that the inherited myopathy and cardiomyopathy of the BIO 14.6 hamster are most likely not due to mutations in the adhalin gene.

Key words: Adhalin; Cardiomyopathic hamster; Cardiomyopathy; Dystroglycan; Dystrophin–glycoprotein complex; Muscular dystrophy

1. Introduction

Duchenne muscular dystrophy results from mutations in the gene encoding dystrophin, a muscle membrane cytoskeletal protein [1]. In normal muscle, dystrophin is complexed with several dystrophin-associated proteins (α-dystroglycan, syntrophin, adhalin, β-dystroglycan, 43DAG or A3b, 35DAG, and 25DAP) to form the dystrophin–glycoprotein complex. The N-terminus of dystrophin binds to filamentous actin and α-dystroglycan binds to laminin, indicating that one function of the dystrophin–glycoprotein complex is to link the actin cytoskeleton to the extracellular matrix (reviewed in [2]). Due to the loss of dystrophin, all dystrophin-associated proteins are greatly reduced in abundance in skeletal muscle from Duchenne muscular dystrophy patients [3,4], *mdx* mice [5], and dystrophic golden retrievers [6].

An identical or antigenically similar complex of proteins has been identified in cardiac muscle [7,8]. Cardiac dystrophin–glycoprotein complex colocalizes with laminin to the cardiac sarcolemma and T-tubule system [8], in contrast with skeletal muscle in which the dystrophin–glycoprotein complex is absent from T-tubules [9]. The dystrophin-associated proteins are preserved in *mdx* cardiac muscle, presumably by forming a complex with utrophin, an autosomal homologue of dystrophin, or Dp71, a C-terminal transcript from the dystrophin gene [7].

Cardiomyopathies develop in patients suffering from Duchenne muscular dystrophy and in patients having some other forms of muscular dystrophy [10,11]. Additionally, the inbred BIO 14.6 hamster develops both an autosomal recessive muscular dystrophy and cardiomyopathy [12–14]. The cardiomyopathic hamster (CMH) is a widely studied animal model of hypertrophic cardiomyopathies in which cardiac cellular necrosis accompanied by a loss of sarcolemmal integrity begins 30–40 days after birth in 100% of animals homozygous for the disease [12]. Primary cell damage ceases by 80 days of age, but calcium deposits and excess connective tissue remain [12]. Progressive hypertrophy followed by dilatation leads to congestive heart failure and death at less than half the normal life span [14]. Several biochemical abnormalities in the CMH heart have been described, but the genetic defect in the BIO 14.6 strain has not been identified.

Dystrophin is present at nearly normal levels in the cardiomyopathic hamster, but the link between dystrophin and WGA-binding glycoproteins is totally disrupted in CMH skeletal [15] and cardiac [16] muscle. All dystrophin-associated proteins are decreased in abundance in CMH cardiac muscle as determined by immunofluorescence [15,16]. However, in CMH skeletal muscle dystrophin and all dystrophin-associated proteins are present at normal levels except adhalin, the 50-kDa dystrophin-associated glycoprotein, which is totally deficient [15–17], and 35DAG, which is reduced in abundance [15,17]. Interestingly, this is the same pattern of staining observed in patients with severe childhood autosomal recessive muscular dystrophy [3].

The cDNA encoding adhalin has been cloned from rabbit [18] and human [19] skeletal muscle. Adhalin mRNA [18,19] and protein [20] is expressed almost exclusively in skeletal and cardiac muscles. The human adhalin gene is located on chromosome 17q, and various missense, nonsense, and frameshift mutations in the adhalin gene have been identified in multiple families with autosomal recessive muscular dystrophy ([19]; and F. Piccolo, S.L.R., K.P.C., J.-C. Kaplan, et al., unpublished data). Another form of autosomal recessive muscular dystrophy is caused by mutations in an unidentified gene on chromosome 13q [21], and at least some patients linked to markers on chromosome 13q have a deficiency of adhalin in skeletal muscle [22]. Thus, at least two genetic defects can lead to adhalin deficiency.

In this report we investigated whether mutations in the adhalin gene might cause the myopathy and cardiomyopathy of BIO 14.6 cardiomyopathic hamsters. However, adhalin mRNA was expressed at normal levels in cardiomyopathic hamsters and no mutations in the coding sequence of adhalin were identified by sequence analysis. Thus, the inherited muscular dystrophy and cardiomyopathy of the BIO 14.6 hamster are most likely not due to mutations in the adhalin gene.

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2. Materials and methods

Male F1B control and BIO 14.6 cardiomyopathic golden Syrian hamsters were obtained from Bio Breeders, Fitchburg, MA. 8-Week-old male CHF146 and CHF147 cardiomyopathic hamsters and male and female golden Syrian and albino control hamsters were obtained from Canadian Hybrid Farm, Centreville, N.S., Canada. Animals were housed and sacrificed in accordance with institutional guidelines. The affinity-purified sheep antibody against adhalin [5] and monoclonal antibody IIH6 against dystroglycan [23] were previously described. Skeletal muscle (7 μm) cryosections were immunostained as described [15]. Cardiac membranes were prepared as described [5] from age-matched hamsters and fractionated on 3–12% gradient SDS-polyacrylamide gels, transferred to nitrocellulose, and stained with antibodies as previously described [9].

Total skeletal or cardiac muscle RNA was isolated from F1B or BIO 14.6 hamsters using RNAzol (Tel-Test), and poly(A)$^+$ RNA was purified using the mRNA Separator kit (Clonetech). For cDNA library construction, F1B or BIO 14.6 hamster skeletal muscle cDNA was synthesized with *NotI*/*Eco*RI adapters using a kit from Pharmacia, cDNA was ligated into λZAPII (Stratagene), and bacteriophage DNA was packaged using Gigapack Gold II (Stratagene). Libraries were screened by hybridization according to Stratagene's protocol using rabbit adhalin cDNA to screen the F1B library and subsequently the F1B hamster adhalin clone to screen the BIO 14.6 library. Polymerase chain reaction was performed using Taq polymerase from Boehringher-Mannheim and a Perkin-Elmer thermal cycler. Sequencing was performed using an Applied Biosystems automated sequencer. Northern blot analysis was performed as described [18].

3. Results

To more clearly determine whether adhalin was a reasonable candidate for causing the myopathy and cardiomyopathy of the BIO 14.6 hamster, the expression of adhalin was examined at various time points in young hamsters. As shown in Fig. 1, the loss of adhalin was observed prior to and throughout the time course of myocytolysis in the cardiomyopathic hamster. Additionally, adhalin was undetectable in a 3-day-old cardiomyopathic hamster bred from BIO 14.6 hamsters in our laboratory (data not shown). Thus, loss of adhalin precedes the onset of myopathy and cardiomyopathy, suggesting that the deficiency of this protein may play a role in the pathogenesis of these disease states.

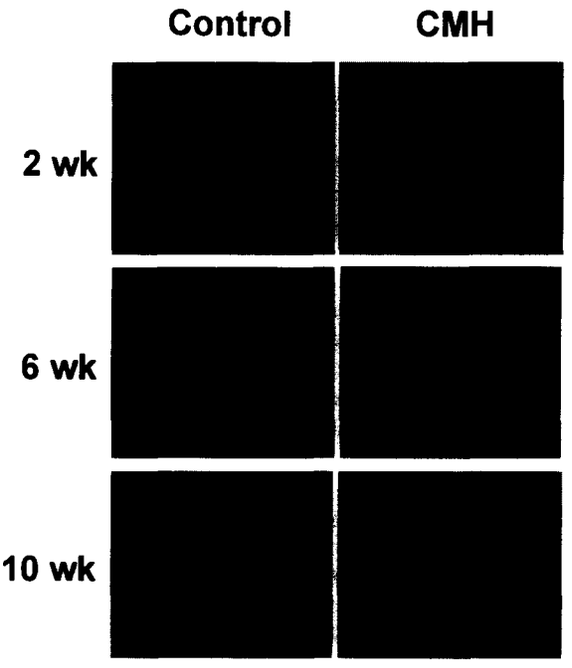

Fig. 1. Deficiency of adhalin in BIO 14.6 hamsters at early ages. Transverse skeletal muscle cryosections from 2-, 6-, or 10-week-old normal (Control) or BIO 14.6 cardiomyopathic (CMH) hamsters were labeled by indirect immunofluorescence with an affinity-purified sheep antibody against adhalin.

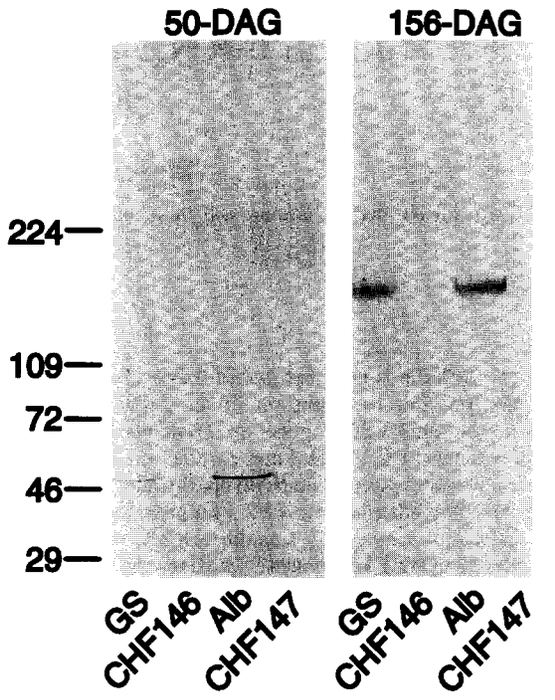

Fig. 2. Adhalin is deficient in two independent strains of cardiomyopathic hamsters derived from the BIO 14.6 line. Identical immunoblots of cardiac membranes from Golden Syrian (GS), cardiomyopathic line CHF146, albino (Alb), and cardiomyopathic line CHF147 hamsters were stained with an affinity-purified sheep antibody against adhalin or monoclonal antibody IIH6 against α-dystroglycan. Molecular weight standards ($M_r \times 10^{-3}$) are indicated.

Because our previous work [15] describing the deficiency of adhalin in cardiomyopathic hamsters used BIO 14.6 hamsters from Bio Breeders, we felt the need to confirm that adhalin was deficient in other colonies of cardiomyopathic hamsters. The BIO 14.6 strain of golden Syrian hamsters was derived from the original myopathic BIO 1.50 line [24]. Subsequently, a UM-X7.1 line was established at the University of Montreal by breeding BIO 14.6 hamsters with a strain of healthy animals [14]. Colonies of cardiomyopathic hamsters from a common origin are maintained at various laboratories around the world. Because of this, adhalin deficiency should not necessarily be maintained if it is not directly related to the disease process.

We analyzed two strains of cardiomyopathic hamsters from Canadian Hybrid Farm in Nova Scotia: the CHF146 line derived from BIO 14.6 and the CHF147 line derived from UM-X7.1, along with two healthy hamster lines. Adhalin was deficient in cardiac membranes from both cardiomyopathic lines but was easily detectable in normal hamsters (Fig. 2). α-Dystroglycan was greatly reduced in abundance in cardiac membranes

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RABBIT   MAAAALLWLPLLVGCLAGPGGTEAQQTTLYPLVGRVFVHTLEPASFLHLP   50
HUMAN    MAET-LFWTPLLVVLLAGLGDTEAQQTTLHPLVGRVFVHTLDHETFLSLP   49
HAMSTER  MAAT-LTWILLFVGLLAGLRDTKAQQTTLYPLVGRVFVHPLEHATFLRLP   49
         **   * *  *.*  ***   * ****** ********* *.  .** **

RABBIT   EHAA-PATIPVTYHAHLQGHPDLPRWLRYTQRSPHHPGFLYGAATPEDRG   99
HUMAN    EHVAVPPAVHITYHAHLQGHPDLPRWLRYTQRSPHHPGFLYGSATPEDRG   99
HAMSTER  EHIAVPPTVRLTYQAHLQGHPDLPRWLRYTQRSPYSPGFLYGTPTPEDRG   99
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RABBIT   RQVIEVTAYNRDSFDTAGQSLVLLIRDPEGSPLPYQTEFLVRSHDVEEVL  149
HUMAN    LQVIEVTAYNRDSFDTTRQRLVLEIGDPEGPLLPYQAEFLVRSHDAEEVL  149
HAMSTER  RQVIEVTAYNRDSFDTTRQRLLLLIEDPEGPRLPYQAEFLVRSHDVEEVL  149
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RABBIT   PPTPASHFLTALAGLWEPGELKLLNITSALDRGGRVPLPIGGQKEGVYIK  199
HUMAN    PSTPASRFLSALGGLWEPGELQLLNVTSALDRGGRVPLPIEGRKEGVYIK  199
HAMSTER  PSTPANRFLTALGGLWELGELQLLNITSALDRGGRVPLPIEGRKEGVYIK  199
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RABBIT   VGSASPFSTCLKMVASPDSHARCARGQPPLLSCYDTLAPHFRVDWCNVSL  249
HUMAN    VGSASPFSTCLKMVASPDSHARCAQGQPPLLSCYDTLAPHFRVDWCNVTL  249
HAMSTER  VGSATPFSTCLKMVASPDSYARCAQGQPPLLSCYDSLAPHFRVDWCNVSL  249
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RABBIT   VDTSVPEPVDEVPTPGDGILEHDPFFCPPTEATARDFLADALVTLLVPLL  299
HUMAN    VDKSVPEPADEVPTPGDGILEHDPFFCPPTEAPDRDFLVDALVTLLVPLL  299
HAMSTER  VDKSVPEPLDEVPTPGDGILEHDPFFCPPTEATGRDFLADALVTLLVPLL  299
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RABBIT   VALLLALLLAYIMCCRREGRLKRDLATSDIQMVHHCTIHENTEELRQMAA  349
HUMAN    VALLLTLLLAYVMCCRREGRLKRDLATSDIQMVHHCTIHGNTEELRQMAA  349
HAMSTER  VALLLTLLLAYIMCCRREGQLKRDMATSDIQMVHHCTIHGNTEELRQMAA  349
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RABBIT   SREVPRPLSTLPMFNVRTGERMPPRVDSAQVPLILDQH   387
HUMAN    SREVPRPLSTLPMFNVHTGERLPPRVDSAQVPLILDQH   387
HAMSTER  RREVPRPLSTLPMFNVRTGERLPPRVDSAQVPLILDQH   387
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Fig. 3. Alignment of rabbit, human, and hamster adhalin amino acid sequences. Amino acids that are identical among all three species are indicated by an asterisk; conservative substitutions are indicated by a dot. Two extracellular potential N-linked glycosylation sites, five extracellular cysteines, and an intracellular consensus site for phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase are conserved. The nucleotide sequence reported in this paper has been submitted to GenBank/EMBL Data Bank with Accession No. U21677.

isolated from both strains of cardiomyopathic hamsters, as it is in BIO 14.6 hamsters [15]. However, α-dystroglycan is present at normal levels in cardiomyopathic hamster skeletal muscle [15], suggesting that the dystroglycan gene is unlikely to be affected. These results demonstrate that three separately maintained strains of cardiomyopathic hamsters from a single genetic origin share a deficiency of adhalin and α-dystroglycan in cardiac muscle.

Normal hamster adhalin cDNA was isolated by constructing and screening a hamster skeletal muscle cDNA library using rabbit adhalin cDNA [48] as a probe. The longest characterized clone contained 1332 bp, including 4 bp of 5′ untranslated sequence and 167 bp of 3′ untranslated sequence (including a polyadenylation signal) based on homology to rabbit and human adhalin.

The deduced amino acid sequence of hamster adhalin is shown in Fig. 3. Hamster, human, and rabbit adhalin are 80% identical at the amino acid level, and homology is 86% when including conservative substitutions. Two consensus sites for N-linked glycosylation, a potential phosphorylation site for Ca^{2+}/calmodulin-dependent protein kinase, and five extracellular cysteines are conserved in all three species. Four of these cysteines are closely spaced in a manner bearing some homology to extracellular domains of entactin and nerve growth factor receptor, suggesting that adhalin may bind to an extracellular matrix component or other cell-surface molecule.

Adhalin mRNA was present at normal levels and at apparently normal size in cardiomyopathic hamster cardiac muscle (Fig. 4). This suggests that either the adhalin gene is unaffected in BIO 14.6 hamsters or that mutations in the adhalin gene do not affect transcription, splicing, or stability of adhalin mRNA and must not involve large deletions or insertions within transcribed regions. Thus, screening for adhalin mutations must involve searching for point mutations or small deletions or insertions within the translated mRNA, and thus cDNA, sequence.

To search for mutations in adhalin cDNA, a BIO 14.6 hamster skeletal muscle cDNA library was constructed and screened using normal (F1B) hamster adhalin cDNA as a probe. One 1301-bp clone was obtained having its 5′ end at the sixth translated nucleotide and having 145 bp of 3′ untranslated sequence. Additionally, BIO 14.6 hamster adhalin cDNA was amplified by PCR from reverse-transcribed BIO 14.6 skeletal muscle poly(A)$^+$ RNA using oligonucleotides corresponding to nucleotides −43 to −24 of human adhalin 5′ untranslated sequence [19] and hamster adhalin nucleotides 1300–1322.

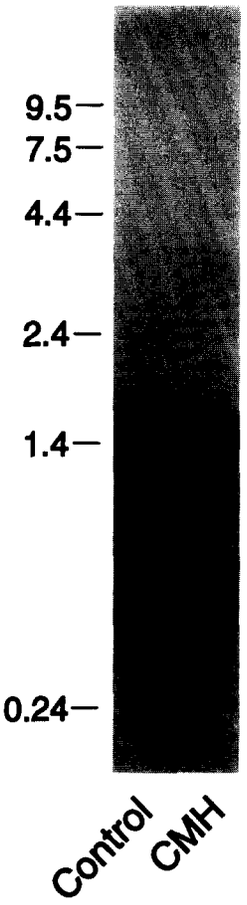

Fig. 4. Adhalin mRNA is present at normal levels in BIO 14.6 hamster cardiac muscle. A Northern blot containing 5 μg per lane of F1B (Control) or BIO 14.6 (CMH) hamster cardiac total RNA was hybridized with a hamster adhalin cDNA probe. The autoradiograph was exposed for 4 days. RNA size standards (kilobases) are indicated.

Sequencing of BIO 14.6 hamster adhalin cDNA obtained by both methods revealed that cardiomyopathic hamster adhalin cDNA was identical to that from normal hamsters.

4. Discussion

Adhalin is deficient in BIO 14.6 cardiomyopathic hamsters prior to and after the onset of myocytolysis. Additionally, adhalin deficiency has been confirmed in three separately maintained strains of cardiomyopathic hamsters, indicating that it would be a reasonable candidate for being the primary cause of muscular dystrophy and cardiomyopathy in this strain of hamsters. Consistent with this hypothesis is the recent finding that mutations in the human adhalin gene can cause muscular dystrophy [19]. However, no mutations were found in the coding region of BIO 14.6 hamster adhalin cDNA, and Northern blot analysis indicated that adhalin mRNA is transcribed normally and is stable in cardiomyopathic hamster heart. Because the complete 5′ untranslated region has not been isolated, it remains strictly possible that mutations upstream of known sequence could adversely affect the translation of adhalin. However, it appears very unlikely that mutations in the adhalin gene are responsible for the myopathy and cardiomyopathy of BIO 14.6 hamsters.

Interestingly, adhalin deficiency in human muscular dystrophies has been observed not only in patients with mutations in the adhalin gene [19], but also in patients linked to an unidentified gene on chromosome 13q [22]. It is possible that the defective gene in the cardiomyopathic hamster is homologous to the muscular dystrophy gene on human chromosome 13q. Although cardiomyopathy is not common in individuals with chromosome 13q-linked muscular dystrophy, a few individuals with adhalin deficiency and cardiomyopathy have been identified (Y. Sunada and K.P.C., unpublished data). Regardless, the affected gene in cardiomyopathic hamsters must affect the expression of adhalin in both skeletal and cardiac muscles [15–17]. Such a gene may encode a chaperone protein, an adhalin-binding protein (either intracellular, on the cell surface, or in the extracellular matrix), or another component of the dystrophin–glycoprotein complex. The identification of the defective gene in the BIO 14.6 hamster will likely increase our knowledge of the function of adhalin and the entire dystrophin–glycoprotein complex.

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