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Missense Mutations in the Adhalin Gene Linked to Autosomal Recessive Muscular Dystrophy

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Summary

Adhalin, the 50 kDa dystrophin-associated glycoprotein, is deficient in skeletal muscle of patients having severe childhood autosomal recessive muscular dystrophy (SCARMD). In several North African families, SCARMD has been linked to chromosome 13q, but SCARMD has been excluded from linkage to this locus in other families. We have now cloned human adhalin cDNA and mapped the adhalin gene to chromosome 17q12-q21.33, excluding it from involvement in 13q-linked SCARMD. However, one allelic variant of a polymorphic microsatellite located within intron 6 of the adhalin gene cosegregated perfectly with the disease phenotype in a large family. Furthermore, missense mutations were identified within the adhalin gene that might cause SCARMD in this family. Thus, the adhalin gene is involved in at least one form of autosomal recessive muscular dystrophy.

Introduction

Severe childhood autosomal recessive muscular dystrophy (SCARMD), disease number 253700 in McKusick (1992), resembles Duchenne or Becker muscular dystrophy but affects females as well as males. SCARMD was initially identified in Tunisia (Ben Hamida et al., 1983) and has since been found in Algeria (Azibi et al., 1993), Morocco (El Kerch et al., 1994), and other Middle Eastern population centers (Salih et al., 1983; Farag and Teebi, 1990) as well as in Europe (Fardeau et al., 1993), South America (Passos-Bueno et al., 1993a), Japan (Higuchi et al., 1994), and North America (S. L. R. and K. P. C., unpublished data). Dystrophin, the protein product of the Duchenne muscular dystrophy gene, is unaffected in patients with SCARMD (Ben Jelloun-Dellagi et al., 1990). However, adhalin, the 50 kDa component of the dystrophin–glycoprotein complex, is specifically deficient in skeletal muscle of SCARMD patients (Matsumura et al., 1992a; Azibi et al., 1993; Fardeau et al., 1993; El Kerch et al., 1994).

The dystrophin–glycoprotein complex (Ervasti et al., 1990; Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991) consists of α-dystroglycan, a highly glycosylated 156 kDa extracellular laminin- and merosin-binding protein (Ibraghimov-Beskrovnaya et al., 1992; Ervasti and Campbell, 1993; Sunada et al., 1994); β-dystroglycan, a 43 kDa transmembrane glycoprotein (Ibraghimov-Beskrovnaya et al., 1992); adhalin, a 50 kDa transmembrane glycoprotein (Roberds et al., 1993b); syntrophin, an intracellular 59 kDa protein triplet (Adams et al., 1993; Yang et al., 1994); a 35 kDa transmembrane glycoprotein; a 25 kDa transmembrane protein; and dystrophin, a 427 kDa cytoskeletal protein containing an actin-binding domain at its N-terminus (Hemmings et al., 1992). Thus, one function of the dystrophin–glycoprotein complex in skeletal muscle is to link the extracellular matrix, via merosin, to the actin cytoskeleton, via dystrophin. In Duchenne muscular dystrophy, the absence of dystrophin leads to a 90% reduction in all of the dystrophin-associated proteins in the sarcolemma (Ohlendieck et al., 1993). However, dystrophin-associated proteins are preserved at the neuromuscular junction in both *mdx* mice (Matsumura et al., 1992b) and Duchenne muscular dystrophy patients (Matsumura et al., 1992b; Mizuno et al., 1994) by forming a complex with utrophin. Recent studies suggest that utrophin and α-dystroglycan form a complex at the neuromuscular junction that may mediate agrin-induced acetylcholine receptor clustering (Campanelli et al., 1994; Gee et al., 1994; Bowe et al., 1994).

Adhalin is absent or nearly absent from skeletal muscle of SCARMD patients, and the 35 kDa dystrophin-associated glycoprotein is slightly reduced in abundance, whereas dystrophin and all other dystrophin-associated proteins are present at apparently normal levels (Matsumura et al., 1992a). The reduction in abundance of the 35 kDa dystrophin-associated glycoprotein may occur due

to its close association with adhalin (Yoshida and Ozawa, 1990; S. L. R. and K. P. C., unpublished data). Recently, SCARMD was linked to the pericentromeric region of chromosome 13q in several Tunisian families (Ben Othmane et al., 1992). The affected gene on chromosome 13q has not been identified. Linkage to this locus has since been found in other families from North Africa (Azibi et al., 1993; El Kerch et al., 1994). However, the 13q locus has been excluded by linkage analysis in other families in Brazil (Passos-Bueno et al., 1993a) and one family in France (Romero et al., 1994). Thus, mutations in at least two independent genes can result in SCARMD with adhalin deficiency. The adhalin gene is a reasonable candidate for being one of these genes.

To determine whether the adhalin gene may be involved in SCARMD, we cloned and sequenced human adhalin cDNA and large portions of the gene and mapped it to the long arm of chromosome 17. We identified a polymorphic microsatellite within an intron of the adhalin gene and used this marker to demonstrate linkage of the adhalin gene with SCARMD in a large family previously shown not to be linked to chromosome 13q. By sequencing adhalin cDNA and genomic DNA from the affected children, we identified two missense mutations, one in each allele of the adhalin gene, that may account for the absence of adhalin in these patients. Thus, genetic defects in at least two components, dystrophin and adhalin, of the dystrophin–glycoprotein complex can independently cause muscular dystrophies, namely Duchenne muscular dystrophy and one form of SCARMD.

Results

Primary Structure and Tissue-Specific Expression of Human Adhalin

Adhalin cDNA was isolated from a human skeletal muscle λZAPII library (Stratagene) using rabbit adhalin cDNA (Roberds et al., 1993b) as a probe (Figure 1). Human and rabbit adhalin are 75% identical at the nucleotide level and 86% identical at the amino acid level. Homology between the rabbit and human proteins is increased to 90% when conservative amino acid substitutions are considered. The conservation of adhalin between human and rabbit is notably less than that of dystroglycan, which is 93% identical and 99% conserved at the amino acid level between human and rabbit (Ibraghimov-Beskrovnaya et al., 1993). Human adhalin has a hydrophobic signal sequence and a single transmembrane domain, two potential sites for N-linked glycosylation, and one consensus site for phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase, all of which are also conserved in rabbit adhalin. The extracellular domain of adhalin contains four closely spaced cysteines bearing limited homology to domains of entactin and nerve growth factor receptor, suggesting that adhalin may serve as a receptor for an extracellular matrix protein.

Northern blot analysis revealed that human adhalin mRNA was most abundant in skeletal muscle but was also expressed in cardiac muscle and, at much lower levels, in lung (Figure 2A). Based on the predominantly muscle-specific expression of adhalin in human and rabbit (Roberds et al., 1993b), adhalin mRNA in lung is likely to arise from smooth muscle cells present in airways or blood vessels. The predominant adhalin message in all tissues was approximately 1.5 kb, although a smaller, less abundant transcript was detected in skeletal muscle. The smaller transcript may arise from alternative splicing of the adhalin gene or from the use of alternative transcription initiation or polyadenylylation sites. Among five fetal tissues examined, human adhalin mRNA was most highly expressed in cardiac muscle and at lower levels in lung (Figure 2B) but was also detected in liver and kidney upon prolonged exposure of the autoradiograph. Notably, adhalin mRNA was not detected in brain. The muscle-specific expression of adhalin contrasts with that of dystroglycan (Ibraghimov-Beskrovnaya et al., 1992, 1993), which is widely expressed in both muscle and nonmuscle tissues throughout development. Hybridization of human adhalin cDNA to Southern-blotted genomic DNA was consistent with the presence of a single adhalin gene (data not shown).

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                                                                    CCCCTGTCTCTGTCACTCACCGGGCGGGCCAGGCCGGGCAGCC   -1
                                                                              ↓1100
ATGGCTGAGACACTCTTCTGGACTCCTCTCCTCGTGGTTCTCCTGGCAGGGCTGGGGGAC   60
 M  A  E  T  L  F  W  T  P  L  L  V  V  L  L  A  G  L  G  D   20

ACCGAGGCCCAGCAGACCACGCTACACCCACTTGTGGGCCGTGTCTTTGTGCACACCTTG  120
 T  E  A  Q  Q  T  T  L  H  P  L  V  G  R  V  F  V  H  T  L   40
                                                    ↓90
GACCATGAGACGTTTCTGAGCCTTCCTGAGCATGTCGCTGTCCCACCCGCTGTCCACATC  180
 D  H  E  T  F  L  S  L  P  E  H  V  A  V  P  P  A  V  H  I   60

ACCTACCACGCCCACCTCCAGGGACACCCAGACCTGCCCCGGTGGCTCCGCTACACCCAG  240
 T  Y  H  A  H  L  Q  G  H  P  D  L  P  R  W  L  R  Y  T  Q   80
                                                          *
CGCAGCCCCCACCACCCTGGCTTCCTCTACGGCTCTGCCACCCCAGAAGATCGTGGGCTC  300
 R  S  P  H  H  P  G  F  L  Y  G  S  A  T  P  E  D  R  G  L  100
          ↓200
CAGGTCATTGAGGTCACAGCCTACAATCGGGACAGCTTTGATACCACTCGGCAGAGGCTG  360
 Q  V  I  E  V  T  A  Y  N  R  D  S  F  D  T  T  R  Q  R  L  120
                      ↓340
GTGCTGGAGATTGGGGACCCAGAAGGCCCCCTGCTGCCATACCAAGCCGAGTTCCTGGTG  420
 V  L  E  I  G  D  P  E  G  P  L  L  P  Y  Q  A  E  F  L  V  140

CGCAGCCACGATGCGGAGGAGGTGCTGCCCTCAACACCTGCCAGCCGCTTCCTCTCAGCC  480
 R  S  H  D  A  E  E  V  L  P  S  T  P  A  S  R  F  L  S  A  160
                                                          *
TTGGGGGGACTCTGGGAGCCCGGAGAGCTTCAGCTGCTCAACGTCACCTCTGCCTTGGAC  540
 L  G  G  L  W  E  P  G  E  L  Q  L  L  N  V  T  S  A  L  D  180
                                              ↓520
CGTGGGGGCCGTGTCCCCCTTCCCATTGAGGGCCGAAAAGAAGGGGTATACATTAAGGTG  600
 R  G  G  R  V  P  L  P  I  E  G  R  K  E  G  V  Y  I  K  V  200

GGTTCTGCCTCACCTTTTTCTACTTGCCTGAAGATGGTGGCATCCCCCGATAGCCACGCC  660
 G  S  A  S  P  F  S  T  C  L  K  M  V  A  S  P  D  S  H  A  220

CGCTGTGCCCAGGGCCAGCCTCCACTTCTGTCTTGCTACGACACCTTGGCACCCCACTTC  720
 R  C  A  Q  G  Q  P  P  L  L  S  C  Y  D  T  L  A  P  H  F  240
                         ↓900
CGCGTTGACTGGTGCAATGTGACCCTGGTGGATAAGTCAGTGCCGGAGCCTGCAGATGAG  780
 R  V  D  W  C  N  V  T  L  V  D  K  S  V  P  E  P  A  D  E  260

GTGCCCACCCCAGGTGATGGGATCCTGGAGCATGACCCGTTCTTCTGCCCACCCACTGAG  840
 V  P  T  P  G  D  G  I  L  E  H  D  P  F  F  C  P  P  T  E  280

GCCCCAGACCGTGACTTCTTGGTGGATGCTCTGGTCACCCTCCTGGTGCCCCTGCTGGTG  900
 A  P  D  R  D  F  L  V  D  A  L  V  T  L  L  V  P  L  L  V  300
                                                    ↓290
GCCCTGCTTCTCACCTTGCTGCTGGCCTATGTCATGTGCTGCCGGCGGGAGGGAAGGCTG  960
 A  L  L  L  T  L  L  L  A  Y  V  M  C  C  R  R  E  G  R  L  320
                      ↓>1600
AAGAGAGACCTGGCTACCTCCGACATCCAGATGGTCCACCACTGCACCATCCACGGGAAC 1020
 K  R  D  L  A  T  S  D  I  Q  M  V  H  H  C  T  I  H  G  N  340

ACAGAGGAGCTGCGGCAGATGGCGGCCAGCCGCGAGGTGCCCCGGCCACTCTCCACCCTG 1080
 T  E  E  L  R  Q  M  A  A  S  R  E  V  P  R  P  L  S  T  L  360

CCCATGTTCAATGTGCACACAGGTGAGCGGCTGCCTCCCCGCGTGGACAGCGCCCAGGTG 1140
 P  M  F  N  V  H  T  G  E  R  L  P  P  R  V  D  S  A  Q  V  380
                                  ↓260
CCCCTCATTCTGGACCAGCACTGACAGCCCAGCCAGTGGTTCCAGGTCCAGCCCTGACTT 1200
 P  L  I  L  D  Q  H                                          387

CATCCTCCCTTCTCTGTCCACACCACGAGTGGCACATCCCACCTGCTGATTCCAGCTCCT 1260
GGCCCTCCTGGAACCCAGGCTCTAAACAAGCAGGGAGAGGGGGTGGGGTGGGGTGAGAGT 1320
GTGTGGAGTAAGGACATTCAGAATAAATATCTGCTGCTCTGCTCACCAATTGCTGCTGGC 1380
AGCCTCTCCCGTCAAAAAAAAAAAAAAAAA                               1410
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Figure 1. Human Adhalin cDNA and Deduced Amino Acid Sequence

Human adhalin cDNA is shown with the adenine of the initiating ATG numbered 1 and deduced amino acid sequence shown below. The predicted hydrophobic signal sequence and transmembrane domain are underlined. The positions of introns within the adhalin gene are indicated by arrows and followed by the approximate size of each intron. Nucleotides mutated in the family described in this paper are indicated by asterisks.

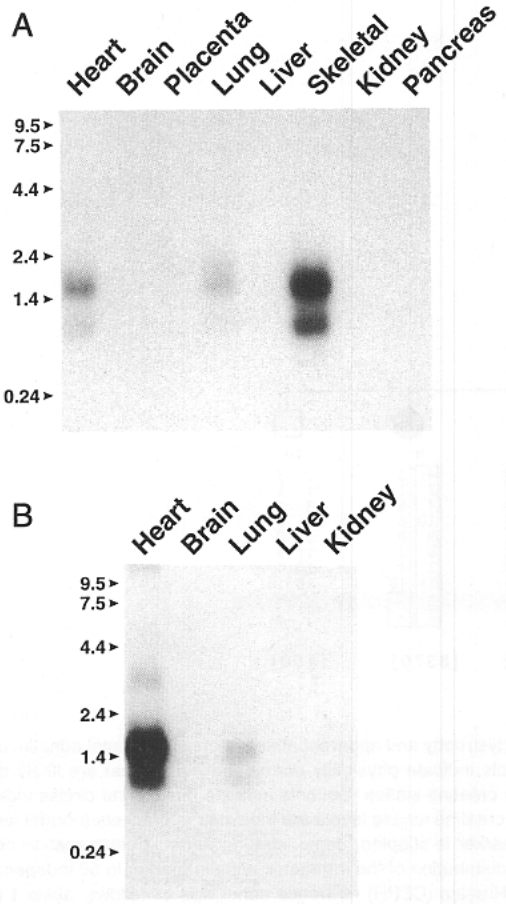

Figure 2. Tissue-Specific Expression of Adhalin mRNA in Adult and Fetal Human Tissues

(A) An RNA blot (Clonetech) containing 2 μg of poly(A)$^+$ RNA from each of eight human tissues, as indicated, was hybridized with a human adhalin cDNA probe. The autoradiograph was exposed for 48 hr.

(B) An RNA blot (Clonetech) containing 2 μg of poly(A)$^+$ RNA from each of five fetal human tissues, as indicated, was hybridized with a human adhalin cDNA probe. The autoradiograph was exposed for 6 days.

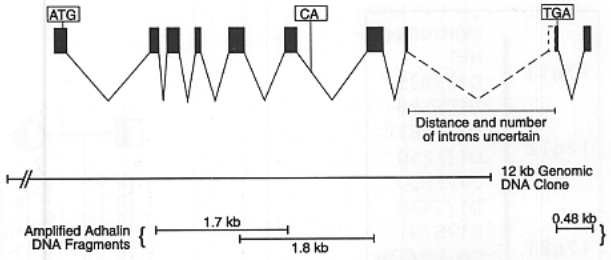

Figure 3. Exon and Intron Structure of the Human Adhalin Gene

Exons are indicated by boxes and introns are indicated by lines, drawn to scale. Closed exons and introns have been sequenced in full. Because the exon indicated by a dashed box has not yet been sequenced within genomic DNA, it may be interrupted by additional introns. Similarly, because the intron indicated by the dashed line has been only partially sequenced, its size is unknown. The positions of the initiation codon (ATG), intragenic polymorphic microsatellite (CA), and termination codon (TGA) are indicated. The relative positions of three genomic DNA fragments amplified from the affected family are indicated to scale.

Structure of the Adhalin Gene and Its Localization to Chromosome 17q12-q21.33

A 12 kb portion of the human adhalin gene was cloned by screening a human genomic DNA library using human adhalin cDNA as a probe. Portions of the adhalin gene were also cloned by polymerase chain reaction (PCR) amplification of 0.5–1.8 kb fragments from human genomic DNA. Nine introns have been identified within the adhalin gene at this time (Figure 3). A 32 bp portion of exon sequence, representing nucleotides 984–1015 of the adhalin cDNA sequence, has not been amplified from genomic DNA in many attempts using different combinations of primers, but greater than 1600 bp of intronic sequence has been obtained 3′ of nucleotide 983. Thus, at least one large intron exists preceding nucleotide 984, and additional introns have not been ruled out between nucleotides 984 and 1015.

To determine the chromosomal localization of the adhalin gene, human adhalin-specific primers were used to amplify fragments of human genomic adhalin DNA using PCR from DNA isolated from a panel of human–rodent somatic cell hybrids containing various human chromosomes. An adhalin DNA fragment was amplified from both of the two hybrids containing chromosome 17 but from no hybrids that did not contain chromosome 17. Thus, the adhalin gene is located on human chromosome 17. To localize the adhalin gene further, we used adhalin-specific primers to screen DNA isolated from human–rodent somatic cell hybrids containing various fragments of chromosome 17. Adhalin DNA fragments were amplified from all hybrids containing the q12-q21.33 region but not from hybrids that lacked this region, thus localizing the adhalin gene to 17q12-q21.33.

To search for possible polymorphic markers within the adhalin gene, we used Southern blot analysis to screen adhalin genomic DNA fragments for the presence of microsatellite markers using oligonucleotide probes encoding a dinucleotide (CA) repeat and several tetranucleotide repeats. No tetranucleotide repeat probes hybridized to adhalin DNA. However, hybridization of the CA repeat oligonucleotide to an adhalin DNA fragment followed by DNA sequencing identified a CA dinucleotide repeat within intron 6.

Linkage of the Adhalin Gene to the SCARMD Locus in a Large Family

We previously described a large French family with SCARMD that did not demonstrate linkage to markers on chromosome 13q (Romero et al., 1994). Adhalin was absent from skeletal muscle of the four affected siblings in this family, whose symptoms included proximal muscle weakness beginning at approximately 10 years of age, calf hypertrophy, and markedly elevated serum creatine kinase levels. As determined by indirect immunofluorescence, dystrophin and all the other dystrophin-associated proteins were present at normal levels except the 35 kDa dystrophin-associated glycoprotein, which was slightly reduced in abundance (Romero et al., 1994), as is character-

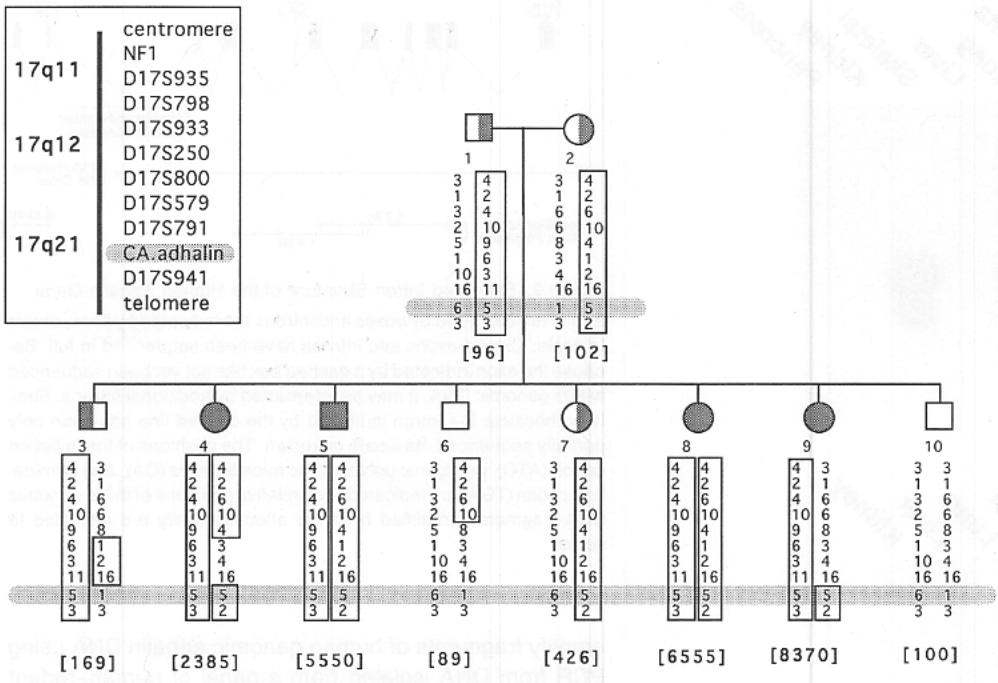

Figure 4. Pedigree of Family H with Haplotypes

Stippled symbols indicate subjects with clinical and pathological signs of muscular dystrophy and apparent absence of sarcolemmal adhalin, open symbols indicate apparently normal individuals, and half-open/half-stippled symbols indicate physically normal individuals that are likely to be heterozygotes based on their being parents or being children with elevated serum creatine kinase. Squares indicate males, and circles indicate females. Children are presented in order of age, with the oldest on the left. Serum creatine kinase levels are indicated in brackets (normal serum creatine kinase is 25–95 U). The parental haplotype is boxed, and the adhalin marker is stippled, demonstrating perfect cosegregation of the disease with allele 5 of the intragenic adhalin marker from each parent. The allele distribution of the intragenic adhalin marker in 50 independent chromosomes taken from the parents of the Centre d'Études du Polymorphisme Humain (CEPH) reference panel was as follows: allele 1 (170 bp), 0.1; allele 2 (168 bp), 0.2; allele 3 (166 bp), 0.02; allele 4 (164 bp), 0.04; allele 5 (162 bp), 0.36; allele 6 (160 bp), 0.24; allele 7 (158 bp), 0.04. (Inset) Relative map positions of the markers used based upon genotypic data from CEPH reference families (Weissenbach et al., 1992). The order shown has a likelihood odds ratio higher than 1000 over all alternative flips of adjacent loci.

istically observed in muscle from SCARMD patients (Matsumura et al., 1992a; Fardeau et al., 1993; Passos-Bueno et al., 1993a).

To determine whether the adhalin gene might be linked to the disease phenotype in this family, we examined several chromosome 17q markers, including the intragenic adhalin CA repeat (Figure 4). The likelihood of linkage between the different chromosome 17q markers and the disease locus was computed under three genetic models: as a simple autosomal recessive trait (model 1); as a pure codominant trait, assuming that heterozygotes show elevated serum creatine kinase levels (model 2); and as a quantitative trait, since half of parents of affected children with SCARMD have moderately elevated serum creatine kinase (model 3). The results were concordant with a chromosome 17q localization of the disease locus. A minimum of one recombination event was seen with every marker except the two most distal markers, the CA repeat within the adhalin gene and *D17S941*, with resulting maximum lod scores of 2.3 under model 1, of 3.6 under model 2 (Table 1), and of 3.1 under model 3. These are the highest values obtainable from this particular pedigree.

Table 1. Two-Point Linkage Analysis of Chromosome 17q Markers with the SCARMD Locus in Family H

θ	0.0	0.001	0.005	0.01	0.05	0.1	0.15	0.2
NF1	−∞	−2.39	−1.02	−0.45	0.70	0.98	1.00	0.91
935	−∞	−2.39	−1.02	−0.45	0.70	0.98	1.00	0.91
798	1.51	1.50	1.49	1.48	1.37	1.23	1.08	0.92
933	−∞	−5.39	−3.32	−2.44	−0.57	0.05	0.30	0.40
800	−∞	−5.09	−3.02	−2.14	−0.28	0.33	0.55	0.61
579	−∞	−8.09	−5.32	−4.14	−1.56	−0.62	−0.20	0.01
791	1.51	1.50	1.49	1.48	1.37	1.23	1.08	0.92
Adhalin	3.61	3.61	3.58	3.54	3.26	2.89	2.51	2.11
941	1.51	1.50	1.49	1.48	1.37	1.23	1.08	0.92

Two-point linkage analysis between the SCARMD locus in family H and each microsatellite marker under the codominant model, assuming full penetrance.

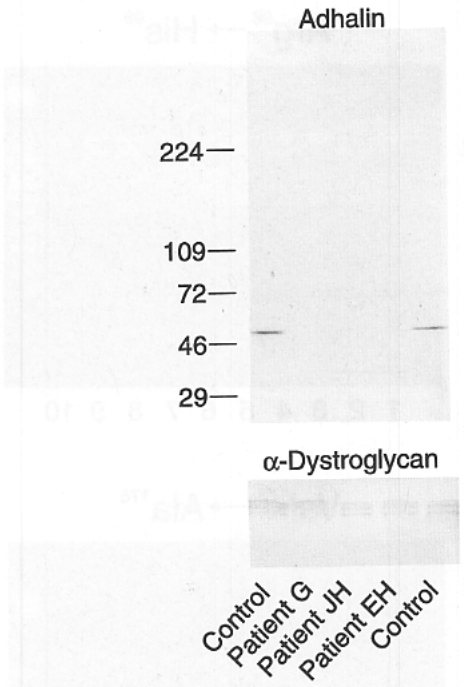

Figure 5. Immunoblot Analysis of Adhalin Expression in Family H

An immunoblot of skeletal muscle SDS extracts from two normal individuals (control), from two affected individuals from family H (patient JH and patient EH), and from one unrelated SCARMD patient (patient G) was stained with an affinity-purified sheep polyclonal antibody against adhalin (Ohlendieck and Campbell, 1991). An identical immunoblot was stained with monoclonal antibody IIH6 against α-dystroglycan (Ervasti and Campbell, 1991).

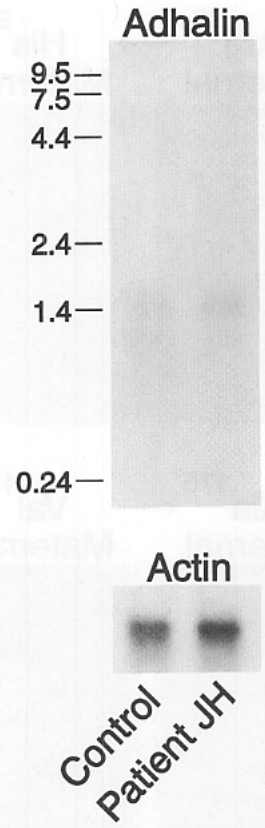

Figure 6. Adhalin mRNA Expression in Family H

An RNA blot of 0.4 μg of human skeletal muscle total RNA per lane from a normal individual (control) and an affected member of family H (patient JH) was hybridized with a human adhalin cDNA probe, and the autoradiograph was exposed for 14 days. After boiling to remove the adhalin probe, the same blot was hybridized with a human β-actin cDNA probe and the autoradiograph was exposed overnight.

Identification of Adhalin Mutations in Individuals with SCARMD

To characterize further the adhalin defect in this family, we subjected total skeletal muscle extracts to immunoblot analysis. Using an affinity-purified sheep polyclonal antibody, we were unable to detect adhalin in skeletal muscle from affected members of this family but clearly detected adhalin in controls (Figure 5). This demonstrates that the deficiency of adhalin observed by immunofluorescence (Romero et al., 1994) did not simply result from a change in the epitope of the monoclonal antibody used in that study. α-Dystroglycan was present at normal levels in affected members of this family as determined by immunoblot analysis (Figure 5), which is consistent with immunofluorescence results (Romero et al., 1994).

To determine whether adhalin mRNA size or abundance was affected in this family, we performed RNA blot analysis on total RNA isolated from a skeletal muscle biopsy from an affected member of this family (Figure 6). Adhalin mRNA was present at normal levels and at an apparently normal size in the affected individual. This strongly suggests that the causative mutation(s) in this family must not involve large deletions or insertions within exons. Additionally, mutations must not grossly affect transcription, splicing, or stability of adhalin mRNA.

To identify possible causative mutations in this family, we amplified three fragments of genomic DNA, as described in Figure 3, from normal and affected members of this family and sequenced them either directly or following subcloning into a plasmid vector. Additionally, adhalin cDNA was amplified following reverse transcription from total RNA prepared from an affected member of this family. The amplified cDNA was sequenced directly, and cDNA representing each allele was sequenced following subcloning.

Sequence analysis of adhalin genomic DNA and cDNA from affected individuals in this family identified two missense mutations. A G→A substitution at nucleotide 293 results in an Arg→His substitution at residue 98 (Figure 7A). Arg-98 is conserved in rabbit (Roberds et al., 1993b), human (see Figure 1), and hamster (S. L. R. and K. P. C., unpublished data). This amino acid substitution may disrupt a predicted coiled-coil structure within the protein, as predicted using the program implemented by Lupas et al. (1991). Disruption of secondary structure may interfere with targeting to the sarcolemma or with the interaction of adhalin with other proteins, either of which might result in shunting of mutated adhalin to a degradation pathway. Restriction analysis with NlaIII of adhalin genomic DNA amplified from all ten individuals of this family demon-

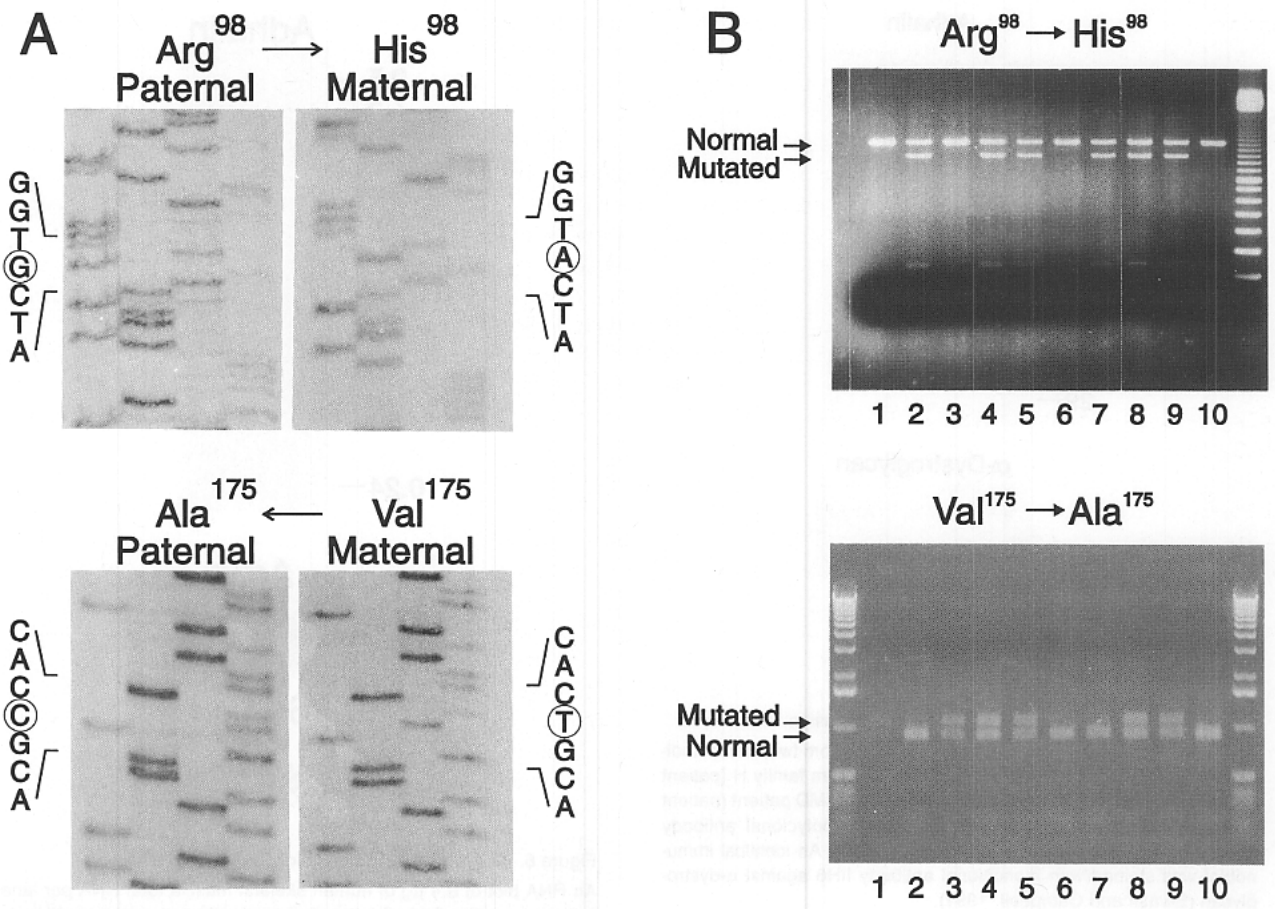

Figure 7. Mutations in the Adhalin Gene in Family H

(A) Sequences of subcloned adhalin cDNA (paternal) or genomic DNA (maternal) fragments representing each allele from patient 9 (see Figure 4) of family H are shown. Maternal sequence from subcloned cDNA was identical to genomic DNA sequence in the regions shown, but the autoradiographs shown were of superior quality. Circled nucleotides were the only bases found to differ between the coding regions of the two alleles. The lanes in each panel are ordered G, A, T, and C, from left to right.

(B) Fragments of genomic DNA from each member of family H, numbered as in Figure 4, were amplified, purified, and digested with a restriction enzyme as described in Experimental Procedures to detect the G→A mutation at nucleotide 293 resulting in an Arg→His substitution at residue 98 (top) and the T→C mutation at nucleotide 524 resulting in a Val→Ala substitution at residue 175 (bottom).

strated that the mutation at nucleotide 293 is on the maternal allele (Figure 7B). Additionally, this mutation was not found in 140 normal chromosomes examined.

The second missense mutation, a T→C substitution at nucleotide 524, results in a Val→Ala substitution at residue 175. Sequencing of subcloned adhalin DNA fragments revealed that the mutation at nucleotide 524 was on the opposite (and therefore paternal) allele from that of the nucleotide 293 mutation (Figure 7A), and this was confirmed by restriction enzyme digestion with HphI (Figure 7B) and by allele-specific hybridization with an oligonucleotide probe (data not shown). This mutation was not found in 200 normal chromosomes examined. Because residue 175 is an isoleucine rather than a valine in rabbit (Roberds et al., 1993b) and hamster (S. L. R. and K. P. C., unpublished data) adhalin, it is unclear how alanine would be harmful at this position. However, Val-175 is positioned between Asn-174 and Thr-176, which form one of the two consensus sites for N-linked glycosylation in adhalin. It is known that adhalin is N-glycosylated in vivo (Ervasti and Campbell, 1991), but it is not known which of the consensus sites is utilized. It is possible that an alanine at this position in adhalin might be unfavorable for glycosylation, leading to an instability of the protein; however, this remains to be tested. Alternatively, this substitution might have an effect on secondary structure unrelated to glycosylation that could result in targeting of mutated adhalin for degradation.

Discussion

The characteristics of affected members of this family, described in detail previously (Romero et al., 1994), differ somewhat from SCARMD patients in North Africa (Ben Hamida et al., 1983; Azibi et al., 1993) by apparent slower progression of symptoms, by less increase of connective tissue, by no immunocytochemically detectable adhalin in contrast with faint sarcolemmal labeling in North African SCARMD, and by French ancestry without likely consanguinity. The perfect cosegregation of the disease with the

polymorphic CA microsatellite within the adhalin gene indicates that mutations in the adhalin gene very likely cause SCARMD in this family. We have identified a missense mutation in each allele of the adhalin gene in this family. These mutations have not been found in unrelated normal individuals. It cannot be known with certainty at this time what effect these mutations might have on adhalin function or stability, and experiments to demonstrate a deleterious functional effect of these mutations in vitro or in vivo are beyond the scope of this report. However, missense mutations in other membrane proteins, such as the cystic fibrosis transmembrane conductance regulator, lead to improper protein processing, resulting in a mislocalized or degraded protein (Cheng et al., 1990; Welsh and Smith, 1993). Thus, a reasonable hypothesis is that mutations in adhalin may result in improper processing of the protein or improper assembly of adhalin with other components of the dystrophin–glycoprotein complex or other proteins that, in turn, leads to rapid turnover of mutated adhalin.

Notably, the SCARMD locus maps to the pericentromeric region of chromosome 13q in some families (Ben Othmane et al., 1992; Azibi et al., 1993; El Kerch et al., 1994) but not in others (Passos-Bueno et al., 1993a; Romero et al., 1994). Because the adhalin gene is located on chromosome 17, the deficiency of adhalin observed in patients linked to chromosome 13q (Azibi et al., 1993; El Kerch et al., 1994) must be secondary to a defect in an as-yet-unidentified gene. One hypothesis is that the SCARMD gene on chromosome 13q encodes a physiological ligand for adhalin. α-Dystroglycan, another component of the dystrophin–glycoprotein complex, binds the extracellular matrix component merosin, the muscle-specific isoform of laminin. Adhalin, a glycoprotein having a large extracellular domain with limited homology to entactin and nerve growth factor receptor, may bind to a second region on laminin or to another extracellular matrix component or to some other protein. The absence or perturbation of a physiological ligand encoded on chromosome 13q may lead to instability of adhalin and subsequently to a disruption of the dystrophin–glycoprotein complex.

A specific deficiency of adhalin has also been observed in skeletal and cardiac muscles of the cardiomyopathic hamster (Roberds et al., 1993a; Iwata et al., 1993; Yamanouchi et al., 1994). Interestingly, these animals suffer from both myopathy and cardiomyopathy, with death occurring due to the latter, whereas SCARMD patients show little or no evidence of cardiac involvement. It is not known whether the difference in phenotype between cardiomyopathic hamsters and SCARMD patients is due to different genetic defects or different pathological manifestations of similar genetic defects between humans and rodents. This cannot be determined until the genetic defect in the cardiomyopathic hamster is identified and transgenic rodents carrying adhalin mutations have been generated.

Even though all other components of the dystrophin–glycoprotein complex appear normal in cardiomyopathic hamster skeletal muscle by immunofluorescence, the loss of adhalin has been shown functionally to disrupt the dystrophin–glycoprotein complex (Roberds et al., 1993a) leading to a loss of linkage between merosin in the extracellular matrix and actin in the subsarcolemmal cytoskeleton. We hypothesize that a similar mechanism is at work in this SCARMD family resulting directly from mutations in the adhalin gene that lead to a deficiency of adhalin. The disruption of the link between merosin and actin also occurs in Duchenne and Becker muscular dystrophy owing to mutations in the dystrophin gene. Thus, genetic defects in two components, dystrophin and adhalin, of the dystrophin–glycoprotein complex can independently cause muscular dystrophies, specifically Duchenne muscular dystrophy and SCARMD.

Clearly, different forms of autosomal recessive muscular dystrophies are difficult to distinguish on the basis of clinical signs. Additionally, it is being discovered that clinically similar, if not indistinguishable, neuromuscular disorders are often caused by mutations in different genes. For example, an autosomal recessive form of limb–girdle muscular dystrophy has been linked to chromosome 15q in some families (Beckmann et al., 1991; Fougerousse et al., 1994), but the same locus has been excluded from linkage in other families (Passos-Bueno et al., 1993b; Bashir et al., 1994).

It is important to distinguish among genetically heterogeneous forms of clinically similar diseases to ensure appropriate genetic counseling, treatment, and (in the future) gene therapy. In the case of SCARMD, at least two genetically heterogeneous forms cannot reliably be distinguished even by biochemical means, as patients with adhalin deficiency may be linked to either chromosome 13q (Azibi et al., 1993) or to the adhalin gene on 17q. In the case of SCARMD with adhalin deficiency, genetic analysis is necessary for an accurate diagnosis. This report provides information necessary for analysis of the adhalin gene in autosomal recessive muscular dystrophy.

Experimental Procedures

Isolation of Human Adhalin cDNA and Blot Hybridization Analyses

A λZAPII human skeletal muscle cDNA library (Stratagene) was screened using ^{32}P-labeled full-length rabbit adhalin cDNA (Roberds et al., 1993b) as a probe. Two partial-length human cDNA clones were obtained spanning nucleotides −3 to 760 and nucleotides 318 to 1393 (with the adenine of the initiating ATG being numbered 1), and a full-length clone was obtained that contained an additional 28 bp immediately preceding the poly(A)$^+$ tail and spanned nucleotides −10 to 1421. These clones were sequenced in full on both strands using an Applied Biosystems automated sequencer or manually using Sequenase II (United States Biochemicals). RNA blot hybridizations were performed as previously described (Roberds and Tamkun, 1991) using a ^{32}P-labeled human adhalin cDNA probe representing nucleotides −3 to +760.

Isolation of Human Adhalin Genomic DNA Clones and Localization of the Adhalin Gene to Chromosome 17q12-q21.33

An EMBL3 human genomic DNA library (Ibraghimov-Beskrovnaya et al., 1993) was screened with a ^{32}P-labeled human adhalin cDNA representing nucleotides 318–1393. The 12 kb clone obtained was digested with EcoRI and BamHI, and fragments were subcloned into Bluescript (Stratagene) for sequence analysis.

Oligonucleotide primers designed in multiple locations along the length of the adhalin cDNA were used to amplify fragments of adhalin genomic DNA from normal and affected family members. The three adhalin DNA fragments of 1.7, 1.8, and 0.48 kb, shown in Figure 3,

were amplified from genomic DNA using primers corresponding to the following regions of human adhalin cDNA sequence, respectively: 99–122 (sense) with 585–605 (antisense), 465–484 (sense) with 849–869 (antisense), and 1016–1035 (sense) with 1200–1221 (antisense). These fragments were subcloned into Bluescript for sequencing of individual alleles.

Oligonucleotides corresponding to human adhalin cDNA nucleotides 99–122 (sense) and 460–483 (antisense) were used in PCR using template DNAs isolated from a panel of 25 human–rodent somatic cell hybrids (BIOS Corporation) containing various combinations of human chromosomes to localize the adhalin gene to chromosome 17. The same primers were used in PCR using DNAs containing deletion mutants of chromosome 17 from the National Institute of General Medical Science Human Genetic Mutant Cell Repository (hybrid numbers GM10660, GM10502, GM10659, GM10657, and GM10501).

Genotyping and Linkage Analysis

The family history was previously described (Romero et al., 1994). Highly polymorphic chromosome 17q microsatellites used include intron *27-NF1* (Lazaro et al., 1993), *D17S250* (Weber et al., 1990), *D17S579*, *D17S791*, *D17S798*, *D17S800*, *D17S933*, *D17S935*, and *D17S941*. Primer sequences, PCR conditions, and other information can be obtained from the Genome Data Base, Johns Hopkins University. Primers used for amplification of the microsatellite within intron 6 of the adhalin gene were 5′-TATCTCCGTCTCTCTGATTGCTCC-3′ and 5′-TTGCGGACTCTGTTGCCCTCTTGT-3′. PCR conditions for this marker were denaturation at 92°C for 40 s and annealing and extension at 68°C for 30 s for 35 cycles. Two-point and multipoint linkage analyses were carried out using the LINKAGE package, version 5.1 (Lathrop et al., 1985).

Immunoblot Analysis

Twenty 20 μm sections from muscle biopsies having approximately 25 mm^2 surface area were homogenized as described (Matsumura et al., 1992a), except DNA was sheared by passage through a small-gauge needle rather than pelleted by centrifugation. Each sample (10 μl) was subjected to SDS–polyacrylamide gel electrophoresis and stained with Coomassie blue. The relative amounts of myosin heavy chain in each sample were determined by densitometry (Molecular Dynamics model 300A). Based on this result, equal amounts of muscle protein from each sample were subjected to SDS–polyacrylamide gel electrophoresis and immunoblot analysis as described (Ohlendieck et al., 1991).

RNA Isolation and cDNA Amplification

Total RNA was isolated using RNAzol (Tel-Test), according to the directions of the manufacturer, from less than 10 mg of skeletal muscle from patient 9 (Figure 4). RNA was fractionated by electrophoresis through a 1% agarose, 3% formaldehyde gel and transferred overnight by capillary action to Biosbrane membranes (BIOS Corporation). The membranes were then hybridized as described above.

Fragments of adhalin cDNA were amplified using various oligonucleotide primers and sequenced directly on both strands using an Applied Biosystems automated sequencer. The 5′-most primer, 5′-CCCCTGTCTCTGTCACTCAC-3′, located 43 bp 5′ of the initiation codon, was based on sequence of an adhalin genomic DNA clone because only 10 bp of 5′ untranslated sequence was obtained from cDNA clones. PCR was performed using a Perkin-Elmer thermocycler with Taq polymerase from Boehringer Mannheim.

Restriction Digests to Confirm Mutations

To detect the G→A mutation at nucleotide 293 resulting in an Arg→His substitution at residue 98, DNA was amplified using primers 5′-CCGTGTCTTTGTGCACACCTTGGA-3′ and 5′-TCCAGAGCCTGGAATCCCAGCCCAG-3′ and digested with NlaIII to detect an NlaIII site created by the mutation. To detect the T→C mutation at nucleotide 524 resulting in a Val→Ala substitution at residue 175, DNA was amplified using primers 5′-GGCTCCTGCTGTGACTCGAATC-3′ and 5′-TAGCCAGAGGAGAGTGGTGA-3′ located within introns 2 and 5, respectively, and digested with HphI to demonstrate removal of an HphI site deleted by the mutation.

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GenBank Accession Number

The accession number for the human adhalin cDNA sequence reported in this paper is U08895.