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β-sarcoglycan: characterization and role in limb-girdle muscular dystrophy linked to 4q12

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β-sarcoglycan, a 43 kDa dystrophin-associated glycoprotein, is an integral component of the dystrophin-glycoprotein complex. We have cloned human β-sarcoglycan cDNA and mapped the β-sarcoglycan gene to chromosome 4q12. Pericentromeric markers and an intragenic polymorphic CA repeat cosegregated perfectly with autosomal recessive limb-girdle muscular dystrophy in several Amish families. A Thr-to-Arg missense mutation was identified within the β-sarcoglycan gene that leads to a dramatically reduced expression of β-sarcoglycan in the sarcolemma and a concomitant loss of adhalin and 35 DAG, which may represent a disruption of a functional subcomplex within the dystrophin-glycoprotein complex. Thus, the β-sarcoglycan gene is the fifth locus identified (LGMD2E) that is involved in autosomal recessive limb-girdle muscular dystrophy.

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The dystrophin-glycoprotein complex (DGC)[1-4] is a large oligomeric complex of sarcolemmal proteins and glycoproteins, consisting of dystrophin, a large F-actin binding intracellular protein[5-7]; syntrophin, a 59 kDa intracellular protein[8,9]; adhalin, a 50 kDa transmembrane glycoprotein[10]; a 43 kDa transmembrane glycoprotein doublet (β-dystroglycan and A3b)[2,3,11]; a 35 kDa transmembrane glycoprotein; a 25 kDa transmembrane protein; and α-dystroglycan, a large extracellular laminin-binding glycoprotein[11-14]. Together, the dystrophin-glycoprotein complex acts as a structural link between the cytoskeleton and the extracellular matrix, and is believed to confer stability to the sarcolemma and protect muscle cells from contraction-induced damage and necrosis[15].

The DGC has been implicated in several forms of muscular dystrophy. In Duchenne muscular dystrophy (DMD), mutations in the dystrophin gene cause the complete absence of dystrophin and a dramatic reduction of its associated glycoproteins at the sarcolemma resulting in severe muscular dystrophy[2,5]. In the milder Becker muscular dystrophy, mutations in dystrophin result in the production of a dysfunctional protein[16]. More recently, some cases of severe childhood autosomal recessive muscular dystrophy (SCARMD or LGMD2D) were shown to be caused by missense and null mutations in the adhalin gene[17,18], which result in the reduction or absence of adhalin at the sarcolemma[19]. Non-Fukuyama congenital muscular dystrophy (CMD) has recently been linked close to the merosin locus on chromosome 6q[20,21], which is likely to be responsible for this disease. Thus, in these muscular dystrophies, mutations in one component of the DGC cause the disruption of the complex and consequently lead to the dystrophic process.

The limb-girdle muscular dystrophies (LGMDs) represent a clinically heterogeneous group of diseases which are characterized by progressive weakness of the pelvic and shoulder girdle muscles[22,23]. These disorders may be inherited as an autosomal dominant or recessive trait, the latter being more common with an estimated prevalence of 1 in 100,000 individuals[24]. Several genes have been implicated in the aetiology of these disorders. The autosomal dominant form, LGMD1A, was mapped to 5q22-q34 (ref. 25), while four genes involved in the autosomal recessive forms were mapped to chromosomes 2p13–p16 (LGMD2B)[26], 13q12 (LGMD2C)[27,28], 15q15.1 (LGMD2A)[29], and 17q12–q21.33 (LGMD2D)[17]. The genes responsible for LGMD2D and LGMD2A have been identified: adhalin[17] and muscle-specific calpain (*CANP3*)[30], respectively.

Cases of autosomal recessive limb-girdle muscular dystrophy among members of the old order of Amish of northern and southern Indiana were described by Jackson and Carey[31] and Jackson and Strehler[32]. Most of the families of these communities are interrelated by multiple consanguineous links and common ancestry which can be traced to the 18th and 19th century in the canton of Bern, Switzerland[31]. In view of the high degree of consanguinity and the similar clinical presentation of all Amish LGMD patients, the demonstration of genetic heterogeneity within this community was unexpected[33]. Although families from northern Indiana were shown to carry the same R769Q calpain 3 mutation[30], involvement of this locus was excluded in

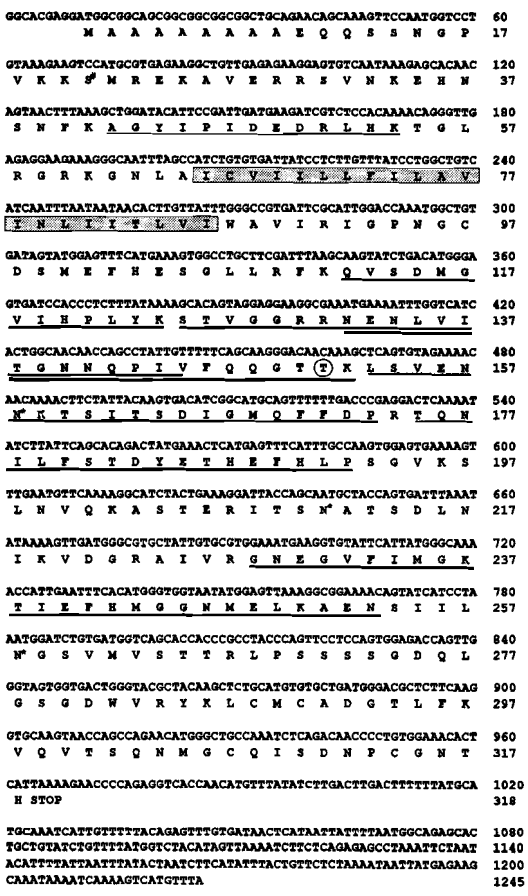

Fig. 1 Human β-sarcoglycan cDNA and translated amino acid sequence. Human β-sarcoglycan cDNA is shown with the deduced amino acid sequence below. The predicted transmembrane domain is indicated by the shaded box. Three consensus sites for N-linked glycosylation are indicated by asterisks, *. The intracellular consensus phosphorylation site by protein kinase C or casein kinase II is indicated by a #. Peptide sequence fragments identified in the amino acid sequence are underlined. The amino acid that is mutated in the Amish limb-girdle muscular dystrophy patients is circled.

Amish families from southern Indiana, as none of the examined patients from these cohorts carried this mutation, even in a heterozygous state (data not shown). Furthermore, the role of known LGMD loci, as well as several other candidate regions involved in other neuromuscular disorders, were all excluded[33]. These results thus implied the existence of yet another locus, LGMD2E, involved in autosomal recessive limb-girdle muscular dystrophy.

Here we report the cloning and characterization of human β-sarcoglycan, a 43 kDa component of the dystrophin-glycoprotein complex, and demonstrate its involvement in LGMD2E. We show that β-sarcoglycan colocalizes with the DGC at the sarcolemma and is expressed ubiquitously, but predominantly in muscle. We report the primary localization of this gene to chromosome 4q12, the identification of an intragenic polymorphic CA repeat, and linkage of LGMD2E to the intragenic β-sarcoglycan microsatellite in southern Indiana Amish LGMD families. We have also identified a missense mutation in the β-sarcoglycan sequence, which is present in a homozygous state in all patients, and is apparently responsible for the muscular dystrophy in all chromosome 4-linked Amish LGMD families screened. By immunofluorescence of affected individuals, we demonstrate a dramatic reduction in the level of the β-sarcoglycan protein at the sarcolemma, as well as a concomitant reduction of adhalin and the 35 kDa DAG, thereby disrupting the integrity of the DGC. Together, these data demonstrate that a defect in β-sarcoglycan is responsible for LGMD2E. Because of this protein's tight association with other components of what has been called the sarcoglycan complex[34], which includes adhalin, this 43 kDa protein, the 35 kDa DAG, and the 25 kDa DAP, the name 'β-sarcoglycan' has been proposed. This name was mutually agreed upon by us and the authors of an accompanying manuscript which describes similar results[35]. Under this nomenclature, α-sarcoglycan becomes synonymous with adhalin.

β-Sarcoglycan cDNA and primary structure

The dystrophin-glycoprotein complex (DGC) was identified in 1989 based on the ability of dystrophin to be retained on a wheat-germ agglutinin column[1,2]. When β-dystroglycan, a 43 kDa DAG, was cloned[11], the translated peptide sequence was compared with peptide sequence fragments obtained from the 43 kDa band of the purified DGC. Of these fragments, only one was found in the primary structure of β-dystroglycan. This suggested the presence of another protein of similar molecular weight, consistent with the observed 43 kDa doublet in the DGC[2,3]. To further investigate this protein, we searched the GenBank database of expressed sequence tags (dbEST) with the unidentified peptide sequences using the TBLASTN search program[36]. Several ESTs encoding peptide sequence fragments of the β-sarcoglycan protein were identified and isolated from a normalized human infant brain cDNA library constructed by Dr. Bento Soares *et al.*[37]. Clones 22297 and 25556, from which ESTs were generated, were received from the IMAGE Consortium at the Lawrence Livermore National Laboratories. The larger of these clones, 25556, was fully sequenced on both strands. In addition, we isolated clones from a λZAPII human skeletal muscle cDNA library using the 1225-bp insert of clone 25556 as a probe. Sequence analysis revealed a single open reading frame that encodes a protein with a predicted molecular weight of 34,777 Da (Fig. 1). Several peptide fragments obtained from sequencing of the 43 kDa doublet were found in the primary structure of the protein. There was no other significant homology with any other known protein or functional protein domains in the database. Additionally, our sequence data provided evidence of alternate polyadenylation. Two distinct poly(A^+) tails were identified, one that is about 300 bases downstream of the stop codon in brain, and one that is approximately 3 kb downstream in skeletal muscle (data not shown).

Hydropathy analysis of the amino acid sequence revealed a single transmembrane domain and no functional signal sequence at the N terminus, despite the presence of eight hydrophobic alanine residues following the initiator methionine. Thus, the small N-terminal domain of the protein is predicted to be intracellular, whereas the large C terminus is extracellular. This membrane topology is consistent with the location of the three putative N-linked glycosylation

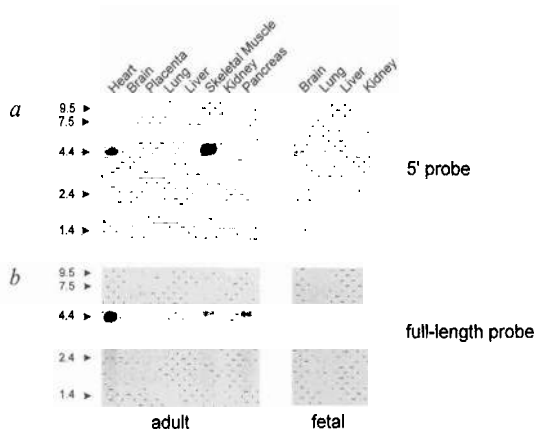

Fig. 2 Expression of β-sarcoglycan in adult and fetal tissues. RNA blots containing 2 μg of poly(A+) RNA from the indicated human adult and fetal tissues were hybridized as follows. Molecular size in kb is indicated on the left. *a*, Northern blots hybridized with a 334-bp 5' segment of the β-sarcoglycan cDNA. *b*, Northern blots hybridized with the 1225-bp insert of β-sarcoglycan clone 25556.

sites, all of which are C-terminal to the transmembrane domain. There are also five extracellular cysteine residues which may form disulfide bonds. There is also one potential intracellular consensus site for phosphorylation by protein kinase C or casein kinase II at Ser21. The predicted membrane organization is similar to that of β-dystroglycan and adhalin, both of which have single transmembrane domains and large extracellular and short intracellular domains[10,11].

Tissue distribution of β-sarcoglycan

To determine the tissue-specific expression of β-sarcoglycan, we performed RNA hybridization analysis. Human adult and fetal multiple tissue northern blots were probed in two different ways: once with a PCR fragment encompassing bases 132-465 of the β-sarcoglycan coding region, and a second time with the 1225 bp insert of clone 25556 described above (Fig. 2). The predominant transcript is approximately 4.4 kb in length; however, there are also weaker signals of 3.0 and 1.35 kb. Sequence data demonstrating evidence of alternate polyadenylation can account for the smallest and largest transcript. β-sarcoglycan RNA is present in all tissues, and is particularly enriched in skeletal and cardiac muscle. This pattern of expression is different from adhalin, which is expressed only in muscle tissue[10,17], but is similar to dystroglycan, which is ubiquitously expressed[11]. Interestingly, the northern blot results are different between the two probes. When probed with a 334-bp PCR fragment corresponding to nucleotides 132-465, the signals in the fetal liver and adult pancreas lanes are weak or absent (Fig. 2*a*). However, when probed with the larger clone, which contains all the coding region and nearly 300 bp of 3' untranslated region, these signals are significantly stronger, particularly in the adult pancreas (Fig. 2*b*). This suggests that the 5' end of the coding region is alternatively spliced among different tissues.

Localization of β-sarcoglycan protein

To confirm that the cloned cDNA represents the 43 kDa dystrophin-associated glycoprotein, we constructed a glutathione-S-transferase (GST) fusion protein (FP-I) containing 64 residues C-terminal to the transmembrane domain (Fig. 3*a*). Anti-FP-I polyclonal antibodies, produced in rabbits, were used to detect β-sarcoglycan in isolated membranes and purified DGC. The antibody specifically recognizes a 43 kDa protein in both crude sarcolemma and purified DGC, but does not recognize GST alone (Fig. 3*b*). Sheep polyclonal antibodies produced against a peptide fragment (residues 42-52) of the β-sarcoglycan protein also recognized a 43 kDa protein in the purified DGC (data not shown). Identification of β-sarcoglycan during the purification of the DGC demonstrates that β-sarcoglycan is an integral component of the DGC.

To determine the subcellular localization of β-sarcoglycan by immunofluorescence, serial transverse cryosections of control human biopsied skeletal muscle were immunostained with anti-FP-I antibody preabsorbed with GST, as well as with antibodies against other components of the DGC including dystrophin, β-dystroglycan, syntrophin, adhalin, and 35 kDa DAG. As shown in Fig. 4, the anti-FP-I antibody labelled throughout the entire sarcolemma and showed colocalization of β-sarcoglycan with other components of the DGC. However, immunofluorescence from DMD muscle biopsy samples demonstrated a loss of the β-sarcoglycan protein at the sarcolemma along with all other components of the DGC (data not shown).

Localization to chromosome 4q12

Primers were designed to amplify a fragment of the human β-sarcoglycan gene from a panel of human-rodent somatic cell hybrids containing various combinations of human chromosomes. Restriction digests of the amplified product with *TaqI* specifically cleaved the human allele, and allowed assignment of the β-sarcoglycan gene to chromosome 4. To further narrow the

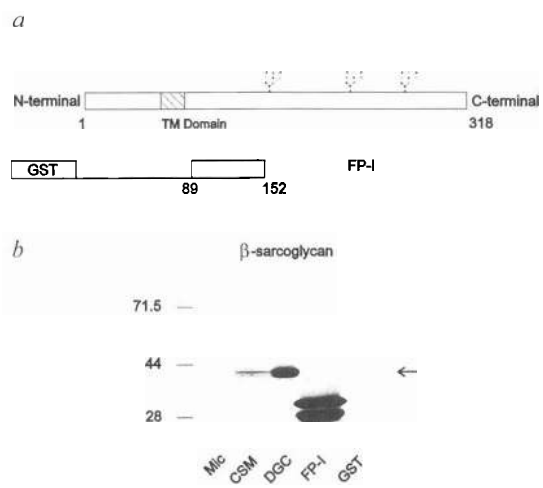

Fig. 3 Immunoblot analysis of β-sarcoglycan. *a*, Schematic model of human β-sarcoglycan cDNA. The transmembrane domain is shaded; three sites of potential N-linked glycosylation sites are indicated. A schematic representation of fusion protein I (FP-I) is shown below. *b*, Nitrocellulose transfer of SDS-PAGE gel stained with anti-FP-I antibody. Membrane contains the following: KCl-washed microsomes (Mic), crude rabbit skeletal muscle sarcolemma (CSM), purified dystrophin-glycoprotein complex (DGC), fusion protein I (FP-I), and glutathione-S-transferase (GST). Anti-GST antibodies were removed prior to staining. Molecular weight in kDa is indicated on the left. Arrow on right indicates position of β-sarcoglycan protein.

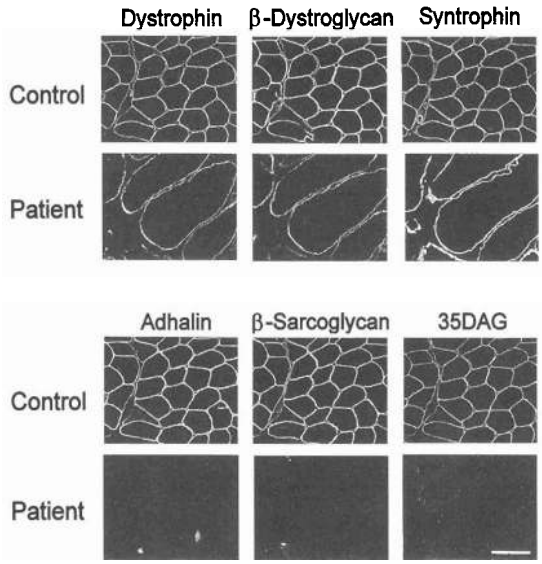

Fig. 4 Immunohistochemical analysis of β-sarcoglycan in normal and Amish LGMD2E muscle. Skeletal muscle biopsy samples were stained with antibodies against DGC components as described in the Methods. DYS, anti-dystrophin; β-DG, anti β-dystroglycan; 35DAG, anti-35 kDa dystrophin-associated glycoprotein. Control, normal subject; patient, Amish LGMD2E patient. Bar, 50 μM.

chromosomal region, the same process was used to analyse DNA isolated from human-rodent somatic cell hybrids containing various fragments of chromosome 4 (ref. 38). β-sarcoglycan fragments could be amplified only from hybrids containing the region 4p14-q21.1, which overlaps the centromere.

We isolated two cosmids spanning approximately 40 kb of the human β-sarcoglycan gene by screening a human chromosome 4 cosmid library. The smaller cosmid, which contained a 28.5 kb insert, was used as a probe for fluorescence *in situ* hybridization (FISH), and resulted in the specific labelling of the centromere on the long arm of chromosome 4, corresponding to band 4q12 (data not shown).

We searched for polymorphic microsatellites within the β-sarcoglycan gene. Southern blots of restriction fragments of the cosmids and genomic PCR fragments were probed with oligonucleotides encoding a dinucleotide (CA) repeat and several tetranucleotide repeats. Only hybridization with the CA repeat oligonucleotide was detected. Sequencing subsequently located a novel CA repeat within an intron of the β-sarcoglycan gene between nucleotides 438 and 439 of the cDNA sequence. This sequence was found to be polymorphic, with seven alleles and an observed heterozygosity of 0.70 based on the study of eight CEPH reference families.

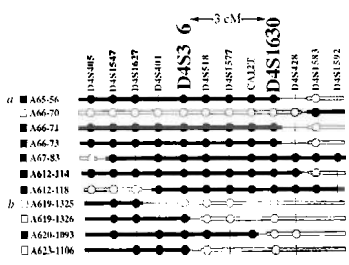

Fig. 5 Recombinant haplotypes in LGMD2E families. The ordered marker loci have arbitrarily been represented as equidistant. Loci bracketing the smallest interval defined by recombination events are noted in larger letters. The intragenic β-sarcoglycan microsatellite is underlined. Solid squares, affected individuals; open squares, healthy carriers. The numbers indicate the family and the individual. Chromosomes segregating with the disease allele or the normal allele are coded as solid or open circles, respectively. Thin horizontal lines represent the recombination interval. Uninformative markers are coded by a line in place of a circle, and nongenotyped markers are left blank. The last two recombinants define the critical interval for the location of the morbid locus. *a*, Previously described Amish LGMD families. *b*, Additional southern Indiana Amish families.

Linkage of the β-sarcoglycan gene to LGMD2E

In the course of the investigation of the β-sarcoglycan protein, six previously-described southern Indiana Amish families[33] were subjected to a systematic linkage search using the highly informative microsatellites described by Gyapay *et al.*[39]. Over 75% of the genome was excluded upon analysis of 320 microsatellite markers until a lod score of 1.12 was obtained at $\theta = 0.001$ with marker *D4S428*, indicative of potential linkage (data not shown). Twenty-nine additional microsatellite markers of the pericentromeric region of chromosome 4 were subsequently genotyped; almost all of them demonstrated linkage to the disease locus in these families (data not shown).

In autosomal recessive disorders, affected individuals from consanguineous families often show homozygosity by descent at the region surrounding a disease locus[40]. Haplotypes were manually constructed for the chromosome 4 markers assuming a minimal number of recombinations. We identified a unique carrier haplotype segregating within all the southern Indiana Amish population (data not shown), suggestive of a unique founder effect, though different from the one found in the northern Indiana and Pennsylvania Amish LGMD2A families[33]. Six affected and one unaffected offspring showed informative crossovers (Fig. 5*a*). This identified *D4S1547* and *D4S1627* as new flanking loci which define a region of approximately 9 cM, based on analyses of CEPH reference families[39].

Five additional southern Indiana LGMD families also showed linkage to this new locus, thereby increasing the number of informative meioses. A maximum lod score of 11.97 at $\theta = 0.0$ was obtained with marker *D4S518* (Table 1*a*). Genotyping of these families with new microsatellite markers allowed a further narrowing of the LGMD2E interval, flanked by markers *D4S396* and *D4S1630* (Fig. 5*b*). Homozygosity mapping and reconstitution of historical crossing over events suggested that the LGMD2E interval is flanked by markers *D4S396* and *D4S428* (data not shown).

Based on physical maps for chromosome 4 (ref. 41 and D. Cox, personal communication), CEPH YACs spanning this region were used to localize the β-sarcoglycan gene inside the LGMD2E interval, between markers *D4S1577* and *D4S1630*. Genotyping of the intragenic microsatellite in LGMD2E families yielded a lod score of 7.21 at $\theta = 0.0$ (Table 1*b*). This lower lod score, as compared to *D4S518*, is due to a lower observed heterozygosity and hence lower informativity of the intragenic marker.

Identification of a mutation in Amish patients

We performed northern blot analysis on total RNA isolated from skeletal muscle biopsies of two affected siblings in these families to determine whether β-sarcoglycan mRNA size or abundance were affected. The major muscle β-sarcoglycan transcript (4.4 kb) was present at normal levels and size in both affected sibs (one of which is shown in Fig. 6) compared to an

Table 1 Linkage of LGMD2E families to chromosome 4 markers

		Z at recombination (θ) of						
	Marker	0.00	0.01	0.05	0.10	0.20	Z_{max} (θ)	One lod support interval
a	*D4S1547*	-∞	8.24	8.51	7.69	5.46	8.64 (0.031)	0.004–0.103
	D4S1627	-∞	2.06	2.96	2.91	2.17	2.99 (0.068)	–
	D4S401	-∞	5.37	5.39	4.82	3.38	5.51 (0.026)	0.001–0.124
	D4S396	-∞	9.62	9.69	8.64	5.98	9.92 (0.026)	0.003–0.089
	D4S1536	3.53	3.48	3.26	2.94	2.20	3.53 (0.000)	0.000–0.160
	D4S518	11.97	11.67	10.45	8.93	5.94	11.97 (0.000)	0.000–0.033
	D4S1577	5.03	4.92	4.47	3.88	2.63	5.03 (0.000)	0.000–0.088
	D4S1630	-∞	5.90	5.84	5.19	3.61	6.02 (0.023)	0.001–0.112
	D4S428	-∞	0.87	1.35	1.37	1.04	1.39 (0.075)	–
	D5S1619	0.77	0.76	0.69	0.60	0.41	0.77 (0.000)	–
	D4S2379	-∞	3.41	5.15	5.03	3.68	5.21 (0.075)	0.018–0.112
	D4S1583	-∞	-0.04	2.12	2.53	2.12	2.53 (0.000)	–
b	CA12T	7.21	7.01	6.23	5.26	3.39	7.21 (0.000)	0.000–0.051
c	T151R mutation	13.43	13.09	11.72	10.01	6.66	13.43 (0.000)	0.000–0.030

a, Maximum pairwise lod scores and their corresponding recombination fractions with one-lod support intervals obtained in ten southern Indiana families between the LGMD2E locus and chromosome 4 markers.
b, The intragenic microsatellite.
c, The T151R mutation. Marker loci are listed according to their order on the regional map of chromosome 4.

unrelated control. This strongly suggested that the causative mutation was most likely to involve a small deletion, insertion, or base substitution.

Fragments of the β-sarcoglycan cDNA were amplified following reverse-transcription from total RNA prepared from biceps brachii muscle biopsies of these two affected sibs. Sequencing the RT-PCR products revealed a single C to G transversion at nucleotide 461 in both patients in a homozygous state (Fig. 6). The codon change is ACA to AGA and results in a Thr-to-Arg substitution at residue 151 (T151R).

Segregation of this mutation was assessed in this family and in other Amish LGMD2E families by sequencing and 'touchdown' PCR[42]. Results showed perfect cosegregation of this missense mutation with the disease in all southern Indiana Amish families tested, as expected from the common haplotype at this locus (Fig. 7). To exclude the possibility that this missense mutation might be a polymorphism, 122 unrelated chromosomes taken from the CEPH reference families were tested; none showed this mutation, nor did any northern Indiana LGMD2A Amish patients (data not shown).

Linkage analysis with the mutation was performed (Table 1*c*). A maximum lod score of 13.43 at $\theta = 0.0$ between the mutation and the disease in these Amish families was obtained. In addition, assuming complete linkage disequilibrium, the calculated lod scores are significantly increased (data not shown), further confirming that the mutation in β-sarcoglycan is responsible for the disease in these patients.

β-sarcoglycan deficiency in LGMD2E muscle

Skeletal muscle biopsy specimens from the two patients described above were examined by immunofluorescence to test the effects of the T151R mutation on β-sarcoglycan expression. Serial frozen sections were stained with antibodies against β-sarcoglycan (anti-FP-I) or other DGC components as described above. Dystrophin, β-dystroglycan, syntrophin (Fig. 4), and laminin α2 chain (not shown) were present at comparable levels with control muscle. However, the immunostaining of β-sarcoglycan was greatly decreased, with a concomitant reduction of adhalin and the 35 kDa DAG. The apparent differences in muscle cell size between control and patient samples is due to cell hypertrophy associated with dystrophic changes.

Discussion

Limb-girdle muscular dystrophy in the Amish of Indiana was first described over 30 years ago[31], but only recently has the nature of the disease been closely studied. The LGMD in the northern Indiana Amish population is caused by mutations in calpain 3, a cysteine proteinase that may be involved in muscle protein processing or signal transduction[30]. However, analysis of southern Indiana Amish revealed no linkage to this locus[33]. The genetic heterogeneity of LGMD in this population was unexpected, because of evidence of common ancestry and multiple consanguineous matings within these populations.

The human cDNA of β-sarcoglycan, a component of the dystrophin-glycoprotein complex, has now been cloned. The identity of this clone is confirmed by the identification of peptide sequences in the primary structure, recognition of a 43 kDa protein in purified DGC by a β-sarcoglycan-specific antibody, and sarcolemmal colocalization of this protein with other components of the DGC. The predicted molecular weight of this translated product is 34,777 Da. Experiments with endoglycosidase F indicate that the discrepancy between the predicted and observed molecular weights is primarily due to the glycosylation of this protein (data not shown).

The β-sarcoglycan gene has been mapped to chromosome 4q12, the same region to which we have linked LGMD in the southern Indiana Amish. In agreement with the common haplotype displayed by these families around the disease locus, a mutation common to all southern Indiana Amish LGMD2E patients tested has been found. This suggests that a unique founder effect may account for LGMD2E in this population, as has already been observed in the LGMD2A families of the northern Indiana and Penn-

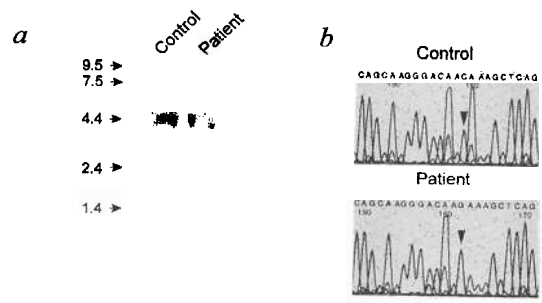

Fig. 6: Northern blot and sequence analyses of β-sarcoglycan gene in family A623. *a*, An RNA blot containing 12 μg of human skeletal muscle total RNA per lane from an unrelated control and an affected member of family A623 hybridized with a human β-sarcoglycan cDNA probe, representing nucleotides 1 to 1225, and exposed overnight. *b*, Direct nucleotide sequence determination of reverse-transcribed total RNA representing nucleotides 1 to 1132 performed on the same individuals described above and on another affected member of the family A623. Arrowheads indicate the position of the mutation. Both affected members of family A623 showed the same mutation.

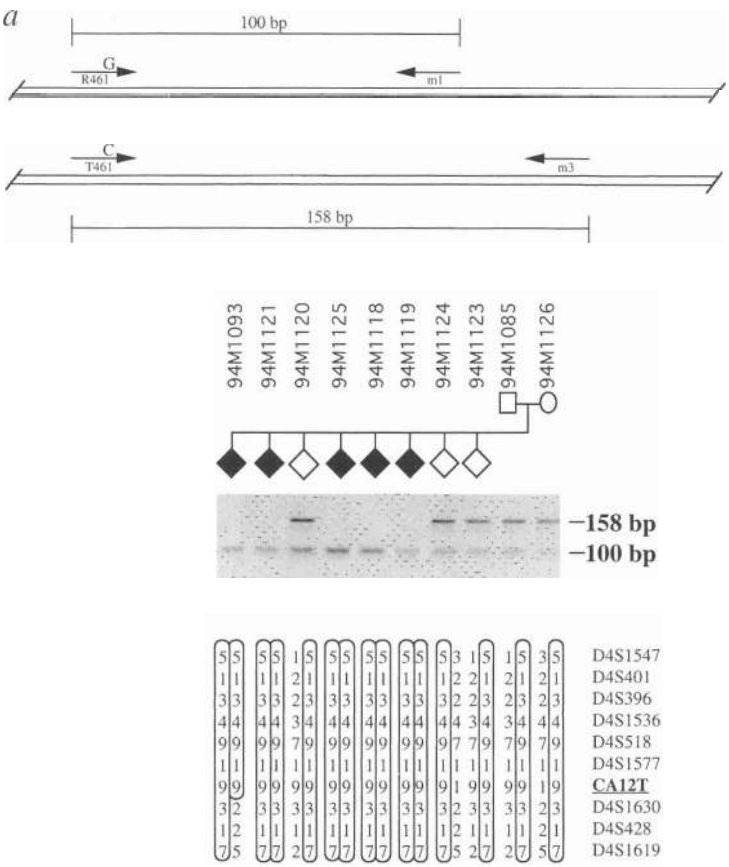

Fig. 7 Illustration of the segregation of the Thr to Arg substitution in the southern Indiana LGMD2E Amish population. *a*, Schematic representation of 'touchdown' PCR amplification. Products of 100-bp and 158-bp are produced using primer R461 coupled with primer m1 and primer T461 coupled with primer m3, respectively. Primer sequences are given in the Methods. *b*, Family A620 pedigree with affected and unaffected individuals indicated by closed and open symbols, respectively. *c*, Duplex deposit of "touchdown" PCR amplification products. *d*, Chromosome 4 haplotypes segregating within the family. Disease-bearing chromosomes are boxed. CA12T represents the intragenic microsatellite.

sylvania Amish. The perfect cosegregation of the mutation with the disease trait within the LGMD2E Amish population and lack of the mutation in over a hundred control chromosomes is strong evidence to support the hypothesis that this base change is responsible for the disease. These genetic data, together with our biochemical results, demonstrate the role of β-sarcoglycan in the aetiology of this disorder. As yet, however, the functional importance of the region in which the mutation occurs has not been defined.

Bönnemann *et al.* have cloned the cDNA of the same protein, and their results are reported in a manuscript published concurrently with this[35]. They have identified a compound heterozygote with mutations resulting in significant truncations of β-sarcoglycan. This patient's clinical presentation is much more severe than that of the Amish LGMD2E patients, indicating that the null mutations in this patient produce a much more deleterious effect. This pattern of severity, compared to the milder clinical course of the Amish patients, is similar to that seen in the spectrum of adhalin mutations that cause LGMD2D[17,18]. Thus, the presence of a missense rather than a nonsense mutation in the southern Indiana Amish population could explain the long survivability and slow disease progression of affected individuals. In the future, it will be interesting to determine whether patients with null mutations exhibit any symptoms related to the truncation of the β-sarcoglycan protein, particularly in the nonmuscle tissues in which the protein is also known to be expressed (Fig. 2).

A feature common to LGMD2D and LGMD2E is a selective absence of β-sarcoglycan, adhalin, and the 35 kDa DAG at the sarcolemma. The close association of these proteins had previously been suggested[34,43,44], and recent immunoprecipitation experiments have further demonstrated the existence of a subcomplex composed of these proteins within the DGC (unpublished data). The loss of all components of this subcomplex in both LGMD2D and LGMD2E indicates that mutations in one component can affect the stability or targeting of other components, further supporting the hypothesis that the missense mutation found in the southern Indiana Amish is the cause of LGMD, since this mutation affects the function of not just β-sarcoglycan, but adhalin and 35 kDa DAG as well. This also suggests yet another mechanism by which LGMD could occur, through a mutation in the 35 kDa DAG gene.

The precise function of β-sarcoglycan and its subcomplex as a whole is currently unknown. In Duchenne muscular dystrophy, disruption of the DGC causes the loss of a functional link between the extracellular matrix and the cytoskeleton, and may result in sarcolemmal instability and greater susceptibility to stress-induced damage. It is likely that the β-sarcoglycan/adhalin/35 kDa DAG complex plays a similar role in maintaining the structural and functional integrity of the cell membrane. In the BIO 14.6 cardiomyopathic hamster, a deficiency of this subcomplex is apparently also responsible for the dystrophic and cardiomyopathic changes in this animal[45,46]. Dystrophin and α-dystroglycan are not tightly associated with the sarcolemma, indicating that disruption of the sarcoglycan complex can lead to the destabilization of the dystroglycan complex as well[45]. Because no mutations have been found in the adhalin cDNA or mRNA of the cardiomyopathic hamster[47], mutations in β-sarcoglycan may be responsible for the loss of this complex and the resulting phenotype. It is also possible, however, that β-sarcoglycan alone serves an important functional role in nonmuscle tissues as well as in muscle, as suggested by the presence of β-sarcoglycan transcript in all tissues studied. Because the pattern of adhalin expression is different from that of β-sarcoglycan, β-sarcoglycan may function in a manner independent of the other components of the complex. In nonmuscle tissues, β-sarcoglycan may be part of another subcomplex. It will be of interest to determine the structure and cellular localization of β-sarcoglycan in these tissues and to identify its associated proteins. The presence of β-sarcoglycan in brain is particularly interesting because of the involvement of dystroglycan in synapse formation[48,49], though its involvement in this or in other processes in the nervous system remains to be determined.

The genetic and clinical heterogeneity of the limb-girdle muscular dystrophies has broad implications for the diagnosis and management of persons afflicted

with this disease. Because defects in several distinct proteins can cause phenotypically similar clinical and biochemical manifestations, a definitive diagnosis can only be achieved through genetic testing of all candidate genes. At the current stage of neuromuscular disease research and therapy, such measures can only provide information on the precise aetiology of LGMD, but this information will be important in directing proper therapy in the future.

Methods

Peptide sequencing and isolation of ESTs. The 43 kDa band of the purified dystrophin-glycoprotein complex[2] was partially sequenced, and several peptide fragments were obtained and are indicated in Fig. 1. Peptide sequences were used to search the GenBank database of expressed sequence tags (dbEST) using the TBLASTN search program[36]. Several overlapping ESTs were identified that represented portions of the β-sarcoglycan cDNA.

Human β-sarcoglycan cDNA cloning and characterization and mRNA analysis. Two of the clones, 22297 and 25556, from which ESTs were generated, were obtained from the IMAGE Consortium at Lawrence Livermore National Laboratories. The larger of these clones, 25556, was fully sequenced on both strands using an Applied Biosystems, Inc. automated sequencer. This clone was determined to contain the full coding region of the β-sarcoglycan cDNA as well as about 300 bp of 3' untranslated region and a poly(A^+) tail. Clones were isolated from a λZAPII human skeletal muscle cDNA library (Stratagene) using the 1225 bp insert of IMAGE clone 25556 as a probe. Primary structure and site detection analyses were performed using PC/Gene software (IntelliGenetics). This sequence data is available from the GenBank database, accession number U29586. CLONTECH adult and fetal human multiple tissue northern blots containing 2 μg of poly(A^+) RNA per lane were probed with a 334-bp PCR-amplified probe that represents nucleotides 132 to 465 of the β-sarcoglycan sequence, and with the 1225-bp insert clone 25556.

Fusion protein construct. A 192-bp region of the β-sarcoglycan cDNA downstream of the predicted transmembrane domain was amplified by PCR using the following primers: sense, 5'–GCCGGGATCCGTGATTCGCATTGGACCAAA–3'; antisense, 5'–GCGCGAATTCCTTTGTTGTCCCTTGCTGAA–3'. Following restriction digest with *Bam*HI and *Eco*RI, the product was subcloned into pGEX2TK[50] and introduced into *E. Coli* DH5α cells. 50 ml overnight cultures were diluted 1:10 and induced with IPTG to promote fusion protein (FP-I) production. Fusion proteins were purified on a glutathione-agarose column[51].

Antibodies. Anti-β-sarcoglycan antibodies were generated by intramuscular and subcutaneous injection of New Zealand white rabbits with 100 μg of purified FP-I in an emulsion of Freund's complete adjuvant. Rabbits were boosted two weeks later with a subcutaneous injection of 500 μg of FP-I in PBS (50 mM sodium phosphate, pH 7.4, 0.9% NaCl). Rabbits were bled two weeks following boost and the serum was tested for the presence of anti-FP-I antibodies. The serum was cleared of anti-GST antibodies with a glutathione column and anti-FP-I antibodies were affinity-purified using Immobilon-P strips containing 250 μg of FP-I. Monoclonal antibodies $VIA4_2$ against dystrophin, and $IVD3_1$ against adhalin were previously characterized[2,52]. Monoclonal antibody 8D5 against β-dystroglycan was kindly provided by Louise Anderson. An affinity-purified rabbit antibody against 35 kDa DAG (D. Jung, unpublished results) was also used in this study. Monoclonal antibody against human laminin α2 chain was purchased from Chemicon.

Western blot and immunofluorescence. KCl-washed microsomes, crude rabbit skeletal muscle sarcolemma, and purified DGC were prepared as previously described[2,53]. Proteins were resolved on a 3%–12% SDS polyacrylamide gel[54] and transferred to nitrocellulose by electroblotting[55]. Blots were incubated overnight in a 1:20 dilution of affinity-purified anti-FP-I antibody in Blotto (5% nonfat dried milk in TBS [20 mM Tris-HCl, 200 mM NaCl, pH 7.4]), then incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Boehringer Mannheim) for 1 hr. Antibody staining was detected with H_2O_2 in TBS with 4-chloro-1-naphthol as a substrate. For immunofluorescence, 7 μm transverse cryosections were prepared from control and Amish LGMD muscle. The following procedures were performed at room temperature. Sections were treated with AB blocking solutions (Vecter), blocked with 5% BSA in PBS for 30 min, and then incubated with a 1:20 dilution of affinity-purified anti-FP-I antibody for 90 min. Antibodies against the following components of the DGC were also tested: dystrophin, laminin α2 chain, β-dystroglycan, syntrophin, adhalin, and 35 kDa DAG. After extensive washing with PBS, sections were incubated with biotinylated secondary antibodies (1:500) for 30 min, washed with PBS, then incubated with FITC-conjugated streptavidin (1:1000) for 30 min. After rinse with PBS, sections were mounted with FITC-guard (Testog) and observed under a Zeiss Axioplan fluorescence microscope. Photographs were taken under identical conditions with the same exposure time.

β-sarcoglycan gene localization to chromosome 4q12. Primers corresponding to human β-sarcoglycan cDNA nucleotides 291-312 (sense) and 413-429 (anti-sense) were used in PCR using DNA from a panel of 25 human-rodent somatic cell hybrids (BIOS Corporation) containing various combinations of human chromosomes. Subsequent restriction digest of the PCR products by *Taq*I was used to distinguish between the human and rodent alleles. Use of a somatic cell hybrid panel containing various regions of chromosome 4 (ref. 38) further narrowed the location of the gene using the same approach described above.

A chromosome 4 cosmid library (kindly provided by Jeffrey Murray) was screened with a ^{32}P-labelled PCR product representing nucleotides 135-429. Two cosmids with inserts of 28.5 kb and 35 kb were obtained. CsCl-purified DNA from the smaller cosmid was used for fluorescence *in situ* hybridization mapping which was performed by Genome Systems.

Families. Six previously described LGMD Amish families from southern Indiana (52 individuals, 13 affected)[33] were analysed in the linkage search. Subsequently, DNA from 5 additional southern Indiana families were included in this study (39 individuals, 13 affected). All of these kindreds show multiple consanguineous links (data not shown). In these families, 11 patients were clinically reevaluated after exclusion of the calpain 3 locus. All patients presented with proximal symmetrical weakness and atrophy of limb and trunk muscles. Clinical onset was at 7.6 years of age (range 4 to 12 years), loss of walking at 26 years (range 12 to 38 years), with marked intrafamilial variability. Calf hypertrophy was similar to that observed in BMD and LGMD2D patients, but different from Reunion Island LGMD patients[56] and LGMD2A patients from the northern Indiana Amish community.

Genotyping and linkage analysis. Markers were selected from the microsatellite panel described[39] or from CHLC maps[57]. DNA (50 ng) was used as templates in a 50 μl polymerase chain reaction as described[58]. Southern blots of restriction fragments of cosmids were probed with $(CA)_n$ and tetranucleotide oligonucleotide repeats labelled with γ-^{32}P ATP. The positive fragment was subcloned and sequenced. The identified intragenic polymorphic CA repeat was amplified using the following primers: sense (5'–TATCTTCTAATGTCTTCTGTCTAT–3')

and antisense (5'–GAAACAAGAATAACATGCCATTT–3'). PCR conditions for this marker were — denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, for 30 cycles. Primer sequences, PCR conditions, and other information concerning the highly polymorphic microsatellites used in this study can be obtained from the Genome Database, John Hopkins University. Two-point and multipoint linkage analyses were carried out using the LINKAGE software package, version 5.1 (ref. 59) assuming fully penetrant autosomal recessive inheritance with a gene frequency of 0.001.

RNA isolation and reverse-transcription PCR. Total RNA was extracted from 20–30 mg of skeletal muscle from one control and two Amish LGMD2E patients from family A623 (ref. 33) using RNAzol (Tel-Test) according to manufacturer specifications. RNA samples were run on 15% formaldehyde/1.5% agarose gels and transferred to Hybond N^+ membrane (Amersham). Membranes were then hybridized with the PCR labelled cDNA as described above.

Total RNA (1 μg) was used for reverse transcription with a specific primer representing nucleotides 1113-1132 (antisense) in the β-sarcoglycan 3' cDNA untranslated region, in a reaction mixture containing 6 mM $MgCl_2$, 200 mM dNTP, 50 mM KCl, 10 mM Tris pH 8.2, 40 U RNasin, 10 pmol specific primer, 6 U AMV reverse transcriptase, and incubated for 90 min at 42 °C. PCR on the reverse-transcribed product was performed using the same 3' primer and one of two 5' primers (representing nucleotides 1-18 and 47-68 respectively) to cover the β-sarcoglycan cDNA coding sequence. The RT-PCR amplification products were analysed by agarose gel electrophoresis and by direct sequencing.

Touchdown PCR. DNA (50 ng) was subjected to touchdown PCR[42] in a 50 μl reaction mix containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 mM of each dNTP, 100 ng of each primer, and 2 U *Taq* Polymerase (Perkin Elmer). After 5 min denaturation at 96 °C, amplification cycles were carried out as follows: 40 s denaturation at 94 °C followed by 30 s annealing steps starting at 63 °C with a decrease of 1 °C every two cycles until 59 °C. Twenty-five additional cycles of amplification consisting of 40 s at 94 °C and 30 s at 58 °C, were performed. Primer pairs R461/m1 and T461/m3 were designed to yield, respectively, a 100-bp product from individuals carrying the mutation, and a 158-bp product from individuals not carrying the mutation. Primers sequences were: R461: 5'–GTTTTTCAGCAAGGGACAAG–3'; T461: 5'–GTTTTTCAGCAAGGGACAAC–3'; m1: 5'–TATTTTGAGTCCTCGGGTCA–3'; m3: 5'–CTTTTCACTCCACTTGGCAA–3'. PCR products were mixed in a 1:1 volume ratio and run in a single lane on 4% agarose gels stained with ethidium bromide.

Acknowledgments

We thank M.B. Soares and G. Lennon of the IMAGE Consortium for providing the clones from the normalized infant brain library; J. Murray for the chromosome 4 specific cosmid library and critical comments on this manuscript; J. Chamberlain for the gift of the microsatellite repeat probes; L. Anderson for the 8D5 β-dystroglycan antibody; D. Jung for the 35 DAG antibody; S.A. Cullen for performing the muscle biopsies; C. Leveille, J. Lee, S. Cutshall, D. Venzke, and D.Tischfield for their expert technical assistance; V. Sheffield for helpful consultation; L. Eberly and D. Moser of the University of Iowa DNA Core for their sequencing assistance; N. Chiannilkulchai, D. Fugman, F. Quetier, J. Tischfield, and all members from our respective labs for all their help. We are particularly indebted to all patients and their families. This research was supported by grants from the Muscular Dystrophy Association, L'Association Française contre les Myopathies (AFM), and the Groupement de Recherche Européen sur le Génome. L.E.L. is supported by a grant from the Iowa Affiliate of the American Heart Association. V.A. is funded by a grant from AFM. K.P.C. is an Investigator of the Howard Hughes Medical Institute.

Received 31 July; accepted 4 October 1995.

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