Human ε-sarcoglycan is highly related to α-sarcoglycan (adhalin), the limb girdle muscular dystrophy 2D gene

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Abstract The dystrophin-glycoprotein complex (DGC) is critical for muscle membrane stability. The sarcoglycans are transmembrane proteins within the DGC, and the function of the sarcoglycans is unknown. Mutations in sarcoglycan genes cause autosomal recessive muscular dystrophy. We have identified a new sarcoglycan gene with high homology to α-sarcoglycan highlighting the redundancy of the DGC. This gene, named ε-sarcoglycan, has an identical intron-exon structure to α-sarcoglycan, and is more broadly expressed. The characterization of ε-sarcoglycan should make it possible to determine if it, like the other sarcoglycan genes, is mutated in muscular dystrophy.

Key words: Sarcoglycan; Dystrophin; Muscular dystrophy; Split hand/split foot; Chromosome 7

1. Introduction

Muscular dystrophies arise from mutations in the genes encoding dystrophin and its associated proteins [1,2]. In skeletal muscle, dystrophin associates with a complex of membrane-associated proteins forming the dystrophin-glycoprotein complex (DGC) [3,4]. The DGC is composed of a number of proteins and binds the extracellular matrix protein laminin [5]. It is hypothesized that defects in the DGC disrupt the mechanical link between the cytoskeleton of muscle and the extracellular matrix. This disruption leads to membrane instability, myocyte necrosis and progressive replacement of the muscle by connective and adipose tissue. Over time, the muscle becomes progressively weakened resulting in the clinical spectrum of muscular dystrophy. The exact molecular mechanism that causes myocyte necrosis is unknown. Moreover, it is unknown why some muscles are more affected than others since the DGC appears to be present in all muscles.

One of the major DGC proteins, dystroglycan, binds dystrophin's carboxy-terminus, spans the sarcolemma and interacts with laminin in the extracellular matrix [5]. Sarcoglycan, another major component of the DGC is composed of at least four subunits, α, β, γ and δ. In contrast to dystroglycan, α-, γ- and δ- sarcoglycan are exclusively expressed in striated muscle, and patients with mutations exhibit a muscle phenotype (for reviews, see [1,2]). β-Sarcoglycan mRNA is expressed at low levels in tissues other than striated muscle, but patients with loss of function mutations in the β-sarcoglycan gene also have classic proximal muscle weakness and muscular dystrophy [1,2]. The sarcoglycan subunits are thought to form a subcomplex within the DGC since, in the presence of β-octyl glucoside, the sarcoglycan subunits preferentially associate. Mutations in any one of the sarcoglycan genes lead to a secondary instability of the other sarcoglycan subunits [1,2,6].

In this report, we describe a novel sarcoglycan gene. This gene, ε-sarcoglycan, is homologous to α-sarcoglycan [7,8], but unlike α-sarcoglycan, ε-sarcoglycan is expressed in other tissues in addition to heart and skeletal muscle. The gene structure is conserved between α- and ε-sarcoglycan with nearly identical placement of the intron-exon borders, but significantly larger intronic distances. ε-Sarcoglycan maps to 7q21, and the murine gene for ε-sarcoglycan maps to the syntenic region on mouse chromosome 6. A processed pseudogene, pseudo-ε-sarcoglycan, is present on chromosome 2 in the human. The high homology of ε-sarcoglycan to α-sarcoglycan and its tissue expression argue that ε-sarcoglycan may be a component of the DGC not only in striated muscle but also in non-muscle tissues. The knowledge of the map location, sequence and intron-exon borders should facilitate understanding the role of ε-sarcoglycan in muscular dystrophy.

2. Materials and methods

2.1. Identification of ε-sarcoglycan cDNA and genomic sequences

Primers designed to the EST N44285 (5′-GACGGGAAGGCGTGGAAAAGAGAA-3′ and 5′-AGCACTGTGATGGACCAGTTGG-3′) were used in a PCR reaction with reverse transcribed total RNA from human skeletal and cardiac muscle. The PCR product was reamplified to generate a radiolabeled fragment that was used to screen a human genomic EMBL3 library (Clontech) and a human cardiac cDNA library [8]. A full length cDNA clone was subcloned into Bluescript and sequenced on both strands. DNA from genomic phage clones encoding ε-sarcoglycan was prepared as described [9] and used as a template in cycle sequencing to determine intron-exon borders. Cycle sequencing was performed on an ABI 377 automated sequencer. Sequences were analyzed using Sequencher (GeneCodes), MacVector and GCG programs.

2.2. Northern blot analysis

The identical probe used for cDNA library screening was used to assess the expression of ε-sarcoglycan mRNA in multiple human tissues. The PCR product was amplified in the presence of ^{32}P-α-dCTP. The radiolabeled product was used against 2 μg of poly(A)$^+$ RNA from multiple human tissues (Clontech) as described [8].

2.3. Radiation hybrid mapping

To determine the map location of ε-sarcoglycan the Genebridge 4 radiation hybrid panel (Research Genetics) was amplified as follows: 25 ng of each hybrid was amplified using 5′-CTTAGCAGGATCTC-

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Abbreviations: DGC, dystrophin-glycoprotein complex; SHSF, split hand/split foot

The sequence submitted here is available from GenBank under accession number AF036364.

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TAATTATC-3′ and 5′-GCAAGTGTCCACCTATCAGG-3′ as the primers in a 10 μl reaction volume using 0.1 ng of each primer per reaction using AmpliTaq Gold (Perkin Elmer Applied Biosystems). The entire reaction volume was separated on 2% agarose gels and stained with ethidium bromide. The reactions were scored as positive (1), negative (0) or indeterminate (2). The map position was determined using Whitehead Institute RHmapper (http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl). Analysis was conducted using a LOD score of >3.0. Primers 5′-CATAGAATTCATTGAGATGCAGTC-3′ and 5′-AAGTGAGACACAGGAAGTGTTGATG-3′ were designed to amplify and map pseudo-ε-sarcoglycan. The results were similarly scored.

2.4. Mapping the murine ε-sarcoglycan gene

Primers, 5′-GTTTATGTCATGGTTGGTGCCGATG-3′ (2F) and 5′-TACTTCCTGATAGGTGGACACTTGC-3′ (1R), to the EST AA051289, were used with 100 ng of mouse genomic DNA (C57BL/6J) with Takara (Panvera) according to the manufacturer's recommendations at 68°C and 10 min extensions. A 3 kbp portion of the murine ε-sarcoglycan gene was reamplified and directly sequenced to determine that it contained a portion of ε-sarcoglycan. The 2F primer (listed above was used with a primer directed at intron 6 (5′-TTCCAACAGCAATTCGGGTTCC-3′, primer 3R), in a PCR reaction with the parental strains in the Jackson Labs BSS and BSB mapping panels [10]. 25 ng of DNA was amplified using 10 ng of each primer in a 10 μl reaction volume in the presence of ^{32}P-α-dCTP as described [9]. The products were separated under non-denaturing conditions using 0.5×MDE (FMC Bioproducts), 1% glycerol. A 40 cm gel was run at 7 W until the products had migrated to the center of the gel. Gels were dried and autoradiographed for 12–24 h at −80°C. The results were scored blind and submitted to Jackson Labs for mapping.

2.5. Genotyping

Primers were designed to amplify the CA repeat found 3′ of exon 3 of ε-sarcoglycan. The primers ESG-E3-3C (5′-GTTATCTACTGTGTTTCCATGCAC-3′) and ESGE3-F2 (5′-CAACAATCATTGAGGTAATTTACACC-3′) were used to amplify 50 ng genomic DNA in a 10 μl volume as described [11] with the addition of 8% DMSO. The cycling parameters were as follows: 10 min at 94°C, 1 min at 55°C, 30 s at 72°C and 20 s at 94°C for a total of 35 cycles. The PCR products were separated on an ABI 377 sequencer and the allele sizes were calculated using Genescan and Genotyper (Perkin Elmer Applied Biosystems). The allele frequencies were determined from a panel of 45 normal, unrelated individuals of mixed ethnicity.

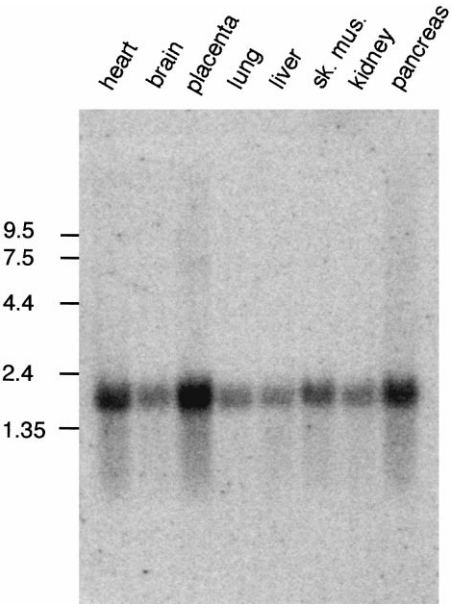

Fig. 1. Expression of ε-sarcoglycan mRNA in multiple human tissues. Shown above is 2 μg of poly(A)$^+$ mRNA hybridized with a portion of the ε-sarcoglycan cDNA. A 1.7 kbp mRNA is seen in all tissues.

3. Results

3.1. ε-Sarcoglycan gene and mRNA expression

Sequences homologous to α-sarcoglycan were detected in the electronic database of expressed sequence tags (ESTs). The plasmid containing the EST N44285 (clone 273055) was obtained and sequenced to confirm its content. This cDNA encoded approximately 500 bp of ε-sarcoglycan and showed high homology to the predicted cytoplasmic domain of α-sarcoglycan. A portion of this cDNA was used as a radioactive probe against a panel of poly(A)$^+$ mRNA from multiple human tissues (Fig. 1). A 1.7 kbp mRNA was detected in all human tissues surveyed. To obtain full length clones encoding

Table 1
Intron-exon boundaries of ε-sarcoglycan

E1	tggacgggacagggtcgggggacacgcaggATGAGCCCCGCG	.....	TCTTGCTGACAGgttagtggcctgcgcgcgacgtggaggtcc
	M S P A		F L L T
E2	ctgtcacttatctttcctctttgttttcagTGTACAGTATTT	.....	ACCCAAAACCTGgtatgttctttgtgaggaaaaaaaagcagt
	V Y S I		Y P K P
E3	ttatgtcttaattctctgtttcttatctagGCGAGATTAGTA	.....	ACAATCATAGAGgtaatttacacctagaaagagcaaaactat
	G E I S		T I I E
E4	ttctttaatatggttttccttttataatagATAACTGCCTAC	.....	TGTCTGCAGAAGgtaggtatgaaaatatgttgatcatttctt
	I T A Y		S A E D
E5	gatatgcatgccctttttcaccaaaattagACTTCCCGTTGC	.....	CCTGAAGGAGGGgtaagcagatctttatctcattgccttaaa
	D F P L		L K E G
E6	ggataacctgagagtttcacatttttacagCGTTTATGTCAT	.....	AAAATTTCATTGgtaagtttttatttgtttttgcaagttta
	V Y V M		K I S L
E7	ttgttgtgtaattgtcttcttttatatcagGTTGATAAAACA	.....	ACGGGAAGGCGTgtgagtactttaaaactaataagataatta
	V D K T		R E G V
E8	tttaattcataaattttgttttgtttctagGGAAAAGAGAAA	.....	GCAAACACCAGAgtaagtgtcttctttcctgttatttttcttc
	E K R N		Q T P D
E9	tatatgttgttttatttctttttgacctagCATCCAACTGGT	.....	GCAAACGCAGCAgtaagtgtccacttagagccataattatta
	I Q L V		Q T Q Q
E11	gttttactcaaaaatgttcatcatttctagGAACTTGCCACA	.....	AGCAGACTACAGgtgagtgctaagaataataaattaatgttt
	N L P H		Q Q T T
E12	cctgcccatattcattcttacacattgcagGTAAATGGTATC	.....	CCTGAagaaagaaaactgactgaagcaatgaatttataatca
	G K W Y		P *

Exon numbers are indicated on the left. Translated nucleotides are shown capitalized. A minority of ε-sarcoglycan transcripts contain exon 10. Exon 10 (boundaries not determined) arises from splicing involving an Alu repeat.

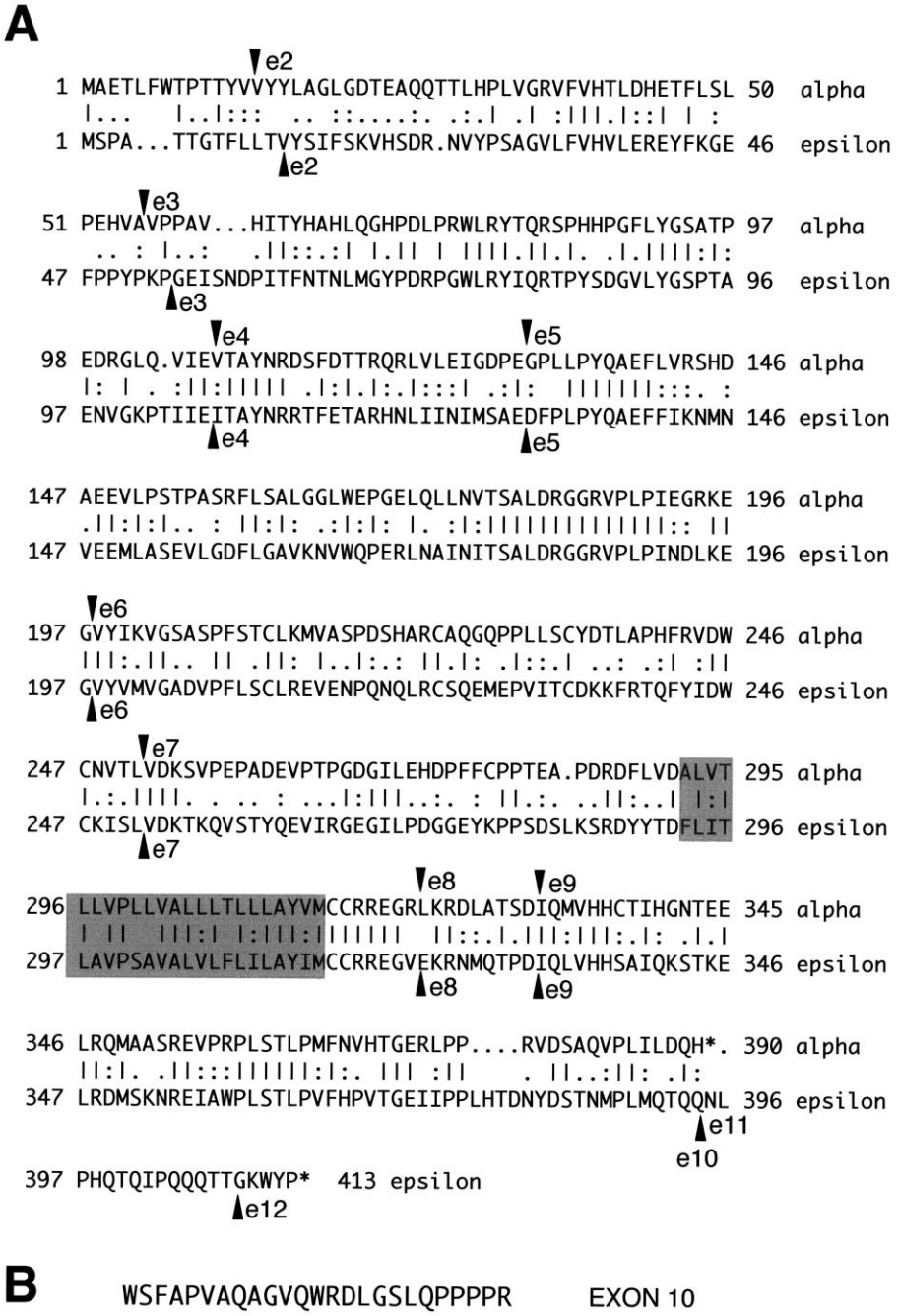

Fig. 2. Comparison of α-sarcoglycan and ε-sarcoglycan predicted protein sequences. The amino acid sequences of α-sarcoglycan and ε-sarcoglycan are shown in A. The placement of the introns is also indicated by arrows. The nearly identical placement of the intron sequences suggests that these two genes may have arisen from a gene duplication event. The hydrophobic transmembrane domain is highlighted. B shows an additional exon that is found to be present in a small percentage of ε-sarcoglycan mRNA.

ε-sarcoglycan, this same probe was used to screen a human heart cDNA library. Four cDNA clones were plaque purified and characterized by restriction digest, PCR and partial sequence data. Of these, one clone was fully sequenced to determine the ε-sarcoglycan nucleotide sequence and this sequence has been deposited in GenBank with the accession number AF036364.

Within the coding region, ε-sarcoglycan is 47% homologous to α-sarcoglycan at the nucleotide level, and 62% similar at the protein level (43% identical). The predicted amino acid sequence of ε-sarcoglycan is shown in Fig. 2. Like α-sarcoglycan, there is a predicted hydrophobic signal sequence followed by a substantial extracellular domain with a conserved consensus site for asparagine-linked glycosylation [7,8]; the site for glycosylation is within the highly conserved region encoded by exon 5. There are four conserved cysteine residues encoded by exon 6. It has been suggested that these residues are important for function since mutations in the homologous cysteine residues in α-sarcoglycan result in muscular dystrophy [1,2]. There is a hydrophobic transmembrane domain followed by a short cytoplasmic domain. An alternative cytoplasmic domain was also seen in a second cDNA and its

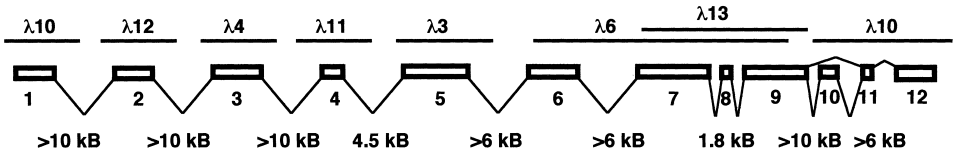

Fig. 3. The gene structure of ε-sarcoglycan. Shown above is a schematic of the intron-exon boundaries of the ε-sarcoglycan gene. Genomic phage encoding exons of ε-sarcoglycan were isolated and sequenced to determine the intron-exon boundaries. Phage that contained overlapping fragments are indicated on the upper line. The size of most of the introns could not be determined using long range PCR which suggests that the minimum size of the ε-sarcoglycan gene is 50 kbp.

sequence is shown in Fig. 2B. This alternative cytoplasmic domain arises from the inclusion of exon 10. Of note, the sequences within exon 10 have homology to the Alu class of repetitive DNA sequences suggesting that the inclusion of exon 10 may result from splicing that includes an Alu repeat. Of the four cDNAs isolated, three represented the splice form without exon 10. The predicted molecular mass of ε-sarcoglycan, without exon 10 and post-translational modifications, is 47 kDa, and the calculated p*I* is 5.78, very close to the predicted p*I* (5.44) of α-sarcoglycan.

3.2. Genomic structure of ε-sarcoglycan

The gene structure of ε-sarcoglycan was determined from genomic phage and is shown in Fig. 3. The positions of the introns are highly conserved between α-sarcoglycan and ε-sarcoglycan as indicated by the arrows in Fig. 2. The sizes of the introns in ε-sarcoglycan were estimated by performing long range PCR reactions with intra-exonic primers as well as looking for overlapping fragments of the genomic phage encoding ε-sarcoglycan (data not shown). The exact size of the α-sarcoglycan gene is not known, but exons 2–9 are found within a 10 kbp region ([7], E.M. McNally and L.M. Kunkel, unpublished results). In comparison, the ε-sarcoglycan gene is estimated to span 50–100 kbp. This increase in size is due to markedly larger introns. The sequences flanking each of the exons are shown in Table 1. A polymorphic dinucleotide CA repeat was found 3′ of exon 3. Primers amplifying this repeat were tested on the genomic DNA from 45 unrelated individuals of mixed ethnicity. This microsatellite repeat was heterozygous in 49% of the individuals tested, and the allele frequencies are shown in Table 2.

3.3. Map position

The map position of ε-sarcoglycan was determined using primers specific for exon 7 and intron 7 against a panel of radiation hybrids (Genebridge 4, Research Genetics). This primer pair was used to avoid amplifying the related locus, pseudo-ε-sarcoglycan (see below). With a LOD score >3.0, the results of the radiation mapping panel is shown in Fig. 4. This places ε-sarcoglycan near the marker D7S644 and D7S657.

A cDNA encoding murine ε-sarcoglycan was identified in the electronic database (EST AA051289, clone 478626) and the murine ε-sarcoglycan gene was partially characterized using long range PCR with primers complementary to coding region in exons 6 and 7. No evidence for an ε-sarcoglycan pseudogene was seen in the mouse genome. The map position of murine ε-sarcoglycan was determined by amplifying a portion of the murine ε-sarcoglycan gene from mouse genomic DNA using primers to exon 6 and intron 6. Species specific PCR products were detected using SSCP analysis. The Jackson Labs Backcross panels BSB (backcross to C57BL/6J) and BSS (backcross to *M. spretus*) were analyzed using this approach. The resulting map position is shown in Fig. 4. Both mapping panels gave similar results. This places the murine ε-sarcoglycan gene near the telomere of chromosome 6. Several other genes for which the human homologs are found at 7q21 are also in this region. The mapping of ε-sarcoglycan extends the synteny of this region.

3.4. Pseudo-ε-sarcoglycan

Two of the phage clones isolated shared overlapping restriction fragments and appeared to contain large fragments of the

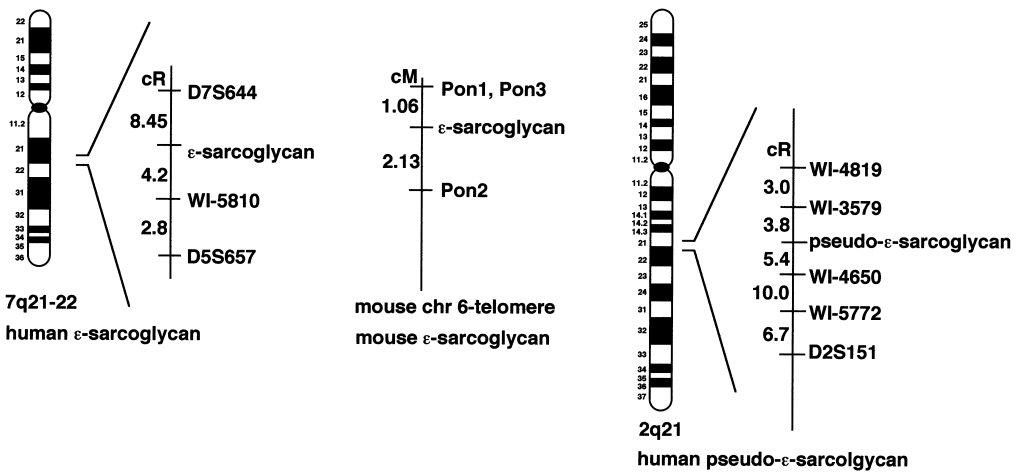

Fig. 4. The map position of human and murine ε-sarcoglycan was determined using radiation hybrid mapping for the human ε-sarcoglycan gene and the Genebridge 4 Radiation Hybrid mapping panel. A processed pseudogene for ε-sarcoglycan was detected on chromosome 2q21, and its map position is shown. The position of the mouse ε-sarcoglycan gene was determined using the Jackson Labs Backcross panel. The mouse ε-sarcoglycan gene maps to the syntenic region of human chromosome 7q21, mouse chromosome 6. cR, centiRay; cM, centiMorgans.

Table 2
Allele frequencies

Allele size (bp)	Allele frequency
162	0.51
164	0.31
168	0.16
170	0.01
172	0.01

ε-sarcoglycan gene based on hybridization with primers to ε-sarcoglycan. Sequence from these clones revealed what appears to be a processed pseudogene for ε-sarcoglycan. Several contiguous exons are present but an intact open reading frame is not present (data not shown). The phage encoding pseudo-ε-sarcoglycan were used in fluorescence in situ hybridization against human metaphase chromosomes and showed hybridization to chromosome 2q21 (data not shown). To confirm the map position of the pseudo-ε-sarcoglycan, primers designed specifically to amplify the pseudogene, as opposed to the chromosome 7-encoded ε-sarcoglycan, were used against the Genebridge 4 Radiation hybrid panel. With a LOD score >3.0, pseudo-ε-sarcoglycan maps near markers on chromosome 2. The results of the radiation hybrid mapping are shown in Fig. 4.

4. Discussion

We have isolated a gene, ε-sarcoglycan, that predicts a protein highly related to α-sarcoglycan [7,8]. Sequence analysis of cDNAs encoding ε-sarcoglycan suggests that ε-sarcoglycan and α-sarcoglycan share a similar structure. Both sequences predict a hydrophobic signal sequence followed by an extracellular domain with highly conserved cysteine residues. There is some divergence in the predicted cytoplasmic domains suggesting that these proteins may bind different partners in the cytoplasmic side of the membrane. The overall gene structure is conserved with nearly identical placement of introns suggesting that these genes arose from a gene duplication event. The lower homology seen at the nucleotide level than the amino acid comparisons suggests that there has been selective pressure on the protein sequence. The redundancy in the DGC is striking and includes {dystrophin/utrophin} [12,13], {γ-sarcoglycan/δ-sarcoglycan} [14,15], {α-syntrophin/β1-syntrophin/β2-syntrophin} [16,17] and, now, {α-sarcoglycan/ε-sarcoglycan}. It is surprising that this system, with it marked redundancy, can be the target of mutations that give rise to such severe phenotypes as those seen in the muscular dystrophies. These proteins, while similar in structure, appear to have critical and unique roles for muscle function.

Mutations in α-, β-, γ- and δ-sarcoglycan have been described in patients with autosomal recessive muscular dystrophy [1,2]. It may be that ε-sarcoglycan is similarly mutated in patients with muscular dystrophy. However, unlike α-, γ- and δ-sarcoglycan, ε-sarcoglycan mRNA appears to be highly expressed in many human tissues [7,8,14,15]. In particular, the mRNA for ε-sarcoglycan is abundant in human placenta suggesting that this gene and its product may be important in human development. The dystrophin-associated protein, dystroglycan, shares a similar expression pattern to ε-sarcoglycan [5], and no patients have been described with dystroglycan gene mutations. Mice homozygously lacking dystroglycan die at embryonic day 6.5 [18]. Immunostaining with anti-dystroglycan antibodies suggests that, normally, dystroglycan is abundantly present in the extraembryonic structure, Reichert's membrane [18]. This membrane is disrupted in animals lacking dystroglycan, and this may lead to the early embryonic lethal phenotype. Because ε-sarcoglycan is so highly expressed in non-muscle tissues, like dystroglycan, mutations in this gene may have a more profound phenotype than mutations in the genes expressed more exclusively in muscle tissue. The similar expression pattern of ε-sarcoglycan and dystroglycan suggests that these proteins may constitute the DGC as it exists outside muscle.

The human map location of the ε-sarcoglycan gene suggests a possible involvement in the Split Hand/Split Foot deformity syndrome (SHSF). In some cases, this is an autosomal dominant disorder that has been refined to a locus involving the marker D7S644 [19]. Based on the map location, ε-sarcoglycan may be deleted in some SHSF patients. DLX5, a member of the distal-less homeobox protein family, has been mapped to the critical interval for SHSF [19]. However, the variably severe phenotype argues that more than one gene may be implicated in the development of this disorder. Haploinsufficiency has not been seen to result in a phenotype in the heterozygous parents of patients with known sarcoglycan gene mutations. The information presented here should facilitate the study of this gene and its protein product in patients with muscular dystrophy and other developmental disorders.

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