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Primary Structure and Muscle-specific Expression of the 50-kDa Dystrophin-associated Glycoprotein (Adhalin)*

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The 50-kDa dystrophin-associated glycoprotein (50-DAG) is a component of the dystrophin-glycoprotein complex, which links the muscle cytoskeleton to the extracellular matrix. 50-DAG is specifically deficient in skeletal muscle of patients with severe childhood autosomal recessive muscular dystrophy and in skeletal and cardiac muscles of BIO 14.6 cardiomyopathic hamsters. The lack of 50-DAG leads to a disruption and dysfunction of the dystrophin-glycoprotein complex in these diseases. The cDNA encoding 50-DAG has now been cloned from rabbit skeletal muscle. The 50-DAG deduced amino acid sequence predicts a novel protein having 387 amino acids, a 17-amino acid signal sequence, one transmembrane domain, and two potential sites of *N*-linked glycosylation. Affinity-purified antibodies against rabbit 50-DAG fusion proteins or synthetic peptides specifically recognized a 50-kDa protein in skeletal muscle sarcolemma and the 50-kDa component of the dystrophin-glycoprotein complex. In contrast to dystroglycan, which is expressed in a wide variety of muscle and non-muscle tissues, 50-DAG is expressed only in skeletal and cardiac muscles and in selected smooth muscles. Finally, 50-DAG mRNA is present in *mdx* and Duchenne muscular dystrophy (DMD) muscle, indicating that the down-regulation of this protein in DMD and the *mdx* mouse is likely a post-translational event.

The dystrophin-glycoprotein complex (DGC)[1] links the actin cytoskeleton to laminin in the extracellular matrix in skeletal and cardiac muscle (1–9). The DGC is composed of dystrophin; an intracellular 59-kDa protein triplet; three transmembrane glycoproteins of 50, 43, and 35 kDa; a 25-kDa transmembrane protein; and an extracellular 156-kDa glycoprotein. The 156- and 43-kDa dystrophin-associated glycoproteins are encoded by a single gene (7). The 97-kDa dystroglycan precursor protein is processed into an extracellular, highly glycosylated 156-kDa proteoglycan (α-dystroglycan) and a 43-kDa glycoprotein (β-dystroglycan) having one transmembrane domain (7). α-Dystroglycan binds specifically to laminin in the extracellular matrix (7, 9), and dystrophin interacts with F-actin in the cytoskeleton (9, 10).

Biochemical characterization of the dystrophin-glycoprotein complex indicates that the 50-kDa dystrophin-associated glycoprotein (50-DAG, previously identified as SL50) is localized to the sarcolemma of skeletal muscle (5, 11), exhibits an increase in electrophoretic mobility following treatment with *N*-glycosidase F, is not removed from membranes by alkaline extraction, and is labeled by the hydrophobic probe 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (5). Taken together, these data suggest that 50-DAG is an integral membrane glycoprotein. Additionally, a monoclonal antibody against 50-DAG quantitatively immunoprecipitates the entire DGC (5), indicating that 50-DAG is a stoichiometric component of the complex.

In Duchenne muscular dystrophy (DMD) and in the *mdx* mouse, the absence of dystrophin (12) leads to a decrease in the abundance of all dystrophin-associated proteins at the sarcolemma (2, 6, 13, 14). In severe childhood autosomal recessive muscular dystrophy (SCARMD), 50-DAG is specifically decreased in abundance at the sarcolemma despite the presence of normal levels of dystrophin (15). Thus, the deficiency of 50-DAG may be the common denominator leading to muscle cell necrosis in DMD and SCARMD. An identical or antigenically similar dystrophin-glycoprotein complex exists in cardiac muscle (8, 9). We have previously shown that a specific deficiency of 50-DAG in skeletal and cardiac muscles of the cardiomyopathic hamster leads to a disruption of the dystrophin-glycoprotein complex, which likely causes this animal's muscular dystrophy and cardiomyopathy (16).

We have determined by cloning of 50-DAG cDNA that 50-DAG is a novel transmembrane glycoprotein. The identity of the cDNA clone has been confirmed using antibodies against fusion proteins and synthetic peptides, all of which specifically recognize the 50-kDa component of the DGC. 50-DAG is expressed almost exclusively in skeletal and cardiac muscles. Finally, 50-DAG mRNA is present in *mdx* and DMD muscle, indicating that the down-regulation of this protein in DMD and the *mdx* mouse is likely a post-translational event.

EXPERIMENTAL PROCEDURES

Cloning of 50-DAG cDNA—Affinity-purified sheep antibodies against 50-DAG (6) were used to screen a rabbit skeletal muscle cDNA library in λgt11 (7) using standard protocols (17). Phage DNA was isolated as described (17), and the 50–1 cDNA was subcloned into Bluescript (Stratagene) and sequenced on both strands using the Sequenase II protocol (U. S. Biochemical Corp.). To isolate a full-length clone, a λZAP II rabbit skeletal muscle cDNA library (Stratagene) was screened according to the manufacturer's protocol using ^{32}P-labeled 50–1 cDNA. Two hybridizing plaques were isolated, and the phage were rescued into Bluescript using Stratagene's protocol. Following initial sequence analysis, only one clone (50–2) demonstrated homology to 50–1 and was sequenced in full on both strands. PCGene (IntelliGenetics) software was used for sequence analysis. For sequencing of 50-DAG tryptic peptides, purified DGC (18) was separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose, and the region of the blot containing 50-DAG was excised and processed as described (7, 19).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U01117.

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[1] The abbreviations used are: DGC, dystrophin-glycoprotein complex; BSA, bovine serum albumin; 50-DAG, 50-kDa dystrophin-associated glycoprotein; DMD, Duchenne muscular dystrophy; SCARMD, severe childhood autosomal recessive muscular dystrophy.

Fusion Protein and Antibody Production—Glutathione *S*-transferase fusion protein constructs were generated by subcloning two PCR-amplified regions of rabbit 50-DAG cDNA into pGEX-2T (20). Polymerase chain reaction reagents were from Perkin-Elmer Cetus. The amplified regions were nucleotides 903–1121 (FP-G) and 1188–1474 (FP-H) encoding amino acids 217–289 and 312–387, respectively. Fusion proteins were purified by glutathione affinity chromatography (21). Each fusion protein was tested for cross-reactivity with existing anti-50-DAG or anti-DGC antisera and was then used to affinity purify anti-fusion protein antibodies from reactive antiserum. Anti-FP-G was affinity-purified from guinea pig anti-50-DAG serum (5) and FP-H from guinea pig anti-DGC serum (5). To produce anti-C-terminal antibodies, a rabbit was immunized with a synthetic peptide representing the 15 C-terminal amino acids of rabbit 50-DAG conjugated to keyhole limpet hemocyanin (Pierce Chemical Co.) as described (22). The C-terminal antibody was affinity-purified using BSA-conjugated C-terminal peptide (23).

Immunoblot Analysis—Rabbit skeletal muscle crude surface membranes (4) and dystrophin-glycoprotein complex (18) were prepared as previously described. For antibody characterization, crude surface membranes (150 μg of protein/lane), dystrophin-glycoprotein complex (4 μg/lane), fusion proteins (2 μg of protein/lane), and BSA-conjugated peptides (2 μg of protein/lane) were fractionated on 3–12% gradient SDS-polyacrylamide gels (24), transferred to nitrocellulose (25), and stained with antibodies as previously described (4).

Northern Blot Analysis—Total RNA was isolated using RNAzol (Tel-Test, Friendswood, TX) according to the manufacturer's directions and fractionated by electrophoresis through a 1.2% agarose, 3% formaldehyde gel. Following overnight transfer to Biosbrane membranes (BIOS Corp.) by capillary action, the filters were hybridized as described (26) with a ^{32}P-labeled full-length rabbit 50-DAG cDNA (clone 50–2). Filters were washed three times for 30 min at 65 °C in 0.2 × SSC, 1% SDS before autoradiography.

RESULTS AND DISCUSSION

Affinity-purified sheep antibodies against 50-DAG (6) were used to screen a rabbit skeletal muscle cDNA expression library in λgt11. An initial clone (50–1) was found to contain regions of identity with peptide sequences obtained from two proteolytic fragments of 50-DAG. The 50–1 clone was used as a probe for hybridization screening of a λZAPII rabbit skeletal muscle cDNA library to obtain a full-length 50-DAG cDNA clone (50–2). The 1474-base pair 50–2 cDNA contained an open reading frame of 1161 base pairs encoding 387 amino acids (Fig. 1). The assigned initiating residue was the first methionine following the first termination codon upstream of an open reading frame. Nine of ten nucleotides surrounding the first methionine matched those of the vertebrate consensus site for initiation of translation (GCC(A/G)CCATGG) (27). Five regions of homology to 50-DAG peptide sequences were identified in the deduced amino acid sequence (Fig. 1, *underlined* amino acids).

A difference in three nucleotides between clones 50–1 and 50–2 resulted in a difference in residues 358 and 359, which were Ser-Thr in 50–1 but Phe-Pro in 50–2. The residues were also identified as Ser-Thr in a tryptic fragment of rabbit skeletal muscle 50-DAG. Therefore, Ser-Thr is shown in Fig. 1. The difference is likely due to allelic variation, as 50–1 and 50–2 were isolated from different cDNA libraries. Searches performed on all data bases used by the National Center for Biotechnology Information BLAST E-mail server (28, 29) failed to identify any significantly homologous nucleotide or protein sequences, indicating that 50-DAG is a novel protein.

Hydropathy analysis (30) of the deduced amino acid sequence predicted a single membrane-spanning domain and an N-terminal signal sequence, suggesting that the N-terminal 290 amino acids of 50-DAG are extracellular. Cleavage of the signal sequence at the predicted site (31) would yield a mature protein of 370 amino acids and a molecular mass of 40,789. Two consensus sites for *N*-linked glycosylation (32) were identified in the putative extracellular domain, which is consistent with biochemical evidence that 50-DAG is *N*-glycosylated *in vivo* (5). Given that rabbit skeletal muscle 50-DAG is *N*-glycosylated,

Met Ala Ala Ala Ala Leu Leu Trp Leu Pro Leu Leu Val Gly Cys 15
Leu Ala Gly Pro Gly Gly Thr Glu Ala Gln Gln Thr Thr Leu Tyr 30
Pro Leu Val Gly Arg Val Phe Val His Thr Leu Glu Pro Ala Ser 45
Phe Leu His Leu Pro Glu His Phe Phe Leu Ala Thr Ile Pro Val 60
Thr Tyr His Ala His Leu Gln Gly His Pro Asp Leu Pro Arg Trp 75
Leu Arg Tyr Thr Gln Arg Ser Pro His His Pro Gly Phe Leu Tyr 90
Gly Ala Ala Thr Pro Glu Asp Arg Gly Arg Gln Val Ile Glu Val 105
Thr Ala Tyr Asn Arg Asp Ser Phe Asp Thr Ala Gly Gln Ser Leu 120
Val Leu Leu Ile Arg Asp Pro Glu Gly Ser Pro Leu Pro Tyr Gln 135
Thr Glu Phe Leu Val Arg Ser His Asp Val Glu Glu Val Leu Pro 150
Pro Thr Pro Ala Ser His Phe Leu Thr Ala Leu Ala Gly Leu Trp 165
Glu Pro Gly Glu Leu Lys Leu Leu **Asn** Ile Thr Ser Ala Leu Asp 180
Arg Gly Gly Arg Val Pro Leu Pro Ile Gly Gly Gln Lys Glu Gly 195
Val Tyr Ile Lys Val Gly Ser Ala Ser Pro Phe Ser Thr Cys Leu 210
Lys Met Val Ala Ser Pro Asp Ser His Ala Arg Cys Ala Arg Gly 225
Gln Pro Pro Leu Leu Ser Cys Tyr Asp Thr Leu Ala Pro His Phe 240
Arg Val Asp Trp Cys **Asn** Val Ser Leu Val Asp Thr Ser Val Pro 255
Glu Pro Val Asp Glu Val Pro Thr Pro Gly Asp Gly Ile Leu Glu 270
His Asp Pro Phe Phe Cys Pro Pro Thr Glu Ala Thr Ala Arg Asp 285
Phe Leu Ala Asp Ala Leu Val Thr Leu Leu Val Pro Leu Leu Val 300
Ala Leu Leu Leu Ala Leu Leu Leu Ala Tyr Ile Met Cys Cys Arg 315
Arg Glu Gly Arg Leu Lys Arg Asp Leu Ala Thr Ser Asp Ile Gln 330
Met Val His His Cys *Thr* Ile His Glu Asn Thr Glu Glu Leu Arg 345
Gln Met Ala Ala Ser Arg Glu Val Pro Arg Pro Leu Ser Thr Leu 360
Pro Met Phe Asn Val Arg Thr Gly Glu Arg Met Pro Pro Arg Val 375
Asp *Ser* Ala Gln Val Pro Leu Ile Leu Asp Gln His 387

FIG. 1. **Primary structure of rabbit 50-DAG.** Rabbit 50-DAG deduced amino acid sequence is shown in three-letter code. The predicted signal sequence and transmembrane domain are *double-underlined*. The regions of deduced amino acid sequence that match protein sequence obtained from five trypsin-digested fragments of rabbit 50-DAG are *underlined*. Consensus sites for *N*-linked glycosylation (32) are shown in *boldface*, and intracellular consensus sites for phosphorylation by casein kinase II (33) or Ca^{2+}/calmodulin kinase (34) are in *italics*.

the finding that the only *N*-linked glycosylation sites found within the 50-DAG sequence lie N-terminal to the membrane-spanning domain is further evidence that the N terminus must be extracellular.

Five extracellular cysteines were identified in rabbit 50-DAG. Interestingly, the binding of the anti-50-DAG monoclonal antibody $IVD3_1$ on immunoblots requires nonreducing conditions, suggesting that at least one intramolecular disulfide bond is present in the native protein. Disulfide bond formation may be important to this protein's function. Four of the extracellular cysteines are arranged in an intriguing pattern: $CX_{12}CX_9CX_{12}C$ (amino acids 199–245). This pattern was identified in other proteins, such as metallothionein and keratin, that are very cysteine-rich. The pattern was also identified in the epidermal growth factor-like region of nerve growth factor receptor precursor and entactin. It will be interesting to determine in the future whether this region of 50-DAG is involved in receptor activity of some sort or in cell-cell or cell-extracellular matrix interactions. In experiments performed to date, 50-DAG has demonstrated no detectable affinity for several extracellular matrix components *in vitro* (9).

Two consensus sites for phosphorylation were identified intracellularly, one for casein kinase II (33) at Thr^{336} and one for Ca^{2+}/calmodulin kinase (34) at Ser^{377}. It is not known if these sites are utilized *in vivo*. The calculated isoelectric point of rabbit 50-DAG is 5.72, which is in good agreement with recent results of isoelectric focusing experiments of Yamamoto *et al.* (35).

To confirm biochemically that the cloned cDNA encoded 50-DAG, two glutathione *S*-transferase fusion proteins were con-

structed covering the C-terminal half of 50-DAG except the transmembrane domain (Fig. 2*A*). Antibodies against FP-G (fusion protein G) and FP-H were affinity-purified from antisera against 50-DAG or against the dystrophin-glycoprotein complex. The affinity-purified antibodies specifically recognized 50-DAG in both purified dystrophin-glycoprotein complex and in rabbit skeletal muscle crude surface membranes (Fig. 2*B*, *FP-G* and *FP-H*).

Additionally, a synthetic peptide corresponding to residues 354–363 of rabbit 50-DAG (based on clone 50–1 and peptide sequence) was used to affinity purify antibodies from sheep antiserum against the dystrophin-glycoprotein complex. Additionally, a synthetic peptide corresponding to the C-terminal 15 amino acids of rabbit 50-DAG was used to generate antibodies in rabbits. Both anti-peptide antibodies also specifically recognized 50-DAG (Fig. 2*B*, *Peptide* and *C-Term*).

Thus, the cloned cDNA was shown to encode 50-DAG by several independent methods. First, protein sequences obtained from five tryptic fragments of 50-DAG were present in the deduced amino acid sequence (Fig. 1, *underlined* amino acids). Next, antibodies that were affinity-purified using fusion proteins or a synthetic peptide corresponding to internal protein sequence specifically recognized the 50-kDa component of the dystrophin-glycoprotein complex (Fig. 2*B*). Finally, polyclonal antiserum generated against the 15 C-terminal amino

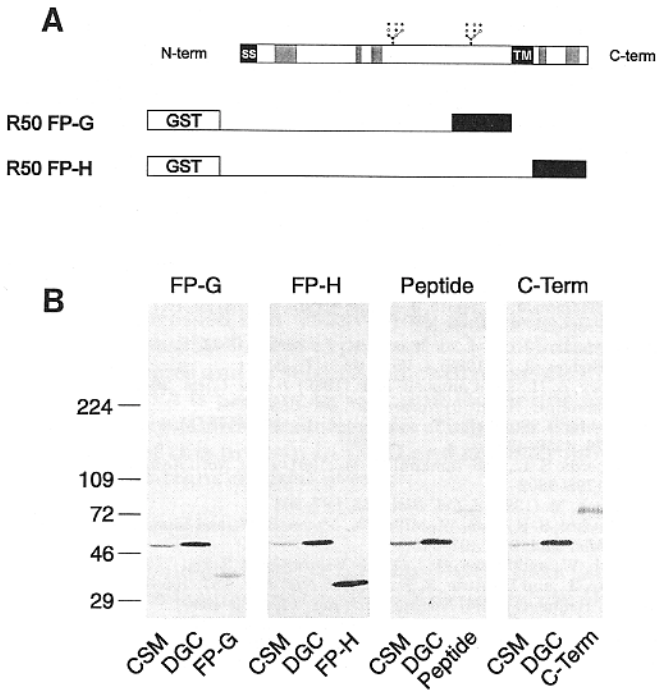

FIG. 2. **Antibodies affinity-purified against rabbit 50-DAG fusion proteins or synthetic peptides specifically recognize the 50-kDa component of the DGC.** *A*, diagram indicating the relative positions of 50-DAG glutathione *S*-transferase (*GST*) fusion proteins, represented by the *black boxes*, to the native protein. The relative positions of the signal sequence (*SS*), transmembrane domain (*TM*), regions matching tryptic peptide sequences (*gray boxes*), and *N*-linked glycosylation sites are indicated. *B*, immunoblots of skeletal muscle crude surface membranes (*CSM*), purified DGC, and glutathione *S*-transferase fusion proteins corresponding to regions of rabbit 50-DAG (*FP-G* and *FP-H*) were stained with antibodies affinity-purified against each of the fusion proteins from anti-50-DAG (FP-G) or anti-DGC (FP-H) polyclonal guinea pig antiserum. For anti-peptide antibodies, immunoblots of crude surface membranes, DGC, and BSA-conjugated peptide (*Peptide* and *C-term*) were stained with polyclonal antibodies affinity-purified against an internal peptide corresponding to amino acids 354–363 or against a peptide corresponding to the C-terminal 15 amino acids of rabbit 50-DAG. Molecular weight standards ($M_r \times 10^{-3}$) are indicated.

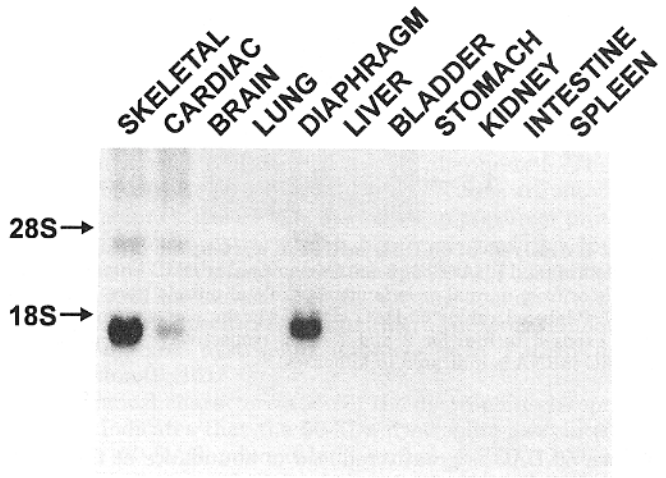

FIG. 3. **Tissue-specific expression of 50-DAG mRNA.** A Northern blot of total RNA (20 μg/lane) from 11 rabbit tissues as labeled (intestine indicates small intestine) was hybridized with ^{32}P-labeled rabbit 50-DAG cDNA. The autoradiograph was exposed for 2 days. The positions of the 28 and 18 S RNA ribosomal subunits are indicated.

acids of deduced amino acid sequence also recognized the 50-kDa component of the dystrophin-glycoprotein complex (Fig. 2*B*). The antibodies used did not cross-react with glutathione *S*-transferase or BSA (data not shown). Thus, the cDNA clone described here encodes rabbit skeletal muscle 50-DAG.

To examine the tissue-specific expression of 50-DAG, Northern blot analysis was performed using total RNA isolated from 11 rabbit tissues (Fig. 3). Expression of 50-DAG mRNA was highest in skeletal muscle, diaphragm, and cardiac muscle. Lower amounts of 50-DAG transcripts were detected in bladder and small intestine, indicating that 50-DAG may be expressed in smooth muscle cells in these tissues. However, immunohistochemistry or *in situ* hybridization will be required to precisely identify the cell type of origin. The muscle-specific expression of 50-DAG mRNA is consistent with 50-DAG protein expression as determined by immunoblot analysis.[2] 50-DAG cDNA hybridized primarily to a transcript of approximately 1.5 kilobases. However, larger transcripts of approximately 3.5 and 7 kilobases were also detected, suggesting that incompletely processed transcripts were present or that alternative splice sites were used.

The tissue-specific distribution of 50-DAG mRNA contrasts strikingly with that of dystroglycan mRNA (36), which encodes the 43- and 156-kDa components of the dystrophin-glycoprotein complex. It is interesting that β-dystroglycan also has a single transmembrane domain, *N*-linked glycosylation sites, and a relatively short intracellular domain. It is possible that the structural similarities between the 43- and 50-kDa dystrophin-associated glycoproteins indicate that they may share one or more functional features. However, this remains to be tested experimentally.

Dystroglycan, which binds to laminin (7, 9), may play a role in linking the extracellular matrix to the subsarcolemmal cytoskeleton via dystrophin, dystrophin-related protein, and dystrophin isoforms in a wide variety of muscle and non-muscle tissues. In contrast, 50-DAG must apparently play a more muscle-specific role. This is consistent with the observation that in severe childhood autosomal recessive muscular dystrophy and in the cardiomyopathic hamster, in which 50-DAG is deficient (16), only skeletal and cardiac muscles are severely affected (37, 38).

Due to the absence of dystrophin in DMD and the *mdx*

[2] J. M. Ervasti and K. P. Campbell, unpublished data.

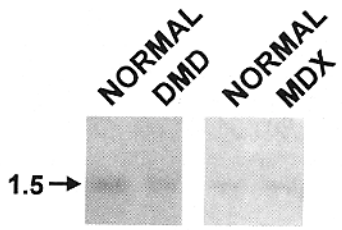

FIG. 4. **Analysis of 50-DAG mRNA in dystrophic muscle.** Northern blots of total RNA (20 μg/lane) from normal or DMD human skeletal muscle or from normal or *mdx* mouse skeletal muscle were hybridized with ^{32}P-labeled rabbit 50-DAG cDNA. The human and mouse blots were exposed to film for 2 and 7 days, respectively. The position of 50-DAG mRNA is indicated in kilobases.

mouse, 50-DAG is greatly reduced in abundance at the sarcolemma (6, 13, 15). This reduction is also observed when using the anti-FP-H or anti-C-terminal antibodies described in this report (data not shown). To determine if down-regulation of 50-DAG is due to alterations in 50-DAG mRNA levels, Northern blot analysis was performed on total RNA isolated from normal and DMD human skeletal muscle and normal and *mdx* mouse skeletal muscle (Fig. 4). A slight decrease in 50-DAG mRNA was observed in DMD muscle, and no change was detected in *mdx* muscle. Thus, the 80–90% reduction of 50-DAG in DMD (13) and *mdx* (6) muscle is likely a post-translational event.

50-DAG is undetectable in skeletal muscle of many patients having SCARMD (15). It remains to be determined whether a mutation in the 50-DAG gene may be responsible for the absence of 50-DAG in these patients and how the absence of 50-DAG may cause the disease. The disease in some families having SCARMD has been linked to chromosome 13q12 (39, 40), and three of these families demonstrate a deficiency of 50-DAG (40). However, this locus has been excluded from linkage in other families with 50-DAG deficiency (41). Therefore, it is clear that phenotypically similar forms of SCARMD characterized by 50-DAG deficiency can be caused by mutations in at least two different genes. We hypothesize that, in patients with SCARMD, the deficiency of 50-DAG (whether due to a mutation in the 50-DAG gene or another gene) leads to a disruption of the dystrophin-glycoprotein complex, causing a decrease in the integrity of the link between the extracellular matrix via dystroglycan and the membrane cytoskeleton via dystrophin. We have previously demonstrated that the dystrophin-glycoprotein complex is disrupted in the skeletal muscle of the BIO 14.6 strain of cardiomyopathic hamsters, which are also specifically deficient in 50-DAG (16).

The present work, taken together with biochemical data (5), indicates that 50-DAG is a muscle-specific glycoprotein having a single transmembrane domain. 50-DAG shares a four-cysteine motif with entactin and nerve growth factor receptor, suggesting that this protein may have receptor activity of some sort or be involved in cell-cell or cell-extracellular matrix interactions. This protein plays an important role in maintaining the functional conformation of the DGC as demonstrated by the absence of 50-DAG leading to the disruption of the dystrophin-glycoprotein complex in the cardiomyopathic hamster (16) and SCARMD patients (15). Because the 50-kDa dystrophin-associated glycoprotein was first implicated in the pathogenesis of SCARMD, which is prevalent in Arabic countries, we propose that 50-DAG be named "adhalin," derived from the Arabic word "adhal" for muscle.

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