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Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix

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The primary sequence of two components of the dystrophin–glycoprotein complex has been established by complementary DNA cloning. The transmembrane 43K and extracellular 156K dystrophin-associated glycoproteins (DAGs) are encoded by a single messenger RNA and the extracellular 156K DAG binds laminin. Thus, the 156K DAG is a new laminin-binding glycoprotein which may provide a linkage between the sarcolemma and extracellular matrix. These results support the hypothesis that the dramatic reduction in the 156K DAG in Duchenne muscular dystrophy leads to a loss of a linkage between the sarcolemma and extracellular matrix and that this may render muscle fibres more susceptible to necrosis.

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THE Duchenne muscular dystrophy (DMD) gene encodes dystrophin, a large membrane cytoskeletal protein found in skeletal muscle[1,2]. Dystrophin is localized to the inner surface of the sarcolemma in normal skeletal muscle but is absent in skeletal muscle of DMD patients, mdx mice and xmd dogs[3–6]. Biochemical studies have demonstrated that dystrophin is tightly linked to a large oligomeric complex of sarcolemma glyco-

proteins[7,8]. A dystrophin-glycoprotein complex (DGC) has been isolated and shown to consist of cytoskeletal (relative molecular mass 59,000 (M_r 59K) and dystrophin), transmembrane (50K, 43K, 35K and 25K) and extracellular (156K) components[9]. A model for the organization of the DGC has also recently been proposed[9]. The membrane organization of the dystrophin-glycoprotein complex[9] and the high density of dystrophin in the sarcolemma membrane[10,11] suggest that this complex could have an important structural role in skeletal muscle, but its exact function remains unknown. Furthermore, the absence of dystrophin in skeletal muscle from mdx mice and DMD patients leads to a dramatic loss of all of the components of the dystrophin-glycoprotein complex[8,12]. Because abnormal expression of the dystrophin-associated glycoproteins (DAGs) may have a crucial role in molecular pathogenesis in DMD, it is important to identify the normal function of the dystrophin-glycoprotein complex in skeletal muscle.

We report here the complete amino-acid sequence of 43K and 156K dystrophin-associated glycoproteins deduced from the cDNA sequence. Both components of the dystrophin-glycoprotein complex are encoded by the same 5.8-kilobase (kb) mRNA. Post-translational modification of a 97K precursor protein results in two mature proteins: 43K DAG and 156K DAG. Sequence analysis of cDNAs reveals an open reading frame encoding a precursor polypeptide with no significant sequence similarity with any known proteins. The N-terminal portion of the precursor polypeptide is processed into the mature 156K DAG with a putative protein core of about 56K with potential attachment sites for *O*-linked carbohydrates. The C-terminal portion of the precursor polypeptide is processed into the mature 43K DAG with potential *N*-glycosylation sites, a single transmembrane domain and a 120 amino-acid long cytoplasmic tail. Northern and western blot analyses have demonstrated that the 43K DAG and 156K DAG are expressed in both muscle and non-muscle tissues. The specific mRNA for the 43-156K DAG is expressed at normal amounts in mdx and DMD skeletal muscle, whereas both glycoproteins are greatly reduced in dystrophin-deficient muscle. The dramatic decrease in the 43K DAG occurs in the absence of dystrophin in muscle but not in other tissues of mdx mice. Finally, we show that the 156K DAG binds laminin, a well characterized component of the extracellular matrix. Thus, our results demonstrate that the 156K DAG is a novel laminin-binding glycoprotein and suggest that the function of the dystrophin-glycoprotein complex is to link the subsarcolemmal cytoskeleton to the extracellular matrix. We propose to name the 43-156K dystrophin-associated glycoprotein 'dystroglycan' because of its identification through dystrophin and its extensive glycosylation.

Cloning and sequence analysis

A λgt11 cDNA expression library from rabbit skeletal muscle was screened with affinity-purified guinea pig polyclonal antibodies specific for the 43K DAG. One cDNA clone with a 0.6-kb insert designated R43-A was found and its sequence revealed one open reading frame (Fig. 1*a*,*b*). Overlapping clones covering the entire coding region of the mRNA were isolated by rescreening of rabbit skeletal muscle cDNA libraries using radiolabelled cDNA probes (Fig. 1*a*).

The 4,200-nucleotide cDNA sequence contains a 2,685 nucleotide open reading frame coding for a polypeptide of 895 amino acids with a calculated M_r of 97,029 (Fig. 1*b*). The first 29 amino acids of the open reading frame are predominantly hydrophobic and probably represent a signal peptide (Fig. 1*b*,*c*). Hydropathy analysis identified a single continuous region of 24 amino acids close to the C terminal showing characteristics of a transmembrane domain (Fig. 1*c*). Four potential *N*-linked glycosylation sites and numerous potential phosphorylation sites are found in the 97K polypeptide. No significant sequence homology was detected in the NBRF database between any known proteins and the predicted amino-acid sequence of the 97K polypeptide.

Biochemical characterization of the dystrophin-glycoprotein complex has demonstrated that the 43K DAG has hydrophobic properties characteristic of a transmembrane protein and contains Asn-linked oligosaccharides[9]. These properties are consistent with the predicted sequence of the C-terminal half of the 97K polypeptide which contains a potential transmembrane domain and three out of four potential sites for *N*-glycosylation. The C-terminal origin of 43K DAG was confirmed using an antibody raised in a rabbit against a synthetic peptide corresponding to the 15 C-terminal amino-acid residues of the deduced sequence. This anti-peptide antibody specifically recognized the 43K DAG (Fig. 2*b*). In addition, peptide sequence determined directly from the 43K DAG matched residues 783-793 of the deduced amino-acid sequence of the 97K polypeptide (Fig. 1*b*).

N-terminal domain of the 97K precursor

To identify the N-terminal domain of the 97K precursor polypeptide, antibodies to different regions of the 97K precursor polypeptide were produced by expressing several overlapping cDNAs encoding different regions in the 97K precursor polypeptide. The pGEX vectors for the expression of foreign sequences as glutathione S-transferase (GST) fusion proteins in *Escherichia coli* cells were used to examine specific regions in the 97K precursor protein, corresponding to the 43K DAG, the N-terminal half of the 97K precursor and to sequence overlapping both the N- and C-terminal halves (Fig. 2*a*).

Monospecific antibodies to each fusion protein were affinity purified from the sheep antiserum raised against purified DGC using immobilized fusion proteins. Affinity-purified antibodies were then tested using each fusion protein and purified DGC (Fig. 2*b*). Consistent with the C-terminal domain encoding the 43K DAG, antibodies to FP-A (fusion protein-A) and FP-C specifically stained both bands of 43K DAG doublet (Fig. 2*b*). But antibodies to FP-B stained the 43K DAG and the 156K DAG components of DGC (Fig. 2*b*). Thus, a second product of 97K precursor polypeptide seems to be the 156K DAG. In accordance with this supposition, antibodies to FP-D stain only 156K DAG (Fig. 2*b*). Therefore, post-translational processing of 97K precursor polypeptide gives rise to two components of DGC: 43K DAG and 156K DAG. Biochemical studies have demonstrated that 156K DAG is not an integral membrane protein and contains *N*-linked and *O*-linked glycosylation[9]. These properties are consistent with the predicted N-terminal half of the 97K precursor which does not possess any hydrophobic region, has one potential *N*-linked glycosylation site and many potential *O*-glycosylation sites. Although generation of the 43K and 156K DAG by proteolysis during the preparation of muscle membranes cannot be ruled out, we have never seen a larger protein react with antibodies to the 43K DAG or 156K DAG and the 156K DAG is seen in direct SDS extracts of muscle[8]. Further analysis of 43-156K-specific mRNA translation is needed to show that the cleavage of the 97K precursor occurs during its synthesis and is not occurring during the isolation procedure. The precise point of cleavage in 97K precursor protein used in the formation of the 43K DAG has not been determined because the N terminal of 43K DAG was blocked and thus could not be sequenced. Among the putative cleavage sites, Arg 457 is the best fitting according to consensus sequence for post-translational processing of protein precursors at monobasic sites[13].

Expression of 43–156K DAG

A prominent 5.8-kb transcript was detected in mRNA from rabbit skeletal muscle, cardiac muscle and lung (Fig. 3*a*). A weaker hybridizing transcript of the same size was found in brain (Fig. 3*a*). Northern blot analysis with total RNA from liver, kidney, diaphragm and stomach also detected a 5.8-kb mRNA in all these tissues (data not shown). Thus, the 5.8-kb

transcript for the 43-156K DAG is present in various muscle and non-muscle tissues, most probably originating from the same gene. The 43-156K DAG in muscle and non-muscle tissues was identified using immunoblots of membranes from different tissues and affinity-purified antibodies to FP-B (43-156K specific) (Fig. 3*b*). The 43K DAG was detected in isolated membranes from skeletal muscle, brain, cardiac muscle and lung (Fig. 3*b*). The 156K DAG was detected in skeletal and cardiac muscle membranes, but has a slightly lower M_r in cardiac membranes (Fig. 3*b*). In brain and lung membranes the M_r of the '156K' DAG reactive protein was ~120K. The variability in M_r for the '156K' reactive protein may be due to differential glycosylation of the core protein in muscle versus non-muscle tissues.

Analysis of dystrophic muscle

RNA blot analysis of skeletal muscle mRNA from control and mdx mice of different ages using cDNA probe R43-B to 43-156K mRNA revealed no reduction of 43-156K DAG mRNA in mdx mice versus control mice (Fig. 4*a*). But as previously observed[8,12], the 43K DAG is greatly reduced in mdx skeletal muscle membranes (Fig. 4*b*). Thus, the absence of dystrophin causes no change in the mRNA for the 43-156K DAG but leads to dramatic reductions in the amount of the 43K DAG and 156K DAG in skeletal muscle. Analysis of mRNA from control and DMD skeletal muscle also showed no difference in 43-156K DAG mRNA expression (Fig. 5*a*). In agreement with findings in mdx mouse muscle, indirect immunofluorescence analysis of cryosections from normal and DMD skeletal muscle with 156K-specific (anti FP-D) and 43K-specific (anti FP-A) antibodies demonstrated a drastically reduced density of 43K DAG and 156K DAG in skeletal muscle of a DMD patient (Fig. 5*b*). Thus the 43-156K DAG-encoding gene is transcribed and specific mRNA is still present at the normal amount in dystrophic muscle, but the 43K DAG and 156K DAG are greatly reduced in dystrophic muscle.

Because the 43-156K DAG is expressed in non-muscle tissues we also examined expression of 43K DAG in the non-muscle tissues of control and mdx mice. The 156K DAG could not be tested because polyclonal antibodies to the protein core of rabbit 156K DAG described above do not crossreact with the 156K DAG in mouse muscle. Immunoblot analysis of brain and kidney membranes from control and mdx mice, stained with polyclonal anti FP-A antibodies (43K specific), revealed no reduction in the amount of 43K DAG in these mdx tissues (Fig. 4*b*). Thus, the dramatic reduction of the 43K DAG that is found in mdx mice seems to be restricted to skeletal muscle and is not found in non-muscle tissues.

156K DAG binds laminin

The high density of dystrophin in isolated sarcolemma and the membrane organization of the DGC have suggested a structural function for the DGC[9,11]. That the 156K DAG is an extracellular component of the dystrophin-glycoprotein complex suggests it may interact with the extracellular matrix. To test for the association of the 156K DAG with the extracellular matrix, rabbit skeletal muscle surface membranes and pure DGC were electrophoretically separated, transferred to nitrocellulose membranes and overlaid with ^{125}I-labelled laminin. A single laminin-binding band, corresponding to the 156K DAG, was detected in surface membranes and purified DGC (Fig. 6*a*). Binding of ^{125}I-labelled laminin to the 156K DAG was significantly decreased by a 1,000-fold excess of unlabelled laminin demonstrating the specificity of ^{125}I-labelled laminin binding to the 156K DAG (Fig. 6*a*). ^{125}I-Labelled fibronectin did not label the 156K DAG or any other component of the DGC, nor did a 1,000-fold excess of non-radioactive fibronectin have any effect on the binding of ^{125}I-labelled laminin to the 156K DAG (data not shown). The interaction of 156K DAG with laminin was also shown by coimmunoprecipitation of laminin and 156K DAG (Fig. 6*b*). Anti-laminin antibodies did not precipitate the 156K DAG from alkaline extracts of rabbit skeletal muscle surface membranes (Fig. 6*b*, lane 3). This result was consistent with our observation (not shown) that the surface membranes used were devoid of laminin, merosin[14] or S-laminin[15] as detected on immunoblots using specific antibodies[16]. But anti-laminin antibodies effectively precipitated the 156K DAG from alkaline extracts that had been preincubated with exogenously added laminin (Fig. 6*b*, lane 7). These results suggest that the 156K DAG specifically binds laminin and may mediate interaction of DGC with extracellular matrix.

Discussion

We report here the nucleotide and deduced amino-acid sequences for two components of the dystrophin-glycoprotein complex. We show that the 43K DAG and 156K DAG are encoded by the same mRNA and that post-translational processing of the 97K precursor polypeptide results in two mature proteins: 43K DAG and 156K DAG. The mature 43K DAG contains a single transmembrane domain, three potential sites for *N*-glycosylation and a 120 amino-acid long cytoplasmic tail whereas the 156K DAG contains no transmembrane sequences,

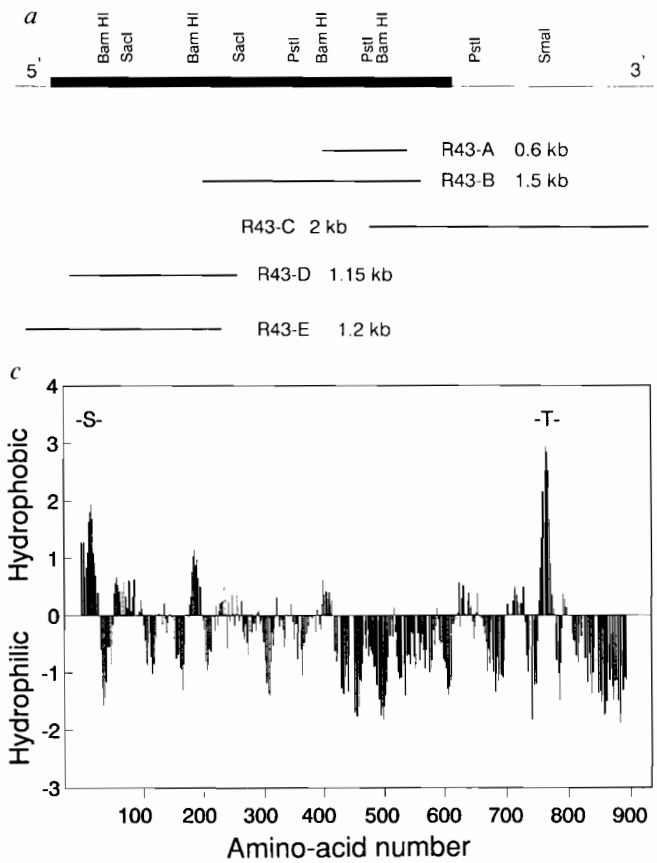
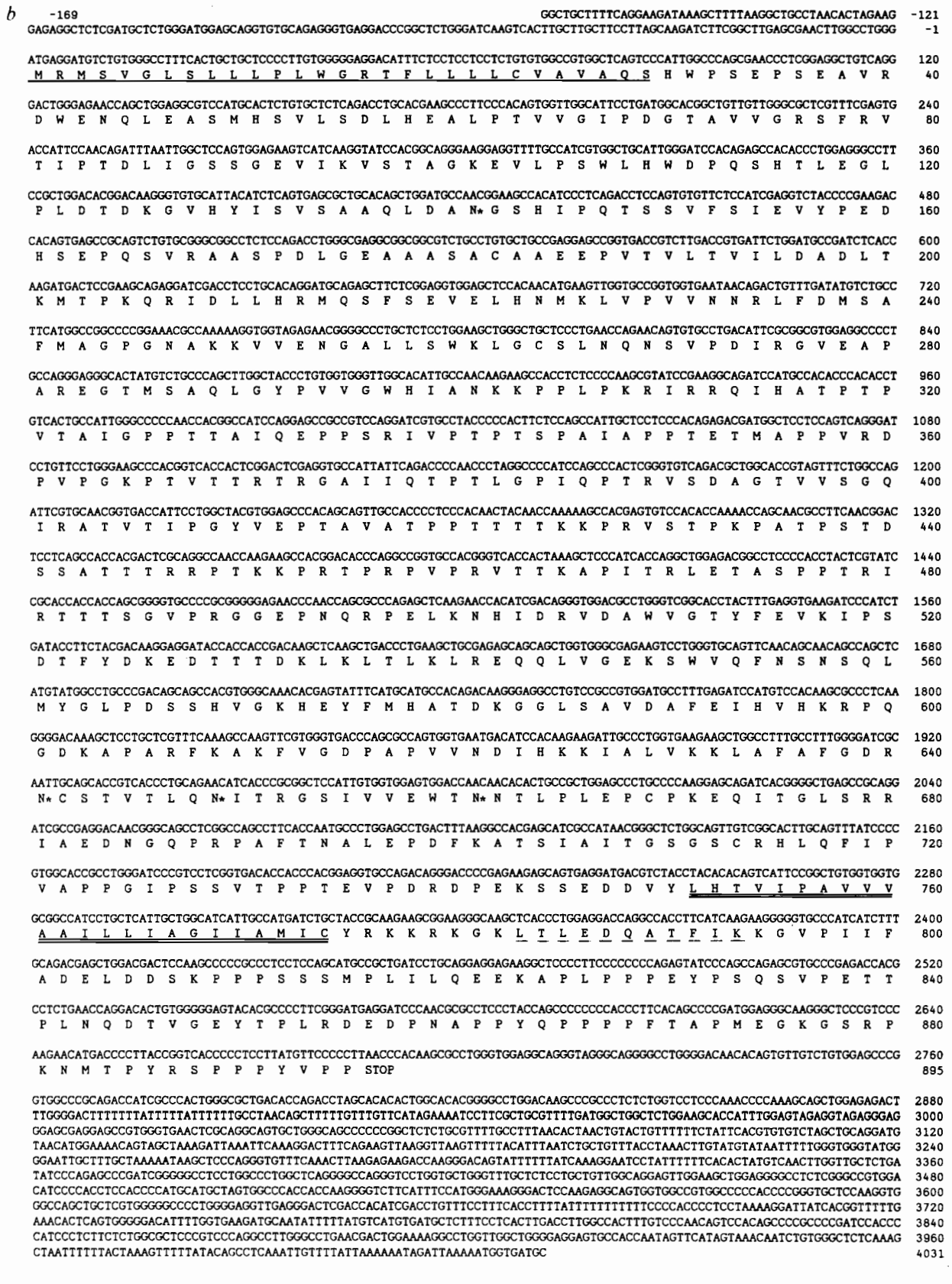


FIG. 1 Cloning and sequence analysis of cDNAs encoding a precursor for the 43-156K DAG. *a*, Restriction map and overlapping cDNA clones encoding the precursor protein for the 43-156K DAG. The protein-coding region is indicated by a solid box. *b*, The nucleotide and predicted amino-acid sequence (single-letter code) of precursor protein for the 43-156K DAG. The putative signal peptide is underlined and the predicted transmembrane domain is double underlined. A sequence matching that of a peptide isolated from the mature 43K DAG is indicated by a dashed line. *, Potential asparagine-linked glycosylation sites. *c*, Hydropathy plot of the 43-156K DAG precursor protein. Hydropathy profile of the 43-156K DAG precursor protein computed according to ref. 29; the window size is 19 residues plotted at one-residue intervals. T, Position for the predicted transmembrane domain; S, proposed signal sequence.

METHODS. Affinity-purified guinea pig polyclonal antibodies to the 43K DAG were prepared as previously described[9] and used to screen 2×10^6 clones ►



of λgt11 expression library. Clone R43-A with a length of 600 base pairs (bp) was isolated from a random primed adult rabbit skeletal muscle λgt11 library[28] by immunoscreening. An oligo-dT-primed rabbit skeletal muscle cDNA library in λzapII (Stratagene) was screened at high stringency with a ^{32}P-labelled cDNA insert from the R43-A clone (random primed labelling kit, Boehringer Mannheim). Clone R43-B overlaps R43-A and extends ~1 kb in the 5′ direction. Further clones were isolated from λgtII libraries[30]: R43-D, random-primed λgt11 library; R43-C, oligo-dT-primed λgt11 library. To isolate cDNA extending to the 5′-end of mRNA (clone R43-E), a rabbit skeletal muscle cDNA library was constructed using random primed cDNA with λgt11 vector (Stratagene). All cDNA inserts were sequenced either on an Applied Biosystems Incorporated Automatic Sequencer or manually by the dideoxy chain termination method[31]. Sequences were analysed with the Genetics Computer Group (Wisconsin package) and PCGene (Intelligenetics) software.

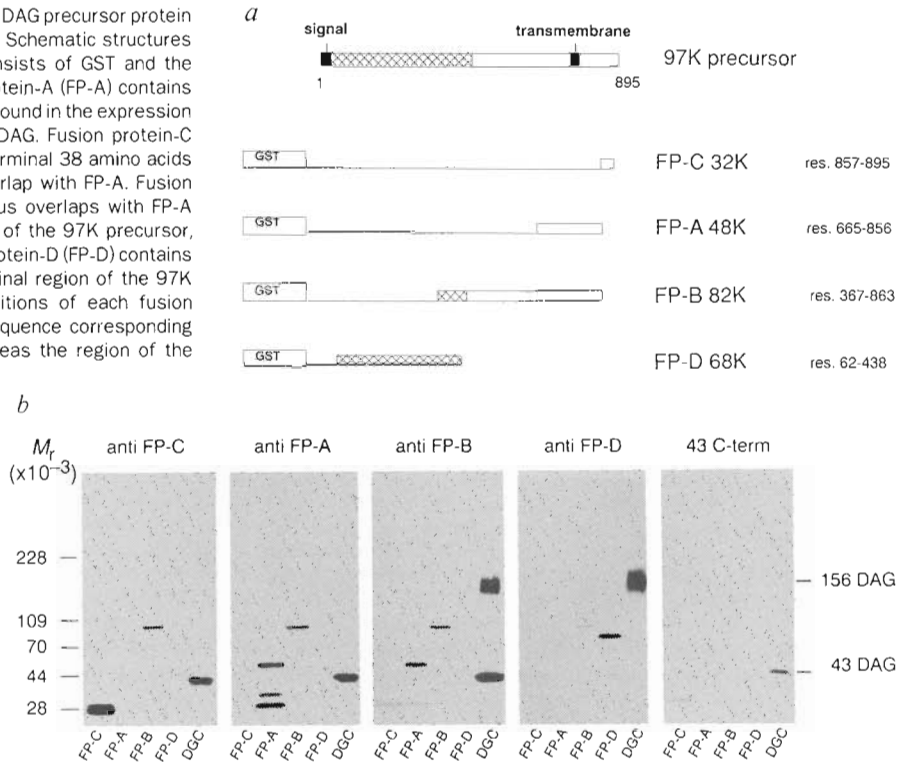


FIG. 2 Expression of different regions of the 43–156K DAG precursor protein as glutathione S-transferase (GST) fusion proteins. *a*, Schematic structures of the GST fusion proteins. Each fusion protein consists of GST and the indicated region of 97K precursor protein. Fusion protein-A (FP-A) contains residues 665–856 corresponding to the cDNA R43-A found in the expression library with affinity-purified antiserum against 43K DAG. Fusion protein-C (FP-C) contains residues 857–895 which are the C-terminal 38 amino acids of the 97K precursor polypeptide, and does not overlap with FP-A. Fusion protein-B (FP-B) contains residues 367–863 and thus overlaps with FP-A and FP-C, and has a portion of the N-terminal region of the 97K precursor, which is not present in the mature 43K DAG. Fusion protein-D (FP-D) contains residues 62–438 and thus contains only the N-terminal region of the 97K precursor polypeptide. The M_r and amino-acid positions of each fusion protein are indicated. The 97K protein precursor sequence corresponding to the 156K DAG is shown as a hatched box whereas the region of the 43K DAG is shown as an open box. Predicted transmembrane and signal sequences are shown as a solid box. *b*, Immunoblot analysis of fusion proteins and the dystrophin–glycoprotein complex (DGC). Samples of partially purified fusion proteins were separated on 3–12% SDS polyacrylamide gels and transferred to nitrocellulose. Nitrocellulose transfers were stained with affinity-purified sheep polyclonal antibodies to the fusion protein constructs: FP-C (anti FP-C), FP-A (anti FP-A), FP-B (anti FP-B), FP-D (anti FP-D) or with a polyclonal rabbit antibody to the synthetic peptide representing the 15 amino acids of the carboxyl terminus of the 97K precursor protein (43 C-term). The M_r standards (×10^{-3}) are indicated on the left and the mature 43K and 156K components of DGC are indicated on the right (43 DAG, 156 DAG).

METHODS. A set of pGEX vectors[32] were used to express various fragments of DNA for the 97K precursor protein as *E. coli* fusion proteins. The correct construction of the recombinant plasmids was verified by restriction mapping. To construct FP-A, the *Eco*RI insert from R43-A clone with the size of 0.6 kb was cloned into the *Eco*RI site of pGEX-1. FP-B was constructed by ligation of the *Eco*RI insert from R43-R clone (1.5 kb) into the *Eco*RI site of pGEX-2T. FP-C was made by ligation into the *Bam*HI site of pGEX-1 the *Bam*HI fragment of cDNA R43-C, containing C-terminal sequence with stop codon, representing the last 38 amino acids. For the FP-D construct, *Eco*RI insert (1.2 kb) from R43-D was inserted into pGEX-2T vector digested with *Eco*RI. Each recombinant molecule was introduced in *E. coli* DH5$_\alpha$ cells. Overnight cultures were diluted 1:10, incubated for 1 h and induced for 2 h with 1 mM IPTG. Cells were resuspended in PBS and sonicated. Fusion proteins were purified from supernatant by affinity chromatography on glutathione-Sepharose (Pharmacia) and eluted with 5 mM glutathione. Dystrophin–glycoprotein complex was isolated as previously described[9]. Sheep polyclonal antibodies to the purified DGC were produced as previously described[12] and anti-fusion protein antibodies were affinity purified from polyclonal antiserum as previously described[9]. A peptide representing the 15 C-terminus amino acids of the 97K cDNA (PKNMTPYRSPPPYVP) was obtained from the HHMI Peptide Facility (Washington University, St. Louis) as the N-terminal *p*-benzolbenzoyl-peptide photoprobe[33]. Peptide was conjugated to keyhole limpet haemocyanin, mixed with Freund's complete adjuvant and injected into a rabbit as described[34]. SDS–PAGE[35] was carried out on 3–12% gradient gels in the presence of 1% 2-mercaptoethanol and transferred to nitrocellulose for immunoblot analysis as previously described[36].

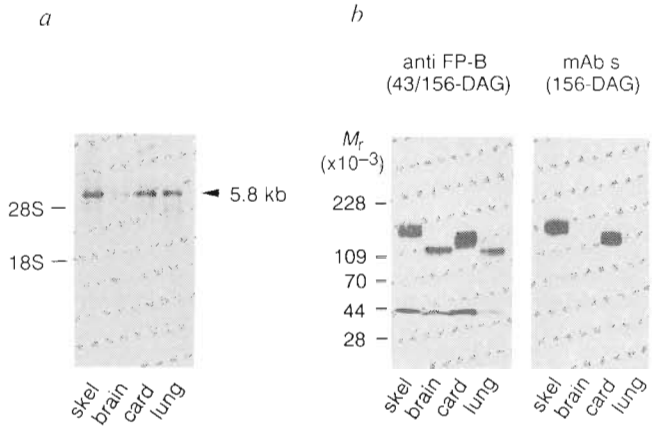


FIG. 3 Tissue distribution of 43–156K DAG. *a*, Northern blot analysis is with 43–156K DAG-specific probe of mRNA from different tissues. Poly(A)$^+$ RNA (4 μg per lane) from rabbit skeletal muscle (skel), brain, cardiac muscle (card) and lung was transferred to nylon filters and hybridized with a ^{32}P-labelled insert from R43-A cDNA clone. The 43–156K DAG-specific mRNA with the size of 5.8 kb is indicated by the arrow. *b*, Immunostaining of identical nitrocellulose transfers of membranes from rabbit skeletal muscle, brain, cardiac muscle and lung stained with anti FP-B antibodies (43–156K specific) and a mixture of monoclonal antibodies VIA4$_1$ and IIH6 (156K DAG specific). M_r standards (×10^{-3}) are indicated on the left.

METHODS. Total RNA was isolated by homogenization in RNAzol (Cinna/Biotecx) followed by chloroform extraction. Poly(A)$^+$ RNA was enriched by oligo-dT cellulose chromatography and resolved on 1.2% agarose gels containing 5% formaldehyde. RNA was transferred to Genescreen Nylon Membranes (NEN Research Products). Prehybridization was done at 42 °C in 5 × SSC, 5 × Denhardt's solution, 50% formamide, 10% dextran sulphate and 100 μg ml^{-1} of salmon-sperm DNA. Membranes were hybridized overnight at 42 °C at a specific activity of at least 1 × 10^6 c.p.m. ml^{-1}. Membranes were washed at 62 °C in 2 × SSC, 0.1% SDS and were exposed to film (X-OMAT AR, Kodak) at −80 °C. Total membranes were prepared from tissues homogenized in 7.5 vols of homogenization buffer (20 mM sodium pyrophosphate, 20 mM sodium phosphate monohydrate, 1 mM $MgCl_2$, 0.3 M sucrose, 0.5 mM EDTA, pH 7.0) using a Polytron PTS-10-S probe (Kinematic GmbH, Luzern) in the presence of a protease inhibitor cocktail[10]. Homogenates were centrifuged for 15 min at 1,100*g* and the supernatant filtered through cheesecloth. The supernatants of four repeated homogenizations were combined and centrifuged for 35 min at 140,000*g*. The final membrane preparations were KCl-washed as previously described[10]. Immunoblot analysis was done as described in the legend to Fig. 2 using 250 μg of skeletal muscle membranes and 500 μg of non-muscle membranes.

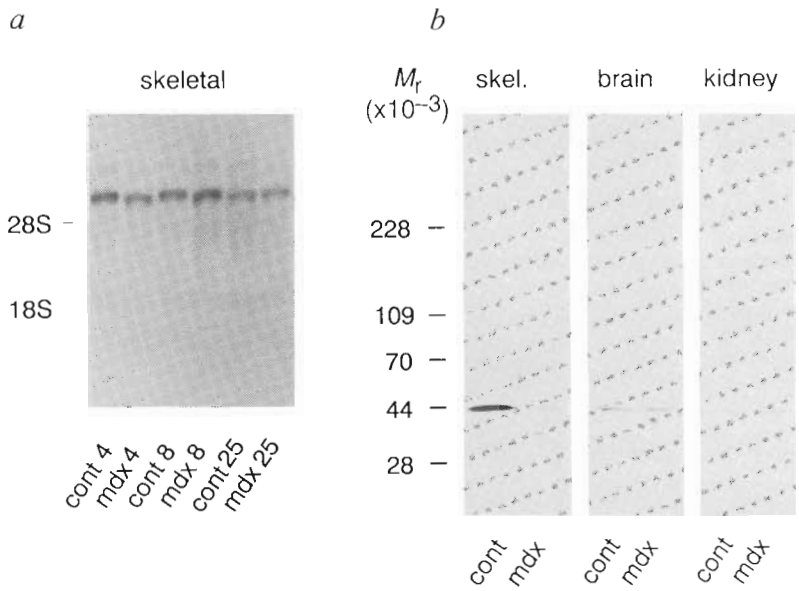


FIG. 4 Comparison of 43-156K DAG expression in muscle and non-muscle tissues of normal and mdx mice. *a*, Skeletal muscle RNA (20 μg per lane) of control mice of ages 4, 8 and 25 weeks (cont 4, cont 8, cont 25) and skeletal muscle RNA of mdx mice of ages 4, 8 and 25 weeks (mdx 4, mdx 8, mdx 25) hybridized with ^{32}P-labelled cDNA inset from R43-B clone. Immunoblot analyses were done as described in the legend to Fig. 3. *b*, Nitrocellulose transfers of skeletal muscle, brain and kidney membranes from control (cont) and mdx mice (mdx) were stained with affinity-purified anti-FP-A (43K DAG specific) antibodies. M_r standards are indicated. Total RNA and membranes from control and mdx mice were prepared as described in Fig. 3.

one site of *N*-linked glycosylation and numerous *O*-linked glycosylation sites. The deduced amino-acid sequence of the N-terminal portion of the precursor protein indicates a ~56K core peptide for 156K DAG. Thus, carbohydrate moieties seem to constitute up to two-thirds of the M_r of the 156K DAG which suggests that the 156K DAG may be a proteoglycan. The exact modifications involved in the processing of the N-terminal portion of the precursor polypeptide to the 156K DAG are not known, but because of the size difference between the 56K protein core and the mature 156K DAG they could involve the addition of glycosaminoglycan chains[17,18].

The predicted features of 156K DAG made it the most probable candidate for interaction with components in the extracellular space. We have demonstrated, using two different techniques, that extracellular 156K DAG binds laminin which is a major component of the extracellular matrix. A number of laminin-binding proteins have been previously identified in skeletal muscle[19–21] but the 156K DAG does not seem to be related to any of these proteins. In addition, the sequence of the 43–156K DAG indicates that it is not related to integrins or cadherin. The exact function of laminin binding by the 156K DAG is not known. It is possible that the 156K DAG is involved in the attachment, spreading and growth of myoblasts on laminin substrate, which are independent of integrin[22].

The broad tissue distribution of the 43–156K DAG precursor argues for an important role of the glycoproteins in membrane organization in different tissues and might indicate the existence of the entire glycoprotein complex in non-muscle tissues. Absence of significant amounts of dystrophin in the examined non-muscle tissues suggests that in non-muscle tissues 43–156K DAG is involved in formation of a different type of complex where dystrophin may be replaced by another cytoskeleton component. The conditions used to identify the 156K DAG as a laminin-binding protein are similar to those used for the identification of cranin as a laminin-binding protein[23]. The apparent M_r of cranin is also very similar to the protein we have identified in brain membranes with the '156K'-specific antibody.

How the deficiency of dystrophin causes muscle cell necrosis and ultimately leads to muscle weakness in DMD is not known. Our findings suggest that the function of dystrophin is to link the subsarcolemma membrane cytoskeleton through a transmembrane complex to an extracellular glycoprotein which binds laminin. Because the absence of dystrophin leads to the loss of all the dystrophin-associated proteins[8,12] our results suggest that dystrophin-deficient muscle fibres may lack the normal interaction between the sarcolemma and the extracellular matrix. The disruption of this linkage between the sarcolemma and the extracellular matrix may be responsible for the increased osmotic fragility of dystrophic muscle[24] or the alteration of specific Ca^{2+} regulatory mechanisms[25] either of which may lead to excessive influx of Ca^{2+} ions in dystrophic muscle[26]. The histopathological analysis of DMD muscle[27,28] supports this hypothesis because a breakdown of the sarcolemma membrane

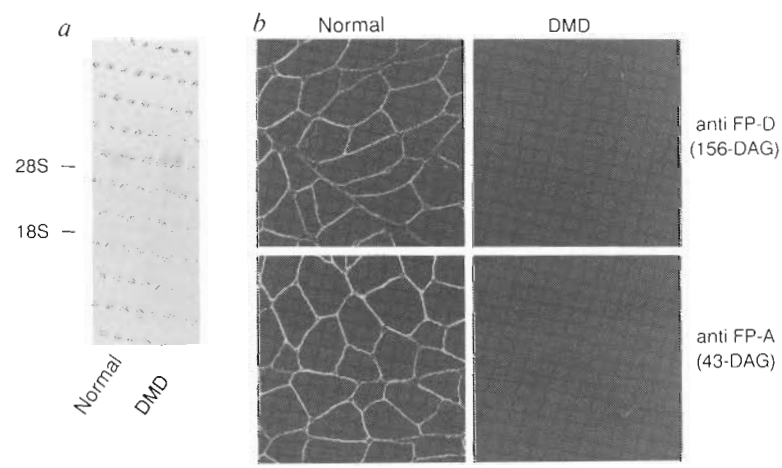


FIG. 5 Characterization of 43–156K DAG in normal and DMD skeletal muscle. *a*, Northern blot analysis of total RNA from normal and DMD skeletal muscle probed with R43-B cDNA. Northern blot analysis with human normal and DMD skeletal muscle RNA was done as described in Fig. 3. *b*, Immunofluorescence analysis of transverse cryosections of normal and DMD skeletal muscle stained with affinity-purified sequence-specific antibodies against FP-D (156K DAG specific) or FP-A (43K DAG specific). Immunofluorescence microscopy of transverse cryosections from normal and DMD muscle was done as previously described[10,12].

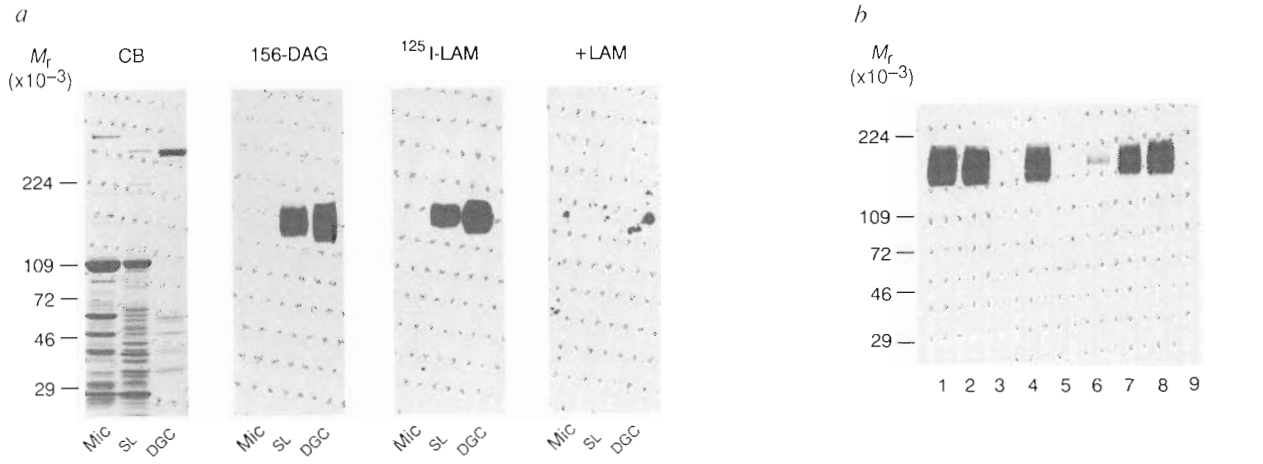


FIG. 6 Laminin binding to the 156K DAG. *a*, Coomassie blue-stained SDS polyacrylamide gel (CB), corresponding immunoblot stained with monoclonal antibody IIH6 to 156K DAG or autoradiograms of identical nitrocellulose transfers of electrophoretically separated proteins of crude rabbit skeletal muscle membranes (Mic), sarcolemma membranes (SL) or dystrophin-glycoprotein complex (DGC) incubated with 0.1 μg ml^{-1} ^{125}I-labelled laminin (~1.7 μCi μg^{-1}) in the absence (^{125}I-LAM) or presence of a 1,000-fold excess of unlabelled laminin (+LAM). *b*, Coimmunoprecipitation of 156K DAG using anti-laminin antibody. Shown is the relative amount of 156K DAG in pH 12 extracts of crude rabbit surface membranes (lane 1), in anti-laminin/protein A-Sepharose voids (lanes 2 and 6), associated with anti-laminin/Protein A-Sepharose (lanes 3 and 7), protein A-Sepharose voids (lanes 4 and 8) or associated with protein A-Sepharose (lanes 5 and 9) in the absence (lanes 2–5) or presence (lanes 6–9) of 50 μg laminin. M_r standards (×10^{-3}) are indicated on the left of each panel.

METHODS. Rabbit skeletal muscle crude membranes, sarcolemma membranes and dystrophin-glycoprotein complex were electrophoretically separated on 3–12% SDS polyacrylamide gels in the presence of 1% 2-mercaptoethanol and transferred to nitrocellulose (see legend to Fig. 2). Purified laminin mouse EHS (Sigma) was iodinated with [^{125}I]NaI using a lactoperoxidase/glucose oxidase reaction by the Diabetes, Endocrinology Research Center at the University of Iowa. The ^{125}I-labelled laminin overlay procedure of Smalheiser and Schwartz[23] was done as described except that nitrocellulose transfers were blocked with 5% non-fat dry milk in 150 mM NaCl, 50 mM sodium phosphate, pH 7.5. To test for coimmunoprecipitation of laminin and the 156K dystrophin-associated glycoprotein, pH 12 extracts[8] of rabbit skeletal muscle surface membranes were incubated for 24 h at 4 °C with gentle mixing in buffer A (0.14 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM triethanolamine-HCl, pH 7.6) in the absence or presence of 50 μg purified mouse EHS laminin (Sigma), then incubated for an additional 24 h at 4 °C with 100 μl of either protein A-Sepharose or anti-laminin/protein A-Sepharose which had been equilibrated with 3% BSA in buffer A and washed four times with buffer A. The Sepharose was pelleted by a brief centrifugation, the supernatant (void) decanted and the Sepharose washed three times with buffer A. Equivalent volumes of the resulting voids and washed Sepharose pellets were analysed by SDS-polyacrylamide gel electrophoresis and immunoblotting using monoclonal antibody IIH6 which is specific for the 156K DAG.

precedes muscle cell necrosis and the basal lamina seems to separate from the sarcolemma in early steps of DMD. In addition, because the extracellular matrix of the adult tissue is a scaffold that is required to allow repair after injury, the absence of the interaction between the sarcolemma and the extracellular matrix may also render dystrophic muscle fibres more prone to injury and less able to repair injury.

The normal production of the mRNA for the 43-156K DAG in dystrophic muscle is important for potential DMD therapies. It was unclear how the absence of dystrophin leads to the loss of the dystrophin-associated glycoproteins but these results indicate that dystrophin-associated glycoproteins are produced in dystrophic muscle. But in the absence of dystrophin the dystrophin-associated glycoproteins may not be properly assembled and/or integrated into the sarcolemma or may be degraded. Our results suggest that restoring dystrophin by myoblast transfer or gene therapy may stabilize and restore normal dystrophin-associated glycoprotein levels in DMD muscle. These results provide strong evidence for the structural role of dystrophin in muscle integrity, demonstrate that the 43-156K DAG (dystroglycan) is a new laminin-binding glycoprotein and suggest that the function of dystrophin-glycoprotein complex is to provide linkage between the sarcolemma and the extracellular matrix. □

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