THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 8, Issue of February 25, pp. 6040-6044, 1994
Printed in U.S.A.

Heterogeneity of the 59-kDa Dystrophin-associated Protein Revealed by cDNA Cloning and Expression*

(Received for publication, September 15, 1993)

Bin Yang‡, Oxana Ibraghimov-Beskrovnaya‡, Carolyn R. Moomaw§, Clive A. Slaughter§, and Kevin P. Campbell‡¶

From the ‡Howard Hughes Medical Institute and Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242 and the §Howard Hughes Medical Institute, Biopolymer Facility, University of Texas Southwestern Medical Center, Dallas, Texas 75235

The 59-kDa dystrophin-associated protein triplet (59-DAP) is a component of the dystrophin-glycoprotein complex which may directly associate with dystrophin. The cDNA encoding one component (59-1 DAP) of the 59-DAP triplet has now been cloned from rabbit skeletal muscle. The deduced amino acid sequence of 59-1 DAP predicts a 505-amino acid polypeptide containing nine potential phosphorylation sites and no predicted transmembrane domains. This is consistent with the 59-1 DAP being a peripheral membrane protein associated with the cytoplasmic face of the dystrophin-glycoprotein complex. Affinity-purified antibodies against rabbit 59-1 DAP fusion proteins only recognize the lowest band of the 59-DAP triplet in skeletal muscle sarcolemma and isolated dystrophin-glycoprotein complex. The tissue-specific expression of 59-1 DAP mRNA, which is most prominent in skeletal and cardiac muscle and is also detected in brain, parallels that of dystrophin but not of utrophin. Levels of 59-1 DAP mRNA are unaffected in *mdx* mouse skeletal and cardiac muscles, although all dystrophin-associated proteins, including 59-DAP, are greatly reduced in *mdx* mouse skeletal muscle. However, in *mdx* mouse cardiac muscle, the up-regulation of utrophin preserves all dystrophin-associated proteins except 59-DAP. Our results suggest that the 59-DAP triplet may contain different protein species and that the 59-1 DAP may associate more specifically with dystrophin than with utrophin.

The dystrophin-glycoprotein complex (DGC)[1] is composed of dystrophin, four sarcolemmal glycoproteins (156-DAG, 50-DAG, 43-DAG, and 35-DAG), a 25-kDa transmembrane protein (25-DAP), and an intracellular 59-kDa protein triplet (59-DAP) in skeletal and cardiac muscle (1–6). The 156-DAG (α-dystroglycan) binds laminin, and the N-terminal domain of dystrophin binds to actin filaments, indicating that one function of the DGC is to provide a link between the extracellular matrix and the actin cytoskeleton (7, 8). The absence of dystrophin in skeletal muscle from *mdx* mice and Duchenne muscular dystrophy (DMD) patients leads to a dramatic reduction of all of the components of the DGC, suggesting that disruption of the structural integrity of the DGC may play a crucial role in the molecular pathogenesis of DMD (2, 5, 9, 10).

Several lines of evidence suggest that dystrophin and 59-DAP may directly interact with one another. Both dystrophin and the 59-DAP triplet dissociate from other DGC components at pH 11, indicating that dystrophin and 59-DAP are not integral membrane proteins (3). 59-DAP can also be cross-linked to dystrophin *in vitro* (6). In *mdx* mice transgenic for normal dystrophin, the 59-DAP is restored and colocalizes with dystrophin in the sarcolemma (11, 12). Furthermore, the 52/58-kDa *Torpedo* dystrophin-associated protein, which is proposed to be the homologue of 59-DAP in skeletal muscle DGC, binds *Torpedo* dystrophin in an *in vitro* overlay assay (13).

We have established the primary structure of one component (59-1 DAP) of the 59-DAP triplet from rabbit skeletal muscle. Analysis of the deduced amino acid sequence suggests that the 59-1 DAP contains several potential phosphorylation sites and no predicted transmembrane domains. The identity of the cDNA clone has been confirmed using antibodies against fusion proteins, all of which specifically recognize the lowest band of the 59-DAP triplet. These data suggest that the 59-DAP triplet contains different protein species. The 59-1 DAP is expressed predominantly in skeletal and cardiac muscle and to a lesser extent in brain. This is an identical pattern to that of dystrophin expression but different from utrophin, which is ubiquitously expressed in various tissues. Northern blot analysis reveals no reduction in the 59-1 DAP mRNA in dystrophic muscle. These results indicate that the down-regulation of the 59-DAP in the skeletal muscle of DMD patients and *mdx* mice is likely a posttranslational event. Finally, we observed coexpression of utrophin and all dystrophin-associated proteins except the 59-DAP in dystrophin-deficient *mdx* cardiac muscle.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing—Affinity-purified sheep polyclonal antibodies to the 59-DAP triplet were prepared as previously described (5) and used to screen 1×10^6 recombinant phages of a 4-day-old rabbit skeletal muscle λgt11 expression library. A 400-base pair cDNA (clone R59-1) was isolated and sequenced. An oligo(dT)-primed rabbit skeletal muscle λZapII cDNA library (Stratagene) was screened at high stringency with ^{32}P-labeled R59-1 cDNA (random-primed DNA labeling kit, Boehringer Mannheim). Several overlapping clones were obtained. Clones R59-1 and R59-9 were completely sequenced on both strands using either an Applied Biosystems Automated Sequencer or manually by the dideoxy chain termination method (14). Sequences were analyzed with the Genetics Computer Group (Wisconsin package) and PC Gene (IntelliGenetics) software. In order to obtain partial amino acid sequence from the 59-DAP triplet, purified DGC (3) was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and the region of the

* This work was supported in part by the Muscular Dystrophy Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number U01243.

¶ Investigator of the Howard Hughes Medical Institute. To whom correspondence should be addressed: Howard Hughes Medical Institute, University of Iowa College of Medicine, 400 EMRB, Iowa City, IA 52242. Tel.: 319-335-7867; Fax: 319-335-6957.

[1] The abbreviations used are: DGC, dystrophin-glycoprotein complex; DAG, dystrophin-associated glycoprotein; DAP, dystrophin-associated protein; 59-DAP, 59-kDa dystrophin-associated protein triplet; DMD, Duchenne muscular dystrophy; kb, kilobase pair(s); FP, fusion protein.

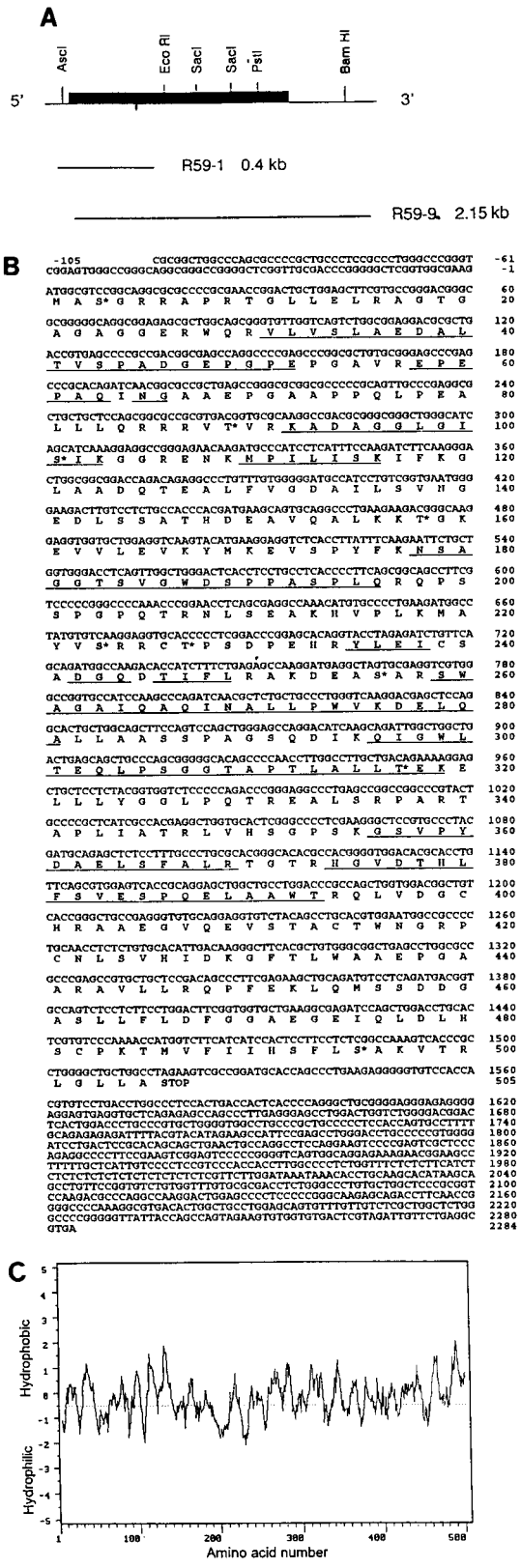

FIG. 1. **Primary structure and hydropathy analysis of rabbit 59-1 DAP.** *A*, diagram of 59-1 DAP cDNA showing the open reading frame (*black box*) and unique restriction enzyme sites. The relative positions and sizes of two unique 59-DAP clones (R59-1 and R59-9) are indicated by lines. *B*, nucleotide and predicted amino acid sequences (*single-letter code*) of 59-1 DAP. The regions of deduced amino acid sequence which match protein sequence obtained from tryptic peptide fragments of purified 59-DAP triplet are *underlined*. Nine potential serine/threonine phosphorylation sites are indicated by *asterisks*. *C*, hydropathy plot of rabbit 59-1 DAP using the method of Klein *et al.* (21) with a window of 9 amino acids.

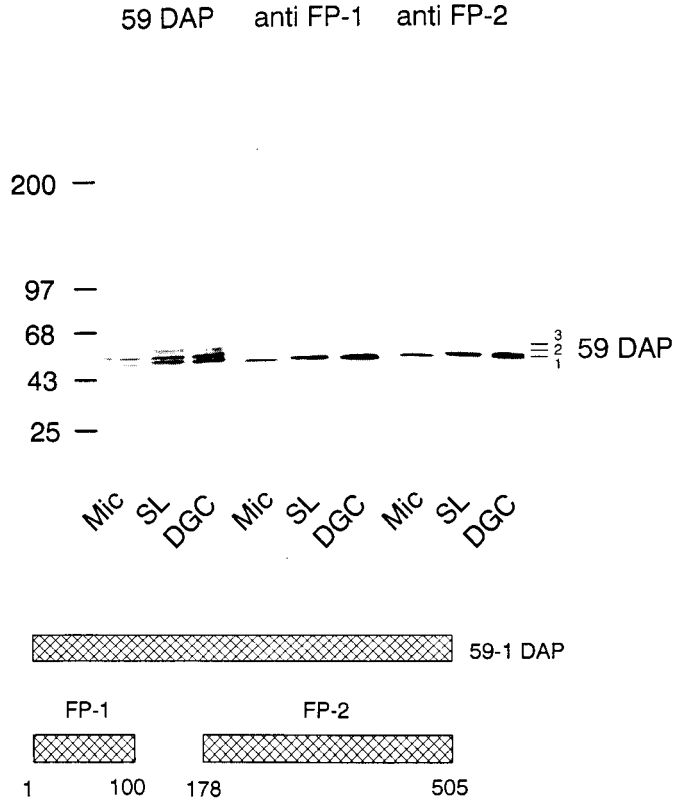

FIG. 2. **Affinity-purified antibodies generated against rabbit 59-1 DAP fusion proteins recognize the lowest band of the 59-DAP triplet.** Immunostaining of skeletal muscle total microsomes (*Mic*), sarcolemma membranes (*SL*), and purified dystrophin-glycoprotein complex (*DGC*) with antibodies affinity-purified against 59-DAP triplet (*59-DAP*), fusion protein 1 (*FP-1*), and fusion protein 2 (*FP-2*). The three bands that define the 59-DAP triplet are indicated on the *right*. M_r standards ($\times 10^{-3}$) are indicated on the *left*. Diagram below indicates the relative positions of the glutathione *S*-transferase fusion proteins to the deduced 59-1 DAP protein sequence.

blot containing the 59-DAP triplet was excised and processed as described (15).

Generation of Fusion Proteins and Antibodies—To construct FP-1, the 400-base pair *Eco*RI insert of clone R59-1 was subcloned into the *Eco*RI site of pGEX-1 (16). To construct FP-2, the 1.8-kb C-terminal *Eco*RI fragment of R59-9 was ligated into the *Eco*RI site of pGEX-3X (16). Each recombinant plasmid was introduced into DH5α cells, and fusion proteins were purified as described (17). Sheep polyclonal antibodies to the purified dystrophin-glycoprotein complex (DGC) were produced as previously described (5). Anti-fusion protein antibodies were affinity-purified from sheep anti-DGC polyclonal antisera as described (7).

Immunoblot Analysis—DGC, skeletal and cardiac muscle total membranes or surface membranes were prepared as described previously (3, 4, 5). All membranes (50 or 250 μg of protein/lane) and purified DGC (8 μg of protein/lane) were separated on 3–12% gradient SDS-polyacrylamide gels and transferred to nitrocellulose. Immunoblot staining was performed as previously described (4, 5).

Northern Blot Analysis—Total rabbit RNA was isolated by homogenization in RNAzol (Cinna/Biotecx) followed by chloroform extraction. Total RNA (10 μg of RNA/lane) was resolved on a 1.2% agarose gel containing 5% formaldehyde and transferred to GeneScreen nylon membrane (DuPont NEN). The membrane was prehybridized, hybridized, and washed as previously described (7). Hybridization was performed using the ^{32}P-labeled 1.8-kb C-terminal *Eco*RI fragment of R59-9 at 2×10^6 cpm/ml. A human multiple tissue mRNA blot (Clontech) was prehybridized at 42 °C in 5 × SSC, 5 × Denhardt's solution, 50% formamide, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA. The membrane was hybridized with ^{32}P-labeled 1.8-kb

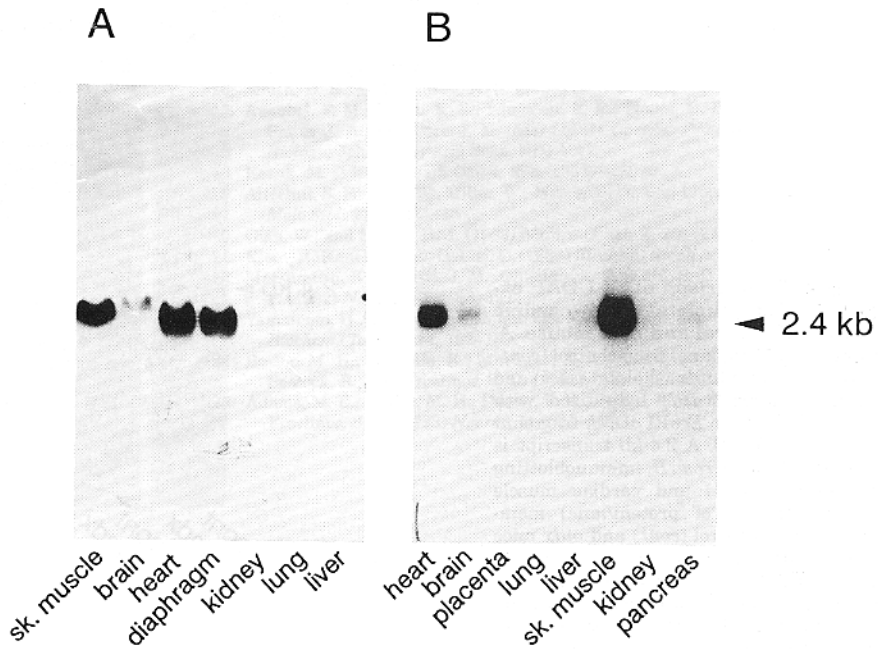

FIG. 3. **Tissue-specific expression of 59-1 DAP mRNA.** *A*, Northern blot of total RNA (10 μg/lane) from seven rabbit tissues as indicated hybridized with ^{32}P-labeled 1.8-kb *Eco*RI fragment from clone R59-9. *B*, Northern blot of poly(A)$^+$ RNA (2 μg/lane) from human tissues hybridized with the same probe as in *A*. The 2.4-kb 59-1 DAP-specific mRNA is indicated by an *arrow*.

C-terminal *Eco*RI fragment of R59-9 at 42 °C at 1 × 10^6 cpm/ml. The membrane was washed three times at 62 °C in 2 × SSC, 0.1% SDS and was exposed to film (X-Omat AR, Kodak) at −80 °C for 16 h.

RESULTS AND DISCUSSION

A rabbit skeletal muscle λgt11 cDNA expression library was screened with affinity-purified sheep polyclonal antibodies specific for the 59-DAP triplet. One positive clone with a 0.4-kb insert designated R59-1 was isolated and sequenced (Fig. 1*A*). Sequence analysis identified one open reading frame. Several overlapping clones covering the entire coding region were isolated by rescreening of a rabbit skeletal muscle cDNA library using ^{32}P-labeled R59-1 insert as a probe. Clone R59-9 was completely sequenced on both strands (Fig. 1*A*).

The 2,389-nucleotide cDNA sequence contains a 1,515-nucleotide open reading frame coding for a 505-amino acid polypeptide with a calculated molecular weight of 53,760 (Fig. 1*B*). 10 out of 14 peptide sequences obtained from tryptic fragments of purified rabbit 59-DAP triplet were found in the deduced amino acid sequence (Fig. 1*B*). The ATG at nucleotides 106–108 in clone R59-1 was identified as the initiating methionine codon, since the nucleotides surrounding this ATG resemble the Kozak translation initiation sequence (18). In addition, clone R59-10 with the identical open reading frame had an in-frame stop codon upstream of this ATG (not shown).

The deduced polypeptide sequence has no significant similarity with any known nucleotide or protein sequences in data bases used by the National Center for Biotechnology Information BLAST E-mail server (19, 20). Hydropathy analysis (21) revealed no predicted transmembrane domains, which is consistent with the biochemical properties of 59-DAP as a peripheral membrane protein (Fig. 1*C*). Nine potential serine/threonine phosphorylation sites (including two protein kinase A, eight protein kinase C, and nine casein kinase II phosphorylation sites) were found in the 59-DAP deduced polypeptide sequence (Fig. 1*B*). The calculated isoelectric point (pI) is 6.41.

To further characterize the relationship between the cloned 59-DAP cDNA and the 59-DAP triplet, two fusion proteins were generated (Fig. 2). Antibodies specific to each fusion protein were affinity-purified from sheep antiserum raised against purified DGC (7) and tested on skeletal muscle microsomes, sarcolemma and purified DGC. Interestingly, antibodies to both fusion proteins recognized only the lowest band of the 59-DAP triplet (59-1 DAP), while antibodies generated against native 59-DAP recognized all components of the 59-DAP triplet (Fig. 2). Thus, we have established the primary structure of one component of the 59-DAP triplet, namely 59-1 DAP, and demonstrated that the 59-DAP triplet consists of antigenically distinct proteins.

59-1 DAP mRNA expression was examined in several rabbit and human tissues. A prominent 2.4-kb transcript was found in skeletal muscle, cardiac muscle, and diaphragm (Fig. 3, *A* and *B*). 59-1 DAP mRNA was present at lower levels in brain and at much lower levels in kidney, lung, liver, and pancreas (Fig. 3, *A* and *B*). Thus, the 59-1 DAP transcript is highly enriched in striated muscle and the tissue distribution of 59-1 DAP mRNA resembles the tissue distribution of dystrophin mRNA but differs from that of utrophin and dystroglycan, which are ubiquitously expressed.

The expression of 59-1 DAP mRNA was also examined in skeletal and cardiac muscle of control and *mdx* mice. Normal expression of 59-1 DAP mRNA was found in *mdx* skeletal and cardiac muscle, although 59-1 DAP protein was not detected with anti-fusion protein antibodies in skeletal and cardiac muscle membranes of *mdx* mice (Fig. 4, *A* and *B*). These data are consistent with our previous hypothesis that abnormal expression of the dystrophin gene causes a posttranslational disruption of the dystrophin-glycoprotein complex. The normal expression of 59-DAP mRNA and other dystrophin-associated protein transcripts is important for potential DMD therapies in which the restoration of dystrophin by gene transfer may restore the dystrophin-glycoprotein complex.

The expression of all components of the DGC was examined in dystrophin-deficient *mdx* cardiac muscle. In contrast to skeletal muscle (5, 11), 59-DAP was the only dystrophin-associated protein that was deficient in *mdx* cardiac muscle membranes (Fig. 4*C*). We have previously demonstrated that a 4-fold increase of utrophin in *mdx* cardiac muscle compared to control mouse could apparently compensate for dystrophin deficiency by retaining DAPs (22). Thus, up-regulation of utrophin expression can lead to the retention of the other DAPs but not 59-DAP in *mdx* cardiac muscle. Thus, the 59-1 DAP may be more specific to the dystrophin-glycoprotein complex than to

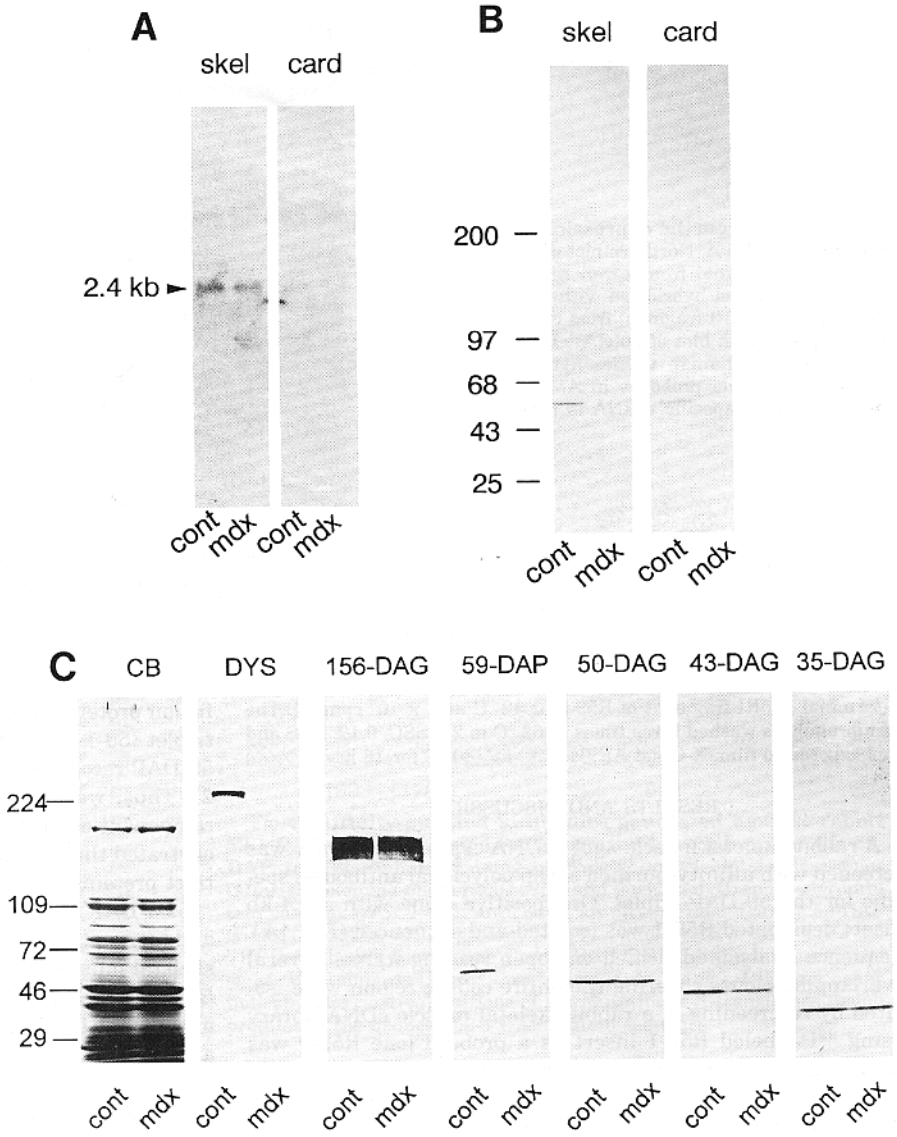

FIG. 4. **Comparison of 59-1 DAP expression in skeletal and cardiac muscle of control and *mdx* mouse.** *A*, total RNA (10 μg/lane) from control (*cont*) and *mdx* mouse (*mdx*) skeletal (*skel*) and cardiac muscle (*card*) hybridized with ^{32}P-labeled 1.8-kb *Eco*RI cDNA fragment from clone R59-9. A 2.4-kb transcript is indicated by an *arrow*. *B*, immunoblotting of skeletal (*skel*) and cardiac muscle (*card*) (250 μg of protein/lane) membranes from control (*cont*) and *mdx* mice (*mdx*) with affinity-purified antibody against FP-2. Molecular weight standards (× 10^{-3}) are indicated on the *left*. *C*, immunoblotting of cardiac muscle membranes from control (*cont*) and *mdx* mice (*mdx*) with affinity-purified antibodies against each individual dystrophin-associated protein (156-DAG, 59-DAP, 50-DAG, 43-DAG, and 35-DAG) and monoclonal antibody XIXC2 against dystrophin (*DYS*). Coomassie Blue (*CB*) staining indicates an equal amount of protein loaded per lane. M_r standards (× 10^{-3}) are shown on the *left*.

the utrophin-glycoprotein complex. It will be interesting to investigate if there is a 59-DAP isoform in the utrophin-glycoprotein complex.

The dystrophin-glycoprotein complex was recently analyzed by two-dimensional PAGE by Yamamoto *et al.* (23). The 59-DAP triplet (called A1 in Ref. 23) was separated into two groups, α-A1 and β-A1, which differed by isoelectric point, molecular weight and antibody reactivity. 59-1 DAP is most likely identical to α-A1 based on molecular weight and isoelectric point. Additionally, the α-A1 and β-A1 contain multiple protein spots along the pH axis, suggesting that at least some potential phosphorylation sites in the 59-1 DAP could be phosphorylated *in vivo*.

In *Torpedo* postsynaptic membranes, dystrophin is complexed with a 52/58-kDa protein, which has been proposed to be the homologue of mammalian skeletal muscle 59-DAP (24). The *Torpedo* 52/58-kDa protein is concentrated at postsynaptic membranes and may be involved in clustering acetylcholine receptors (24). Recently, the cDNA for the 52/58 kDa-protein (named syntrophin) has been isolated from *Torpedo* and mouse (25), and two forms of mouse syntrophin have been identified. Syntrophin-1 is 92% identical to the rabbit 59-1 DAP, supporting the hypothesis that the 52/58-kDa *Torpedo* protein and 59-1 DAP play similar functional roles.

In the present work, we have characterized the primary structure of 59-1 DAP, which is one component of the heterologous 59-DAP triplet in the dystrophin-glycoprotein complex. The identity of the cloned protein product has been confirmed by comparison to the 59-DAP tryptic peptide sequences and by the use of affinity-purified anti-59-1 DAP antibodies. The expression of 59-1 DAP is more prominent in striated muscles and parallels that of dystrophin but not utrophin. 59-1 DAP mRNA is present in *mdx* skeletal and cardiac muscle, indicating that the down-regulation of 59-DAP is likely a posttranslational event. Thus, molecular biological and biochemical studies indicate that the 59-1 DAP is a peripheral membrane protein associated with the cytoplasmic face of the dystrophin-glycoprotein complex and are consistent with the hypothesis that the 59-1 DAP specifically links dystrophin, but not utrophin, to the membrane glycoprotein complex.

Acknowledgments—We thank Dr. James Ervasti for contributions to peptide sequencing of the rabbit 59-DAP triplet and Dr. Kay Ohlendieck for the immunobloting experiments with *mdx* cardiac and sarcolemmal membranes. We also thank Dr. Steven Roberds for helpful comments and Steven Kahl, Cynthia Leveille, Jon Meyer, and Mike Mullinnix for expert technical assistance. We would also like to thank Dr. Stanley Froehner for a preprint of his paper.

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