Volume 329, number 1,2, 227–231 FEBS 12897 August 1993

Defective association of dystrophin with sarcolemmal glycoproteins in the cardiomyopathic hamster heart

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Received 3 June 1993

In ventricular muscle from 30- to 60-day-old Bio 14.6 cardiomyopathic hamsters, dystrophin-associated glycoproteins of 43, 50 and 150 kDa are markedly reduced in abundance. In particular, the 50-kDa glycoprotein is totally deficient in the sarcolemma of myopathic ventricular myocytes as revealed by immunofluorescence microscopy. The dystrophin–glycoprotein complex formation is defective in the cardiomyopathic hamster heart, because dystrophin and the glycoproteins behave independently when digitonin-solubilized ventricular homogenates are fractionated on wheat germ agglutinin beads or anti-dystrophin immunoaffinity beads.

Cardiomyopathy; Cardiomyopathic hamster; Dystrophin; Dystrophin-associated glycoprotein; Muscular dystrophy

1. INTRODUCTION

The cardiomyopathic Syrian hamster develops a genetically determined cardiomyopathy, characterized by spontaneous necrosis of cardiomyocytes occurring at relatively early ages (30 to 50 days after birth), which leads to hypertrophy and ultimate failure of the heart and to death at about 300 days of age [1]. This animal model can be a good, experimental tool for the study of the disease mechanism of human hereditary cardiomyopathy, although it also develops cell necrosis in skeletal muscle. Previous studies suggest that cell necrosis is caused by calcium overloading of myopathic cardiomyocytes which may arise from abnormal calcium handling by these cells [2]. However, the primary defect causing such cell necrosis has not been identified.

A similar progressive muscular dystrophy is known to occur in skeletal muscle of patients with Duchenne muscular dystrophy (DMD), which is caused by the lack of dystrophin, the high molecular weight protein product of the DMD gene [3,4]. In striated muscle cells, dystrophin is localized at the inner surface of the sarcolemma, presumably forming a sub-membrane cytoskeletal network [4–6]. Dystrophin binds to actin at its N-terminal side and a complex of membrane glycoproteins at its C-terminal side [7–9]. Detailed examination revealed that dystrophin binds to the glycoprotein complex at the cysteine-rich domain and the first half of the C-terminal domain [9]. The dystrophin-associated glycoprotein complex consists of at least three integral membrane glycoproteins of 35, 43 and 50 kDa, which were designated A4, A3 and A2, respectively [10], and an extracellular, laminin-binding glycoprotein of 156 kDa [10–12]. Thus glycoproteins link the dystrophin skeleton to the sarcolemma and to the extracellular matrix, structurally stabilizing the sarcolemma.

Recently, we have shown that dystrophin is 50% less abundant in cardiomyopathic hamster ventricle compared with normal control and that despite its low abundance, extraction of dystrophin from the ventricular muscle into the microsomal fraction is increased fourfold in cardiomyopathic hamster [13]. This suggests an altered membrane–dystrophin association in the cardiomyopathic hamster, which could result from a defect in the sarcolemmal glycoproteins that dystrophin anchors. Here we report markedly decreased abundance of the 43-, 50- and 150-kDa glycoproteins, and disruption of the integrity of the dystrophin-glycoprotein complex in myopathic hamster cardiomyocytes.

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Abbreviations: DMD, Duchenne muscular dystrophy; TCA, trichloroacetic acid; WGA, wheat germ agglutinin; LBP, laminin binding protein; NAG, *N*-acetyl-D-glucosamine.

2. MATERIALS AND METHODS

2.1. *Animals*

Thirty- to sixty-day-old Syrian hamsters of the Bio 14.6 strain and age-matched control Golden hamsters were used as experimental animals [13,15].

2.2. *Preparation of TCA homogenates*

A hamster ventricle (0.18 g) was cut into pieces in 10% TCA (1 ml)

and then homogenized three times for 30 s with an ultra high speed Physcotron NS-60 homogenizer (NITI-ON Instruments CO.) at 25,000 rpm. After centrifugation at 5,500 × *g* (max) for 20 min, the pellet was completely dissolved in 1 ml of 0.1 M $NaHCO_3$, 2% SDS and 1 mM EDTA by ultrasonic treatment.

2.3. *Antibodies*

Monoclonal mouse antibodies against the rod domain (NCL-DYS1) and the COOH-terminal region (NCL-DYS2) of human dystrophin were purchased from Novocastle Laboratories. Polyclonal rabbit antiserum to laminin purified from rat yolk sac tumor L2 was obtained from Gibco Laboratories. Polyclonal antibodies against A3a (PA3a) and A2 (PA2), which were the 43- and 50-kDa dystrophin-associated glycoproteins from skeletal muscle [10], were produced in rabbits using synthetic polypeptides [14]. All these antibodies recognized single proteins of the expected molecular weight for the target proteins in the dystrophin–glycoprotein complex purified from rabbit skeletal muscle (data not shown) or in the hamster ventricular homogenates (Fig. 1). Preparation of a polyclonal rabbit antibody against α-subunit of Na^+,K^+-ATPase was described in [16].

2.4. *Immunoblot analysis and detection of laminin binding proteins*

TCA homogenates (60 μg) or samples (20 μl) eluted from WGA-Sepharose or dystrophin immunoaffinity beads were electrophoresed on 4–20% gradient or 5 or 7.5% SDS-polyacrylamide gels according to Laemmli [17]. For immunoblotting, the proteins in the gels were transferred to Immobilon membranes (Millipore) with an efficiency higher than 83% even for dystrophin. Immobilon membranes were treated with a specific antibody and a peroxidase-conjugated secondary antibody as described in [15]. The protein recognized by each specific antibody was quantified by using Amersham ECL immunoblotting detection system also as described [15,16]. For detection of laminin binding proteins, Immobilon transfers were treated with purified rat laminin (1 μg) (TELIOS Pharmaceuticals) for 30 min at 37°C, washed free of unbound laminin and then incubated with polyclonal rat anti-laminin antibody for 30 min at 37°C. The protein-bound laminin recognized by anti-laminin antibody was quantified as described above. Control experiments were done without the laminin overlay.

2.5. *Isolation of dystrophin and its associated proteins using dystrophin immunoaffinity beads or WGA-Sepharose beads*

Hamster ventricle (0.2 g) was homogenized for 30 s three times with Physcotron NS-60 at 25,000 rpm in buffer A (1 ml) (1% digitonin, 0.5 M NaCl, 50 mM Tris-HCl (pH 7.4), 0.25 mM phenylmethanesulfonyl fluoride, 1.5 mM benzamidine, 2.5 μg/ml leupeptin, 2.5 μg/ml aprotinin, and 2.5 μg/ml pepstatin A). After centrifugation at 540,000 × *g* (max), the supernatant (20 mg protein) was incubated overnight at 4°C either with anti-mouse IgG Sepharose beads (50 μl) (Sigma) carrying anti-dystrophin antibody (NCL-DYS2) or with WGA-Sepharose 6 MB beads (100 μl) (Pharmacia-LKB). The beads then were washed extensively with buffer A and eluted with SDS sample buffer (100 μl) containing 4% SDS, 200 mM DTT and 20% glycerol (for immunoaffinity beads) or buffer A (1 ml) containing 0.6 M NAG (for WGA-Sepharose beads). The sample eluted from WGA-Sepharose was precipitated by the addition of 100% TCA (100 μl) and the resultant precipitate was dissolved with SDS sample buffer (50 μl).

2.6. *Immunofluorescence microscopy*

Frozen sections (6 μm) of hamster ventricular muscle were incubated for 30 min in PBS (0.9% NaCl, 50 mM sodium phosphate, pH 7.5) containing 5% defatted dry milk (PBS/milk) and stained for 1 h with a monoclonal antibody against dystrophin (NCL-DYS1) or a polyclonal antibody against the 43-kDa (PA3a) or 50-kDa (PA2) dystrophin-associated glycoprotein. Samples were washed, blocked again for 15 min in PBS/milk, and then treated with FITC-conjugated anti-mouse IgG or FITC-conjugated anti-rabbit IgG (Organon Teknika N.V.-Cappel products). Visualization of samples was performed as described previously [13].

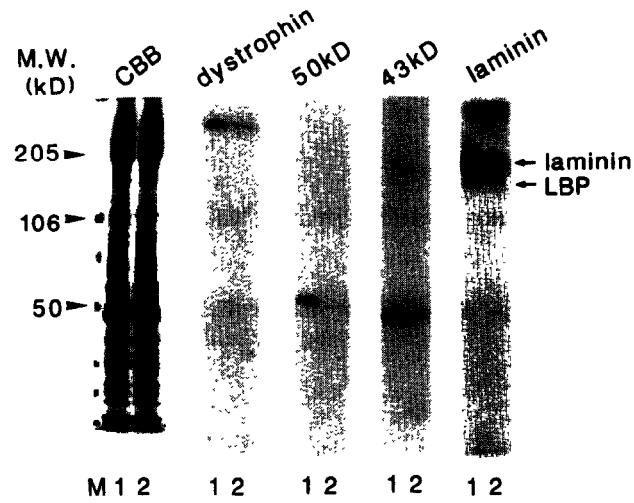

Fig. 1. Immunoblot analysis of ventricular homogenates from control and myopathic hamster. TCA homogenates (60 μg) were subjected to SDS polyacrylamide gel electrophoresis. For CBB, gels were stained with Coomassie brilliant blue. For other gels, proteins were blotted to Immobilon membranes and then immunostained as described in section 2 with antibodies against the indicated proteins (lane 1, samples from normal hamster; lane 2, samples from myopathic hamsters). Arrows show the positions of laminin and the laminin binding protein (LBP). Molecular mass standards are shown on the left.

3. RESULTS

Fig. 1 shows immunoblots of ventricular homogenates from normal and cardiomyopathic hamsters with anti-dystrophin antibody, PA3a, PA2 and anti-laminin antibody. The figure also shows relative abundance of a 150-kDa laminin-binding protein (LBP) in normal and myopathic ventricular homogenates, which was visualized by laminin overlay followed by staining with an anti-laminin antibody (see section 2). This 150-kDa LBP probably is a cardiac form of the 156-kDa dystrophin- associated glycoprotein which was shown to be a major LBP in crude skeletal muscle membranes [12] (see section 4). Immunoblot analysis revealed that dystrophin, the 43- and 50-kDa glycoproteins, and the 150-kDa LBP were markedly reduced in myopathic ventricle compared with normal control. The reductions were 42 ± 13% ($n = 4$) for dystrophin, 85 ± 13% ($n = 5$) for the 50-kDa glycoprotein, 61 ± 8% ($n = 4$) for the 43-kDa glycoprotein, and 92 ± 10% ($n = 4$) for the 150-kDa LBP, as estimated by quantitative immunoblot assay. In contrast, there was no difference in the laminin content between normal and cardiomyopathic hamsters.

Greater reductions of the 43- and 50-kDa glycoproteins and the 150-kDa LBP relative to dystrophin in myopathic ventricular muscle prompted us to investigate the association status of these proteins with dystrophin. We solubilized dystrophin and its associated proteins from ventricular homogenates by treating the latter directly with 0.5 M NaCl and 1% digitonin (see section 2). About 50 and 67% of total dystrophin were

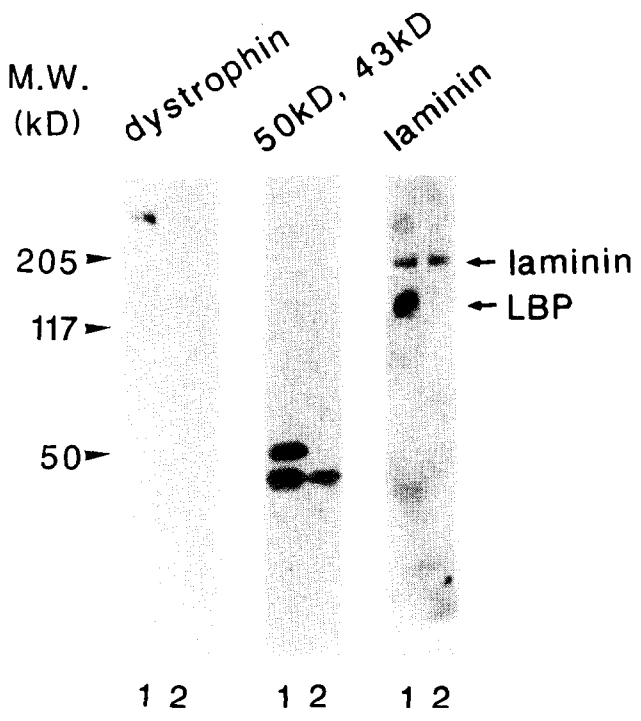

Fig. 2. Isolation of dystrophin and its associated proteins using WGA-Sepharose beads. Digitonin-solubilized ventricular homogenates from normal or myopathic hamsters were loaded onto WGA-Sepharose beads. Proteins were eluted with 0.6 M NAG and subsequently precipitated by the addition of TCA as described in section 2. The TCA precipitate was dissolved with SDS sample buffer and analyzed by SDS-PAGE and immunoblotting (lane 1, samples from normal hamster; lane 2, samples from myopathic hamsters). Immobilon transfers were stained with antibodies to the indicated proteins as in Fig. 1.

extracted into soluble fractions from normal and myopathic ventricular homogenates, respectively. We then incubated the solubilized material with WGA-Sepharose beads. Immunoblot analysis revealed that dystrophin, the 43- and 50-kDa glycoproteins and the 150-kDa LBP from normal hamster ventricle were retained on WGA Sepharose, from which they were eluted with NAG (Fig. 2, lane 1). On the other hand, dystrophin, the 50-kDa glycoprotein, and the 150-kDa LBP from myopathic hamster ventricle were not detectable in the NAG-eluted fraction from WGA-Sepharose under the conditions of Fig. 2 (Fig. 2, lane 2). When the amount of the solubilized material loaded onto WGA-Sepharose was increased threefold, the presence of the 150-kDa LBP but not the 50-kDa glycoprotein became detectable in the NAG-eluted fraction (data not shown). In these NAG-eluted fractions from normal and myopathic hamsters, Na^+,K^+-ATPase was found to be present at equal levels, as estimated by immunoblotting with an antibody against the α-subunit of this enzyme (data not shown). The Na^+,K^+-ATPase was retained by WGA-Sepharose probably via its β subunit. Thus we conclude that dystrophin from myopathic hamster is not retained on WGA-Sepharose, as opposed to the 43-kDa glycoprotein and the 150-kDa LBP from the same hamster. The same results were obtained with succinylated WGA-Sepharose.

Association of dystrophin with its associated proteins was also analyzed by immunoprecipitation of dystrophin (Fig. 3). Dystrophin immunoaffinity beads were incubated with the digitonin-solubilized material from normal or myopathic hamster ventricular homogenates. After extensive washing, proteins were eluted from the immunoaffinity beads with SDS buffer and analyzed by SDS-PAGE and immunoblotting. Dystrophin immunoaffinity beads immunoprecipitated the 43- and 50-kDa glycoproteins and the 150-kDa LBP together with dystrophin from normal ventricular homogenates (Fig. 3, lane 1). In contrast, these dystrophin-associated proteins were not immunoprecipitated from myopathic ventricular homogenates with the same immunoaffinity beads (Fig. 3, lane 2).

We investigated the distribution of dystrophin and its associated proteins in ventricular muscles from normal and myopathic hamsters by indirect immunofluorescence microscopy (Fig. 4). We showed previously that anti-dystrophin antibody uniformly and similarly stained the peripheral sarcolemma and T tubules of both normal and myopathic hamster cardiomyocytes [13]. We confirmed these findings in cross (Fig. 4a and b) and longitudinal sections (data not shown) of ventricular muscles from normal and myopathic hamsters. We found that in normal hamster cardiomyocytes, distributions of the 43- and 50-kDa glycoproteins as stained with PA3a and PA2, respectively, were essentially the same as that of dystrophin (Fig. 4a,c,e). A recent paper by Klietsch et al. presented similar results by using papillary muscles from several animals [18]. The immunostaining patterns of normal and myopathic hamster cardiomyocytes with PA3a were also indistinguishable (Fig. 4e and f). Intriguingly, however, PA2 did not label the sarcolemma of myopathic hamster cardiomyocytes (Fig. 4d). Thus the 50-kDa glycoprotein is deficient in the myopathic sarcolemma. We observed weak staining of the interior of these cells with PA2 (Fig. 4d). However, such staining did not differ significantly from the background staining obtained with preimmune serum (data not shown). Lack of staining of the 50-kDa glycoprotein in the sarcolemma was observed in ventricular myocytes from myopathic hamsters ranging from 30 to 300 days of age (data not shown).

4. DISCUSSION

In this study, we investigated the association of dystrophin with proteins of 43, 50 and 150 kDa in cardiomyopathic hamster heart. These proteins are most likely cardiac equivalents to A3a (the 43-kDa glycoprotein), A2 (the 50-kDa glycoprotein) and the 156-kDa glycoprotein of the dystrophin-glycoprotein complex in skeletal muscle [10–12] for the following reasons; (1) the cardiac 43- and 50-kDa proteins were recognized re-

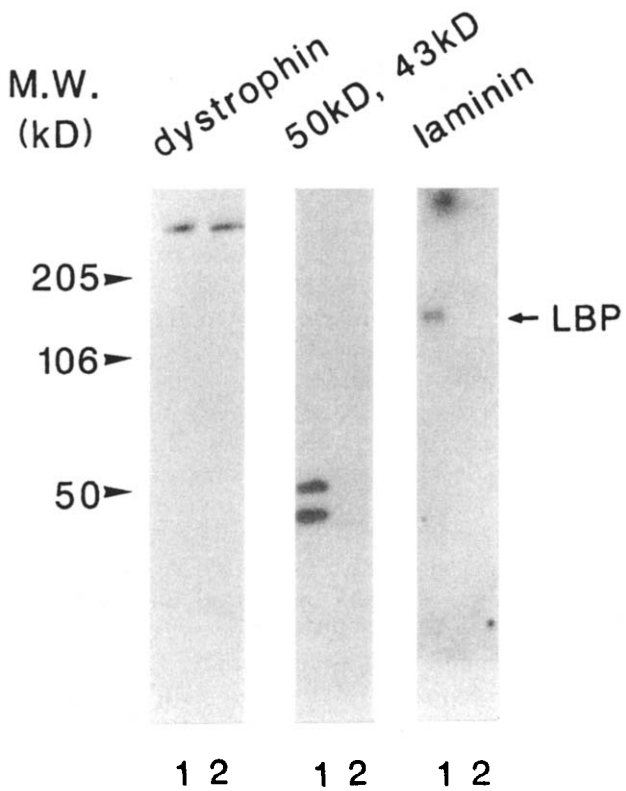

Fig. 3. Isolation of dystrophin and its associated proteins using anti-dystrophin immunoaffinity beads. Digitonin-solubilized ventricular homogenates from normal and myopathic hamsters were loaded onto anti-dystrophin immunoaffinity beads and then proteins were eluted with SDS sample buffer as described in section 2. Eluted proteins were analyzed by SDS-PAGE and immunoblotting (lane 1, samples from normal hamster; lane 2, samples from myopathic hamster). Immobilon transfers were stained with antibodies to the indicated proteins as in Fig. 1.

spectively by antibodies PA3a and PA2 raised against the 43- and 50-kDa components of the skeletal muscle dystrophin–glycoprotein complex and, like dystrophin, localized to the peripheral sarcolemma and T tubules in normal cardiomyocytes (Fig. 4); (2) they were retained on WGA-Sepharose and eluted with NAG and immunoadsorbed to anti-dystrophin immunoaffinity beads, when digitonin-solubilized ventricular homogenates from normal hamster were treated with these beads (Figs. 2 and 3); (3) the cardiac 150-kDa and skeletal 156-kDa LBPs were the only laminin-binding proteins in the dystrophin–glycoprotein complexes from cardiac and skeletal muscles (Fig. 3 and [12]).

Previously we reported that dystrophin is decreased to 50% in myopathic ventricular homogenates and that despite such low abundance, extraction of dystrophin into the microsomal fraction was fourfold greater from myopathic hamster ventricle as compared to normal control [13]. The latter finding suggests that dystrophin may be linked to the sarcolemma much less tightly in the myopathic cardiomyocytes. This study showed that dystrophin from myopathic hamster was not retained on WGA-Sepharose (Fig. 2) and that anti-dystrophin immunoaffinity beads immunoprecipitated none of the above glycoproteins from myopathic hamster (Fig. 3). The results clearly indicate that integrity of the dystrophin–glycoprotein complex in the sarcolemma is disrupted in myopathic cardiomyocytes.

Such disruption could be caused by defect(s) in dystrophin or in the dystrophin-associated glycoproteins. An intriguing finding was that by immunostaining with PA2, the 50-kDa glycoprotein was totally deficient in the myopathic sarcolemma (Fig. 4d). We observed weak positive staining with PA2 in the interior of myopathic cardiomyocytes (Fig. 4d). However, such staining was not distinguishable from the background staining obtained with pre-immune serum (see section 3). We suspect that a small amount of the 50-kDa glycoprotein is present in the interior of myopathic cardiomyocytes, because a small amount of the PA2-reactive 50-kDa protein was detected by immunoblot analysis (see sec-

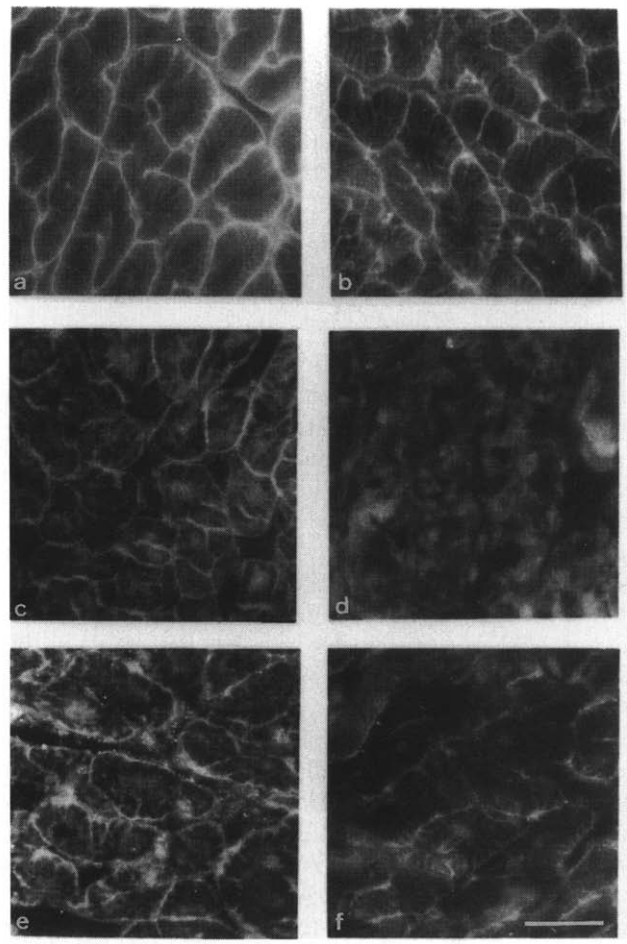

Fig. 4. Immunohistochemical localization of dystrophin, the 43- and 50-kDa glycoproteins in ventricular muscles of normal and myopathic hamsters. Frozen cross sections of normal (a,c,e) or myopathic (b,d,f) ventricular muscle were labeled by indirect immunofluorescence as described in section 2. Sections were incubated with anti-dystrophin antibody (a,b), PA2 (anti-50 kDa glycoprotein) (c,d) and PA3a (anti-43 kDa glycoprotein) (e,f). Bar in f, 20 μm.

tion 3) and because this protein, like dystrophin, exhibited a significant increase in its abundance in the microsomal fraction prepared from myopathic ventricle (Iwata et al., unpublished observation). In contrast to the 50-kDa glycoprotein, the 43-kDa glycoprotein was present in myopathic ventricular homogenates at about 40% of the normal level (see Fig. 1 and section 3) and its immunostaining pattern in the myopathic cardiomyocytes was not different from that for the normal control (Fig. 4e and f).

We found that the 150-kDa LBP was also decreased to 10% of the control level in the myopathic ventricle, as estimated by the measurement of binding of laminin to this protein (see Fig. 1 and section 3). The decrease in laminin binding could result from decreased abundance of the 150-kDa LBP and/or reduced ability of this protein to bind laminin. We tentatively interpret the decreased laminin binding as a reflection of the decreased abundance of the 150-kDa LBP. On the other hand, the laminin content in normal and myopathic ventricles was not different (Fig. 1). Immunofluorescence analysis with an anti-laminin antibody revealed that laminin localizes to the peripheral sarcolemma and T tubules similarly in normal and myopathic cardiomyocytes (Iwata, Y. et al. unpublished observation).

The Bio 14.6 cardiomyopathic hamster is known to develop cell necrosis also in skeletal muscle [1]. Our immunofluorescence analysis revealed that the 50-kDa glycoprotein was also absent in the skeletal muscle sarcolemma from the myopathic hamster (Iwata, Y. et al., unpublished observation). Thus absence of A2 (the 50-kDa glycoprotein) in the sarcolemma may well be responsible for disruption of the integrity of the dystrophin–glycoprotein complex in myopathic cardiomyocytes and myopathic skeletal muscle cells. Such disruption could secondarily cause reduction of dystrophin and some other components of the dystrophin complex observed here. Indeed, in skeletal muscle from DMD patients and mdx mice, specific deficiency of dystrophin was shown to be associated with much reduced abundance of all the dystrophin-associated proteins [12,19]. The possible etiological importance of A2 (the 50-kDa glycoprotein) deficiency in muscle dystrophy has been suggested by a recent report that it is specifically deficient in the skeletal muscle sarcolemma from patients suffering from severe childhood autosomal recessive muscular dystrophy [20], which is a DMD-like progressive muscular dystrophy. Thus muscle dystrophies found in these patients and cardiomyopathic hamsters may share a similar mechanism of pathogenesis.

It should be pointed out that the decreased levels of dystrophin and its associated glycoproteins observed in this study are not the result of muscle cell necrosis in the myopathic hamster ventricle. We reported previously that six integral membrane proteins, i.e. the $\alpha 1$ subunit of L-type calcium channels, Na^+,K^+- ATPase, Na^+-H^+ antiporter, the ryanodine receptor, and sarcoplasmic reticulum Ca^{2+}-ATPase, did not show any difference in their abundance between normal and myopathic ventricular homogenates [16]. In addition, we showed equal abundance of vinculin, a cytoskeletal protein, in normal and myopathic ventricular homogenates [13]. Previous studies of several types of skeletal muscle dystrophy including human DMD have suggested that loss of integrity of the dystrophin–glycoprotein complex may somehow lead to sarcolemmal instability and eventually to muscle cell necrosis. Our results reported in this and previous [13] studies suggest that a defect in the dystrophin–glycoprotein complex may also play a critical role in muscle cell necrosis in the hamster cardiomyopathy.

Acknowledgements: We thank Drs. Toshiaki Imagawa and Yoshiyuki Kijima for helpful discussion.

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