Dystrophin-associated protein A0 is a homologue of the *Torpedo* 87K protein

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Abstract **We raised a monoclonal antibody, MA0, which reacts with A0, a 94-kDa rabbit skeletal muscle dystrophin-associated protein (DAP) bound to the syntrophin-binding domain of dystrophin. The antibody also reacted with the 62-kDa DAP which was moved to the locus close to β-syntrophins by 2-dimensional PAGE, but the DAP did not coincide with any known β-syntrophins. We have cloned a fragment of cDNA which codes the protein reacting with MA0 from a neonatal rabbit heart cDNA library. Based on the coincidence of cDNA sequences and the similarity in molecular mass, we concluded that the proteins reacting with MA0 are rabbit homologues of the *Torpedo* 87K protein.**

Key words: A0; *Torpedo* 87K protein; Syntrophin; Dystrophin-associated protein; Molecular cloning

1. Introduction

Dystrophin is a long slender spectrin-like protein with the molecular mass of 427 kDa [1] and is the protein responsible for Duchenne and Becker muscular dystrophies [2]. It is anchored on the cytoplasmic surface of sarcolemma by a large oligomeric transmembranous glycoprotein complex [3–6]. We recently showed that the glycoprotein complex, which is composed of at least 5 dystrophin-associated proteins (DAPs), is made up of two subcomplexes – the dystroglycan complex composed of α- and β-dystroglycans (156DAG and 43DAG, respectively) and the sarcoglycan complex, which is comprised of three DAPs, adhalin, 35DAG and A3b [7]. A3b is a unique DAP with a molecular mass of 43 kDa but is different from β-dystroglycan, which has a similar molecular mass [7,8]. The sarcoglycan complex is assumed to bind indirectly with dystrophin through the dystroglycan complex [7,9]. In muscles affected by a Dechenne muscular dystrophy-like disease, severe childhood autosomal recessive muscular dystrophy or its animal model, we showed that the sarcoglycan complex is selectively lost, whereas dystrophin and β-dystroglycan, a component of the dystroglycan complex, are expressed normally [10,11].

Besides the DAPs which belong to the glycoprotein complex, there are DAPs (A1 or 59DAP) which are observed as characteristic triplet bands with a molecular mass of 59–62 kDa on a conventional 1-dimensional SDS/PAGE pattern [5,12]. The proteins are assumed to be cytoplasmic peripheral membrane proteins because they were extracted from sarcolemma with alkali [6]. We previously showed that A1s are separated by a 2-dimensional PAGE into two series of spots, the weakly acidic one (α-A1) and the basic one (β-A1) [13]. This observation was supported by three recent reports on the cloning of these A1s [14–16]. These A1s are now called syntrophins [17], since they are mammalian homologues of *Torpedo* syntrophin (the 58-kDa postsynaptic membrane protein) [18]. At present one acidic syntrophin (α-syntrophin) and two basic syntrophins (β1- and β2-syntrophins) are known to be found in the muscles.

It has recently been shown that syntrophins bind with dystrophin in a very limited region corresponding to the alternative splicing-prone site of the dystrophin C-terminus [19,20]. A similar report followed these reports [21]. It was further shown that A0, a less abundant and weakly acidic DAP with a molecular mass of 94 kDa [5], also binds with dystrophin at the same site as β1-syntrophin [19]. In the present study, we raised a monoclonal antibody (MA0) by immunizing mice with a purified rabbit dystrophin-DAP complex partially dissociated with alkali. The antibody reacted with A0 as well as a basic series of spots with a molecular mass similar to those of syntrophins on the 2-dimensional PAGE pattern of the dystrophin-DAP complex. These basic spots, however, did not correspond to known basic syntrophins, namely β1- and β2-syntrophins. We cloned a fragment of cDNA to code the protein which reacts with MA0 and found that it is a rabbit homologue of the *Torpedo* 87K protein.

2. Experimental procedures

2.1. Antibodies

A monoclonal antibody, MA0, was prepared by immunization of BALB/c mice with a purified and alkali-dissociated dystrophin-DAP complex [22]. The antibody stained the sarcolemma of rabbit skeletal and heart muscles (data not shown). The monoclonal antibody MA1–1 was raised against a purified dystrophin-DAP complex and was shown to react with basic syntrophin [13]. The polyclonal antibody Pβ1SYN was raised in rabbits against a bovine-serum-albumin-conjugated synthetic polypeptide, DPPSSQSFSFHRDR, which corresponds to amino acids 226–240 of human β1-syntrophin [14] in the same way as previously described [8]. The following three antibodies were generously provided by Prof. S.C. Froehner: the monoclonal antibody ab 1351 raised against *Torpedo* syntrophin [18], and the affinity-purified polyclonal antibodies ab SYN17 and ab SYN24 raised in rabbits against the synthetic polypeptides corresponding to the internal sequences of mouse α- and β2-syntrophin, respectively [17].

2.2. Electrophoresis and immunoblot

Conventional 1-dimensional SDS/PAGE was performed on a 10% polyacrylamide slab gel in a standard buffer system [23]. Two-dimensional PAGE [24] was performed by the previously described method

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Abbreviations: DAP, dystrophin-associated protein; HRP, horse radish peroxidase.

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[22]. The proteins transferred onto a polyvinylidene difluoride membrane (Millipore Intertech., Bedford, MA) were stained with antibodies and a Vectastain ABC-horse radish peroxidase (HRP) kit (Vector Labs., Inc., Burlingame, CA). Double staining of the blot was performed as follows. The blot was first allowed to react with a mixture of selected antibodies and then with a mixture of appropriate HRP-conjugated and biotinylated secondary antibodies. After color development with H_2O_2 and 3,3'-diaminobenzidine tetrahydrochloride, the blot was washed with the buffer [10 mM sodium phosphate (pH 7.2), 0.15M NaCl and 0.05% Tween 20], and then treated with an ABC-HRP kit. The last color development was made with a Konica immunostaining HRP kit IS-50B (Konica KK., Tokyo).

2.3. Cloning the cDNA

IgG purified from the culture supernatant of the hybridoma producing MA0 was used to screen a neonatal rabbit heart cDNA library in lambda ZAP II according to the manufacturer's protocol (Stratagene cloning systems, La Jolla, CA). One positive plaque was isolated, and the phage was rescued into Bluescript according to the protocol. Single-stranded DNA was recovered according to the protocol and the cDNA A0–1 was sequenced by the dye deoxy primer method with the automated DNA sequencer 373A (Applied Biosystems, Inc., Foster City, CA).

2.4. Others

Dystrophin-DAP complex was purified from rabbit skeletal muscle by the previously described method [25].

3. Results

3.1. Specificity characterization of various antibodies

As shown in Fig. 1, ab 1351, which is a monoclonal antibody against *Torpedo* syntrophin, reacted with the characteristic triplet bands of syntrophin on 1-dimensionally separated SDS/PAGE patterns of purified rabbit dystrophin-DAP complex. On the other hand, Pβ1SYN, a polyclonal antibody of human β1-syntrophin, reacted with the top two bands of the triplet bands. An extra band over these two bands was often stained

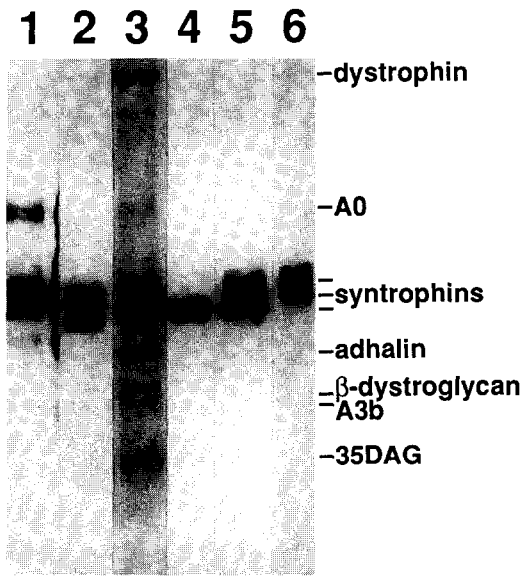

Fig. 1. Immunoblot patterns of dystrophin-DAP complex with various antibodies. The antibodies used are MA0 (anti-A0 antibody, lane 1), ab SYN24 (anti-β2-syntrophin antibody, lane 2), ab SYN17 (anti-α-syntrophin antibody, lane 4), ab 1351 (anti-*Torpedo* syntrophin antibody, lane 5) and Pβ1SYN (anti-β1-syntrophin antibody, lane 6). Lane 3 is stained with Coomassie brilliant blue. From the left lane, 0.12, 6.5, 4, 1.3, 0.1 and 0.5 μg samples were loaded.

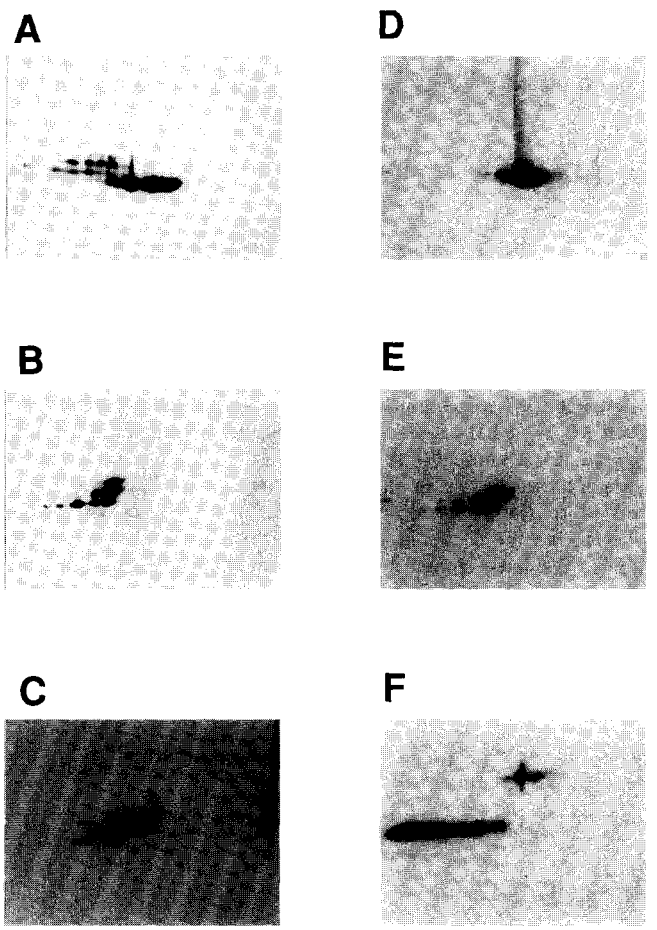

Fig. 2. Immunoblot patterns of dystrophin-DAP complex separated by two-dimensional PAGE with various antibodies. (A) ab 1351 (anti-*Torpedo* syntrophin antibody); (B) MA1–1 (anti-basic syntrophin antibody); (C) ab SYN24 (anti-β2-syntrophin antibody); (D) ab SYN17 (anti-α-syntrophin antibody); (E) Pβ1SYN (anti-β1-syntrophin antibody); (F) MA0 (anti-A0 antibody). One, 2.4, 12, 10, 4 and 1 μg samples were loaded on the gels. Samples were loaded from the basic side except (C), in which β2-syntrophin was focused only when the sample was loaded from the acidic side.

weakly showing that these bands including the extra band correspond to β1-syntrophin. Ab SYN17, the antibody of mouse α-syntrophin (syntrophin-1), reacted with only the bottom band among the triplet bands, showing that it corresponds to α-syntrophin. This result is compatible with another report [16]. On the 2-dimensionally separated PAGE patterns shown in Fig. 2, ab SYN17 and Pβ1SYN reacted with acidic and basic series of protein spots, respectively. These results correspond well to the isoelectric points calculated on the basis of the amino acid sequences of α- and β1-syntrophin [14,16]. On the other hand, ab 1351 reacted with not only α-syntrophin, but also β1-syntrophin of rabbit skeletal muscle. The monoclonal antibody MA1–1, which was previously described as an antibody against basic syntrophin, showed a very similar 2-dimensional pattern to that obtained with Pβ1SYN (Fig. 2). MA1–1 is thus assumed to react with β1-syntrophin.

A newly raised monoclonal antibody MA0, reacted with both A0 (a 94-kDa DAP) and the 62-kDa broad diffuse band which encompasses the β1-syntrophin bands (Fig. 1). On a

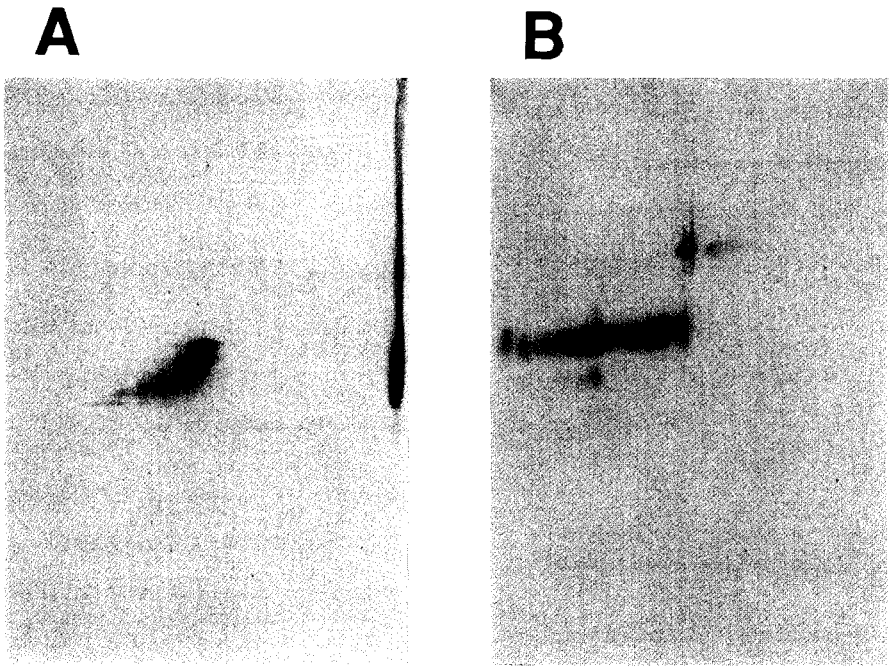

Fig. 3. Immunoblot patterns of dystrophin-DAP complex separated by two-dimensional PAGE with various antibodies. Twelve (A) and 0.6 μg (B) samples were loaded on the gels from the acidic and basic side, respectively. The blots were stained with a mixture of MA1–1 (mouse anti-β1-syntrophin antibody) and ab SYN24 (rabbit anti-β2-syntrophin antibody), (A) and a mixture of MA0 (mouse anti-A0 antibody) and Pβ1SYN (rabbit anti-β1-syntrophin antibody), (B). The blots that reacted with the specific antibodies were allowed to react with HRP-labeled anti-mouse IgG and biotinylated anti-rabbit IgG, and color-developed with H_2O_2 and diaminobenzidine tetrahydrocloride (brown). The blots, after being washed, were then allowed to react with the HRP-labeled avidin–biotin complex and color developed with a Konica stain (blue). Since a much more abundant sample was loaded from the acidic side in (A), the β1-syntrophin spots look denser than those in (B).

2-dimensional PAGE pattern, this antibody reacted with a basic series of spots similar to β1-syntrophin besides A0. However, its staining pattern was much more diffuse than that of Pβ1SYN (Fig. 2). To see whether the 62-kDa protein corresponds to another basic syntrophin, namely, β2-syntrophin, we examined the immunoblot pattern with ab SYN24, which is a polyclonal antibody of mouse β2-syntrophin (syntrophin-2) [15]. The antibody reacted with several bands, most of which apparently overlap the syntrophin triplet bands, but it did not react with A0 (Fig. 1). On a 2-dimensionally separated pattern, this antibody stained many basic tiny spots which are rather similar to those stained with Pβ1SYN (Fig. 2). To find the relative positions of the spots between β1- and β2-syntrophins, we examined the 2-dimensional PAGE pattern by double staining with Pβ1SYN and ab SYN24. As shown in Fig. 3, the average position and molecular size of β2-syntrophin spots were slightly more basic and smaller than those of β1-syntrophin. On the other hand, the basic series of spots stained with MA0 embraced the spots stained with Pβ1SYN, but they showed clearly different patterns from each other. These results suggest that the 62-kDa protein recognized by MA0 is the DAP distinct from β1- and β2-syntrophins.

3.2. *Cloning the cDNA to code the protein which reacts with MA0*

In order to identify the proteins which react with MA0, we screened the neonatal rabbit heart lambda ZAP II cDNA library with the MA0 IgG. One positive plaque was isolated from 9×10^5 plaques examined. The 1.6-kb cDNA A0–1 was obtained and partially sequenced (Fig. 4A). We found that at least the first one hundred amino acids coded by this cDNA are well homologous to those of the *Torpedo* 87K protein with an overall 82% amino acid identity over 106 amino acids (amino acids 167–272) (Fig. 4B).

4. Discussion

The nature of A0, which we described as one of the DAPs [5], has been left undetermined. In the present study, we showed that the monoclonal antibody MA0 reacts with A0 (94 kDa) as well as a 62-kDa protein whose quantity was much larger than that of A0. The 62-kDa protein appears as a diffuse band on SDS-PAGE and as a series of basic, broad and diffuse spots on 2-dimensional PAGE. These obsevations led us think the protein to be one of β-syntrophins. However, we did not see the protein react with the anti-β-syntrophin antibodies suggesting it to be the DAP different from β-syntrophins. We tentatively name the 94- and 62-kDa DAPs α- and β-A0s, respectively. We at present do not know their relationship.

We cloned a fragment of cDNA that codes the protein recognized by MA0 from a neonatal rabbit heart lambda ZAP II cDNA library. Partial sequence analysis of the cDNA clearly showed that the protein was homologous to the *Torpedo* 87K protein [26]. Based on this finding together with the fact that α-A0 has a molecular mass similar to the 87K protein, we concluded that α- and β-A0 are its rabbit homologues. Recently, the proteins that interact with rat syntrophin(s) were examined by immunoaffinity precipitation with ab 1351 raised against *Torpedo* syntrophin [27]. Among the proteins, those with electrophoretic mobilities similar to α- and β-A0 were

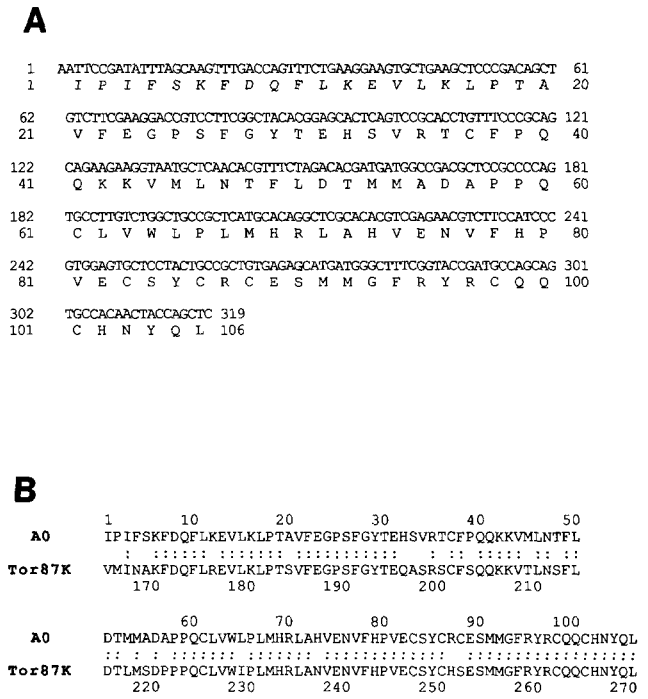

Fig. 4. Partial nucleotide sequence of the cDNA A0–1 and deduced amino acids (A). A comparison of the deduced amino acid sequence of A0–1 with that of the *Torpedo* 87K protein (B).

detected in various tissues by immunoblot analysis with the antibody of the *Torpedo* 87K protein. Interestingly, the quantitative ratio of these proteins detected in the skeletal muscle looks very similar to that of α- to β-A0s contained in dystrophin-DAP complex (Fig. 1). These results demonstrate that rabbit DAPs, A0s, closely resemble the rat homologues of the *Torpedo* 87K protein.

The *Torpedo* 87K protein was first identified as a protein closely associated with the acetylcholine receptor [28], and then found to associate with *Torpedo* syntrophin (58K protein) [26,29]. Recently, the 87K protein was reported as a dystrophin homologue, since it was homologous to the cysteine-rich and C-terminal domains of dystrophin with an overall 27% amino acid identity over 546 amino acids [26]. On the other hand, it was shown that the protein also associates with dystrophin [26–27,29], but it was not clear whether its association was direct or through syntrophin. In the previous reports [9,19], however, we showed that A0 (α-A0) directly associates with dystrophin at a common site with β1-syntrophin. Therefore, the mammalian homologues of the *Torpedo* 87K protein should be considered to be the proteins which directly associate with dystrophin. The nature of A0s may be close to that of β1-syntrophin rather than dystrophin.

Finally, this is the first report showing the presence of β2-syntrophin in the purified dystrophin-DAP complex. β2-Syntrophin was detected as a series of slightly more basic spots than β1-syntrophin. This result coincides well with the isoelectric points of β1- and β2-syntrophins, 9.3 and 9.2, respectively, calculated from the deduced amino acid sequences of cDNAs [14,15]. β2-Syntrophin appears to be a less abundant DAP than the other syntrophins, since it was detected only as a series of tiny spots when a large amount of sample was electrophoresed on a gel. This result is compatible with the immunohistochemical results recently reported that β2-syntrophin was localized only at the neuromuscular junction [17].

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