# Mammalian α1- and β1-Syntrophin Bind to the Alternative Splice-prone Region of the Dystrophin COOH Terminus

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*Abstract.* The carboxy-terminal region of dystrophin has been suggested to be crucially important for its function to prevent muscle degeneration. We have previously shown that this region is the locus that interacts with the sarcolemmal glycoprotein complex, which mediates membrane anchoring of dystrophin, as well as with the cytoplasmic peripheral membrane protein, A0 and β1-syntrophin (Suzuki, A., M. Yoshida, K. Hayashi, Y. Mizuno, Y. Hagiwara, and E. Ozawa. 1994. *Eur. J. Biochem.* 220:283–292). In this work, by using the overlay assay technique developed previously, we further analyzed the dystrophin-syntrophin/A0 interaction. Two forms of mammalian syntrophin, α1- and β1-syntrophin, were found to bind to very close but discrete regions on the dystrophin molecule. Their binding sites are located at the vicinity of the glycoprotein-binding site, and correspond to the amino acid residues encoded by exons 73-74 which are alternatively spliced out in some isoforms. This suggests that the function of syntrophin is tightly linked to the functional diversity among dystrophin isoforms. Pathologically, it is important that the binding site for α1-syntrophin, which is predominantly expressed in skeletal muscle, coincides with the region whose deletion was suggested to result in a severe phenotype. In addition, A0, a minor component of dystrophin-associated proteins with a molecular mass of 94 kD which is immunochemically related to syntrophin, binds to the same site as β1-syntrophin.

Finally, based on our accumulated evidence, we propose a revised model of the domain organization of dystrophin from the view point of protein–protein interactions.

DYSTROPHIN, the protein product of the Duchenne muscular dystrophy (DMD)[1] gene, is a spectrin-like, long slender protein with a molecular mass of 427 kD (Koenig et al., 1987, 1988; Hoffman and Kunkel, 1989). Immunohistochemical and biochemical studies have revealed that it is located on the cytoplasmic surface of the sarcolemma and works as a major component of subsarcolemmal cytoskeleton (Watkins et al., 1988; Arahata et al., 1988; Ohlendieck et al., 1991). Although the defect of this protein has been shown to cause DMD, the molecular mechanism underlying this muscle degeneration process is not well understood yet.

We have focused our attention to the COOH-terminal region of dystrophin, because this region has been suggested to be pathologically important (Beggs et al., 1991). Our previous work has proven that this region is involved in interactions with a number of dystrophin-associated proteins (DAPs), including the components of the sarcolemmal glycoprotein complex (GPC) which mediates membrane anchoring of dystrophin (Yoshida and Ozawa, 1990; Suzuki et al., 1992, 1994). GPC is composed of at least four membrane-spanning glycoproteins (adhalin [50DAG], β-dystroglycan [43DAG], A3b and 35DAG) and one extracellular proteoglycan (α-dystroglycan [156DAG]) (Ervasti et al., 1990; Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991; Ibraghimov-Beskrovnaya et al., 1992, 1993; Roberds et al., 1993). The GPC-binding site on dystrophin is located in a widely extended region encompassing the cysteine-rich domain and the first half of the COOH-terminal domain (Suzuki et al., 1992, 1994), the deletion of which had been shown to obligatorily lead to severe phenotypes (Beggs et al., 1991). Therefore, the lack of interaction between this COOH-terminal region and GPC was suggested to be one of the essential causes of the disease. On the other hand, among the components of GPC, the protein which directly interacts with this GPC-binding site is β-dystroglycan (Suzuki et al., 1994). From our recent studies which showed that β-dystroglycan extracellularly binds to the laminin-binding α-dystroglycan and forms a distinctive subcomplex in GPC named

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1. *Abbreviations used in this paper:* DAP, dystrophin-associated protein; DMD, Duchenne muscular dystrophy; GPC, glycoprotein complex; GST, glutathione-*S*-transferase; SCARMD, severe childhood autosomal recessive muscular dystrophy.

dystroglycan complex, we suggest that the interaction between dystrophin and the dystroglycan complex may serve an essential link between dystrophin and the extracellular matrix (Ibraghimov-Beskrovnaya et al., 1992; Ervasti and Campbell, 1993; Suzuki et al., 1994; Yoshida et al., 1994).

The $NH_2$-terminal domain of dystrophin has been shown to bind actin filaments in vitro (Way et al., 1992; Ervasti and Campbell, 1993). Therefore, it has been postulated that dystrophin mediates the linkage between the extracellular matrix and the actin-based subsarcolemmal cytoskeleton, and the disruption of this linkage results in the damage of the sarcolemma, which, in turn, leads to muscular degeneration (Ervasti and Campbell, 1993; Ohlendieck et al., 1993). However, recent results indicated that, even in the presence of this linkage, muscles may degenerate (Matsumura et al., 1992; Yamanouchi et al., 1994; Mizuno et al., 1994): in the skeletal muscle of the other type of muscular dystrophy (severe childhood autosomal recessive muscular dystrophy [SCARMD]) and its animal model, dystrophin and the dystroglycan complex are expressed almost normally and show normal membrane localization, whereas other components of GPC, namely adhalin, 35DAG and A3b, which form the other subcomplex in GPC named sarcoglycan complex (Yoshida et al., 1994), are selectively reduced. Therefore, it is beginning to appear that, as a complicated protein complex constituted by the dystroglycan and sarcoglycan subcomplexes, GPC not only serves a membrane-anchoring site for dystrophin, but also exerts a more sophisticated function which is crucial for muscle fiber maintenance. In this sense, the COOH-terminal region of dystrophin becomes more important as a locus where complicated protein–protein interactions may occur (Bowe et al., 1994).

In addition to the sarcolemmal glycoproteins in GPC, the cytosolic peripheral membrane proteins, A0 and $\alpha$-/$\beta$-A1 (59DAP), have been reported to be copurified with rabbit skeletal muscle dystrophin (Ervasti et al., 1990; Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991). Among them, $\alpha$- and $\beta$-A1 were recently shown to be the mammalian homologues of a Torpedo 58-kD postsynaptic protein, syntrophin, which has been suggested to be involved in clustering acetylcholine receptors (Froehner 1984, 1987; Adams et al., 1993; Ahn et al., 1994; Yang et al., 1994). In agreement with other laboratories, we renamed $\alpha$- and $\beta$-A1 as $\alpha$1- and $\beta$1-syntrophin, respectively (Peters et al., 1994). In the previous paper, we have shown that A0 and $\beta$1-syntrophin also bind to the dystrophin COOH-terminal region directly, and their binding sites are located within the distal half of the COOH-terminal domain (Suzuki et al., 1994). In the present paper, we advanced the analysis on the binding site for $\beta$1-syntrophin and A0 by using the overlay assay method we developed previously (Suzuki et al., 1994). In the course of this study, we found that not only $\beta$1-syntrophin but also $\alpha$1-syntrophin (syntrophin-1, 59DAP-1), binds directly to dystrophin. The defined binding sites for the two syntrophin isoforms are located very close to each other on the dystrophin molecule but are made up of completely different amino acid sequences. Interestingly, both binding sites are confined within the region which is alternatively spliced out depending on tissue types (Feener et al., 1989; Bies et al., 1992). The results we obtained here may provide important clues for the biological function of syntrophins.

## Materials and Methods

### Expression and Purification of Dystrophin Deletion Mutants as Fusion Proteins with Glutathione-S-Transferase

All deletion mutant constructs shown in Fig. 1 were generated from pGEX/DCT685 described previously (Suzuki et al., 1994) using restriction enzymes. This construct contained the 3.7-kb HindIII fragment of dystrophin encoding the COOH-terminal residues 3026-3685, which we ligated into the EcoRI site of pGEX-3X (Pharmacia LKB Biotechnology, Piscataway, NJ) using HindIII–EcoRI adapters made by mixing EcoRI–SmaI and HindIII–EcoRI adapters (Takara Shuzo Co., Ltd., Kyoto, Japan). During the construction of deletion mutants, the correct colonies were verified by restriction mapping of the recombinant plasmids or immunoscreening with region-specific anti-dystrophin antibodies (see Fig. 1).

A COOH-terminal deletion mutant, pGEX/DCT535, was constructed by a cut of the pGEX/DCT685 at a convenient XbaI site within the 3′ untranslated region (UTR) and at a StuI site, followed by end blunting and religation. For construction of pGEX/DCT263-685 and pGEX/DCT536-685, a 3.2-kb BamHI–EcoRI fragment of pGEX/DCT685 was at first subcloned into pGEX-2T to adjust the reading frame: pGEX/DCT685 was digested with BamHI and EcoRI and the 3.2-kb restriction fragment derived from the insert was purified from an agarose gel and religated into complementary sites in pGEX-2T. The resultant plasmid was digested by BamHI/AccIII for pGEX/DCT264-685 or by BamHI–StuI for pGEX/DCT536-685 and religated after end blunting. pGEX/DCT444-685 was constructed by the use of a NheI site within the COOH-terminal coding sequence. To achieve this, the NheI site in the 3′ untranslated region was first deleted as follows. pGEX/DCT685 was digested with BamHI and religated to remove nucleotides containing the EcoRI site in the 5′ polylinker site (PLS). The cloned plasmid, which was named pGEX/DCT194-685, was then cut at an XbaI site in the 3′ untranslated region, and at an EcoRI site in the 3′ polylinker site to remove nucleotides containing the second NheI site. After the resultant plasmid was blunt ended, religated, and cloned, the construct pGEX/DCT444-685 was generated by a cut of the plasmid with BamHI–NheI and religated after end blunting. To generate pGEX/DCT444-535 and pGEX/DCT444-494, pGEX/DCT194-685 was digested with StuI–SmaI and BglII–SmaI, respectively, blunt ended and religated. This procedure removed the second NheI site in the 3′ untranslated region. Each cloned plasmid was then processed by BamHI–NheI digestion, blunt ended, and religated.

### Expression and Purification of the Fusion Proteins

Recombinant proteins were expressed in *E. coli* and purified from the soluble fraction of cell lysates with the glutathione-Sepharose column essentially as described previously (Suzuki et al., 1994). The $lon^-$ mutant (ME8426) of *E. coli* was also used as a host strain for the expression of DCT685, DCT535, DCT442, DCT264, and DCT264-685, since these were very susceptible to bacterially endogeneous proteases. To remove degradation products with low molecular masses, gel filtration was performed for these constructs on Superose 12 (Pharmacia LKB Biotechnology). For the other fusion proteins which were stably expressed, the strain HB101 was used and the gel-filtration procedure was omitted. As we described previously (Suzuki et al., 1994), the original method (Smith and Johnson, 1988) of purifying the GST-fusion proteins was modified by the addition of a washing procedure of the glutathione-Sepharose resin-adsorbed fusion proteins with PBS (150 mM NaCl, 10 mM $NaH_2PO_4$/NaOH, pH 7.5) containing 3 mM Mg-ATP, 1 mM DTT and proteinase inhibitors (2 $\mu$g/ml leupeptin, 0.5 $\mu$g/ml aprotinin, 1 mM benzamidine and 0.1 mM PMSF). This procedure eliminates contaminating *E. coli* DnaK (the analog of the vertebrate heat-shock protein 70). In the case of DCT264-685 purification, this washing buffer was changed to a casein/Mg-ATP buffer (30 mM Tris/HCl, 50 mM KCl, 5 mg/ml casein, 10 mM $MgCl_2$, 10 mM ATP, 2 mM DTT and inhibitors, pH 7.5), and the washing period was prolonged to more than 20 min at room temperature, because the sample contained *E. coli* GroEL (the analog of the vertebrate heat-shock protein 60) in addition to DnaK.

### Overlay-binding Assay

The overlay-binding assay was also performed as previously described (Suzuki et al., 1994). Briefly, dystrophin-DAP complex (1 $\mu$g) purified from

rabbit skeletal muscle (Yoshida and Ozawa, 1990; Suzuki et al., 1992) was electrophoretically separated on a 10% gel by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. After being blocked with the PBS containing 0.1% casein and 0.1% gelatin overnight at 4°C, the membrane was incubated with purified fusion proteins for 2 h at room temperature in the presence of 1 mM DTT. Bound fusion proteins were detected with anti-dystrophin antibodies.

### *Electrophoresis and Immunostaining Analysis*

For overlay assay, SDS-PAGE and protein transfer to a polyvinylidene difluoride membrane were performed as described (Laemmli, 1970; Kyhse-Anderson, 1984). For characterization of the expressed fusion proteins, we used an automated electrophoresis system, Phast System (Pharmacia LKB Biotechnology), using the 12.5% precast mini-gel PhastGel for convenience and saving samples. Two-dimensional PAGE was performed essentially according to the method of O'Farrell as described previously (O'Farrell, 1975; Yamamoto et al., 1993). Briefly, 1 $\mu$g of dystrophin-DAP complex denatured with 8 M urea and 2% $\beta$-mercaptoethanol was isoelectrophoretically separated on 1% polyacrylamide capillary gel (4 cm × 1 mm i.d.) containing 8 M urea, 0.5% NP-40 and 2% Ampholine pH 3.5-10. After being equilibrated with a solution containing 0.15 M Tris/HCl (pH 6.8), 2% SDS, 5% $\beta$-mercaptoethanol and 10% glycerol, the capillary gel was placed laterally on top of a 10% slab gel and subjected to SDS-PAGE. Immunostaining was carried out by the use of the Vectastain ABC kit (Vector Labs., Inc., Burlingame, CA) for fusion protein characterization or the Elite ABC kit for overlay assay.

### *Antibodies*

The region-specific anti-dystrophin antibodies used in this study were as follows. The polyclonal antibodies P31b, P33c, and P34a, which were previously reported (Tanaka et al., 1989; Suzuki et al., 1992), were raised against the synthetic polypeptides corresponding to the amino acid sequences of human dystrophin 3186-3200, 3373-3391, and 3495-3544, respectively. A new polyclonal antibody, PEX73, was generated in Japanese white rabbits with GST-free DCT444-494 as an antigen. The antigen was prepared by digestion of DCT444-494 with thrombin and purified by SDS-PAGE on a 20% gel followed by a cut of the corresponding band. A monoclonal antibody, Dy8/6C5, which was raised against the synthetic polypeptide corresponding to the last 17 amino acids at the COOH terminus of the human dystrophin was purchased from Novocastra Laboratories, Ltd. (NE2, 4AA, UK)

### *Peptides*

P34 peptide, which corresponds to the amino acid residues 3495-3544 of the human dystrophin sequence, was the same as the one used for preparation of the region-specific anti-dystrophin antibody P34a (Tanaka et al., 1989).

## *Results*

### *Expression and Purification of Dystrophin Mutants*

In our previous work, we constructed a dystrophin fusion protein, DCT685 (Fig. 1), which expresses the dystrophin COOH-terminal amino acid residues corresponding to the cysteine-rich domain and the entire COOH-terminal domain, and found that it specifically binds to three DAPs, $\beta$-dystroglycan (43DAG), A0, and $\beta$1-syntrophin (Suzuki et al., 1994; see Fig. 3 lane *3*). In that work, by using the two COOH-terminal deletion mutants of DCT685, DCT442, and DCT264, we mainly studied the interaction between the GPC-binding site and $\beta$-dystroglycan. In the present work, to focus on the interactions of dystrophin COOH terminus with A0 and $\beta$1-syntrophin and confine their binding site(s), we newly constructed three kinds of $NH_2$-terminal deletion mutants of DCT685, namely, DCT264-685, DCT444-685, and DCT536-685, as well as one additional COOH-terminal deletion mutant, DCT535 (Fig. 1). In Fig. 2 *a* the typical SDS-PAGE pattern of each fusion protein sample used for the following assays is shown. All of them were confirmed to be reasonably recognized by the predictable region-specific anti-dystrophin antibodies shown in Fig. 1 (data not shown).

### *Binding Site(s) for A0 and $\beta$1-Syntrophin Reside within Residues 3444-3535*

Dystrophin fusion proteins were examined for the DAP-binding activity by the blot overlay assay (Fig. 3). As we

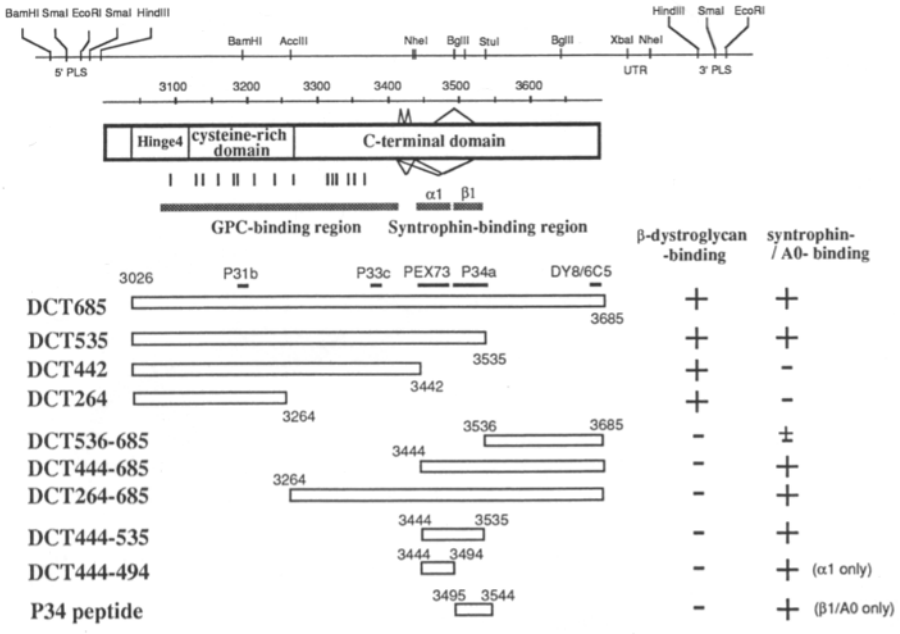


*Figure 1.* A Schematic diagram of dystrophin deletion mutants fused to glutathione-*S*-transferase (GST). The largest bar is a schematic representation of the dystrophin COOH terminus showing the domain organization predicted by Kunkel and co-workers (Koenig et al., 1988; Hoffman and Kunkel, 1989). The restriction endonuclease sites used to construct truncated dystrophin expression vector and the amino acid scale are illustrated above the dystrophin diagram. Vertical lines below the diagram show the position of the cysteine residues. Some of the reported alternative splicing patterns of exons 71-74 are also indicated (Feener et al., 1989; Bies et al., 1992). Shaded bars indicate the locations of GPC- and syntrophin-binding sites defined in the previous (Suzuki et al., 1992, 1994) and the present studies, respectively. A set of smaller bars below shows the locations of the constructed fusion proteins and a synthetic peptide, P34 peptide. The locations of the epitopes for the region-specific anti-dystrophin antibodies used in this study are shown by black bars. The results of the overlay assay method presented here are summarized on the right of each fusion protein.

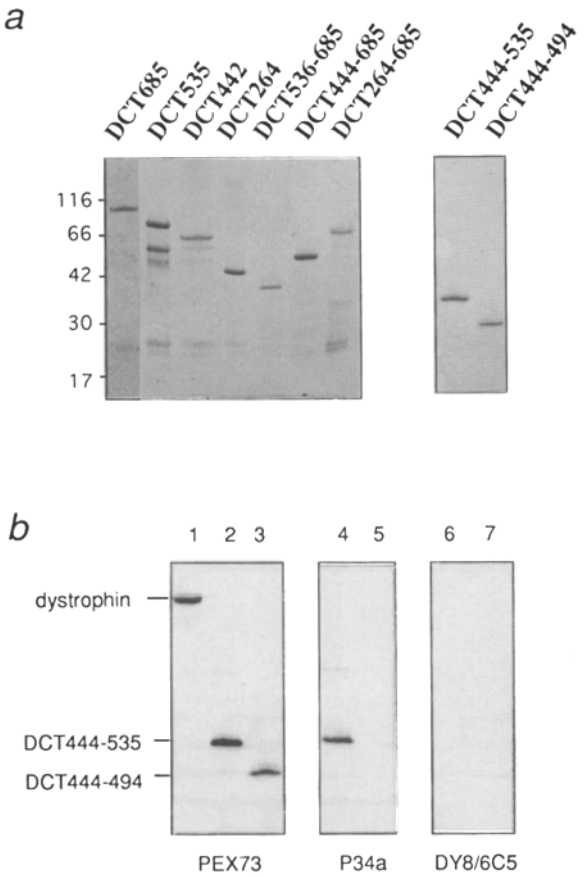


*Figure 2.* Purification and characterization of the constructed fusion proteins. (*a*) The typical SDS-PAGE patterns of the purified fusion proteins stained by Coomasie brilliant blue are shown. (*b*) (Lane *1*) The specificity of the newly prepared anti-dystrophin antibody, PEX73. PEX73 reacts with dystrophin in the purified dystrophin-DAP complex. (lanes *2–7*) Characterization of DCT444-535 (lanes *2*, *4*, and *6*) and DCT444-494 (lanes *3*, *5*, and *7*) with the three region-specific anti-dystrophin antibodies indicated at the bottom.

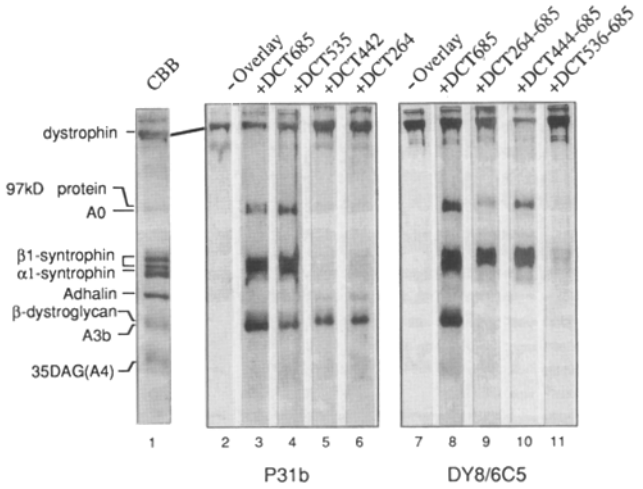


*Figure 3.* The inclusive results of the overlay assays using the constructed dystrophin-deletion mutants. Dystrophin-DAP complex (1 μg) was separated on a 10% gel by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Blot overlay was carried out as described previously (Suzuki et al., 1994). The fusion proteins used are indicated at the top of the lanes, except for CBB, which means a Coomasie blue–stained blot which is not processed by overlay (lane *1*). Some of the lanes are from separate experiments. The protein concentration of each fusion protein sample overlaid (10–20 μg/ml) was adjusted so as to give clear results. The positions of DAPs and the anti-dystrophin antibodies used are indicated on the left and at the bottom, respectively.

showed previously (Suzuki et al., 1994), the binding of dystrophin COOH terminus to A0 and β1-syntrophin is disrupted if the residues downstream from 3443 are deleted (lanes *5* and *6*). On the other hand, a newly constructed COOH-terminal deletion mutant, DCT535 retains the binding ability to both A0 and β1-syntrophin, suggesting that the amino acid residues 3443-3535 are essential for this binding (lane *4*). Next, a series of fusion proteins with progressive $NH_2$-terminal deletions of DCT685 (lanes *8–11*) was examined. $NH_2$-terminal deletions up to residue 3443 did not significantly affect the binding (lanes *9* and *10*), while further $NH_2$-terminal deletion (DCT536-685) resulted in a dramatic reduction in the binding (lane *11*). A low affinity–binding site may exist within the residues 3536-3685, because very weak staining of A0 and β1-syntrophin was observed in lane *11*. However, these deletional analyses of DCT685 from both directions collectively indicated that the essential binding site(s) for A0 and β1-syntrophin resides within the residues between 3444-3535.

### α1-Syntrophin Binds to the Dystrophin COOH Terminus as Well as β1-Syntrophin

In non-skeletal muscle tissues such as heart and brain, four tandem exons, 71-74, of the DMD gene are differentially spliced in at least 10 separate patterns to generate the distinct dystrophin isoforms (Feener et al., 1989; Bies et al., 1992). Interestingly, the essential binding region for A0 and β1-syntrophin defined above closely corresponds to the sequence encoded by exon 73-74 (Fig. 1). Therefore, to confirm the above results and confine the binding site(s) more precisely in relation to the exon structure of dystrophin, we constructed two additional fusion proteins, DCT444-535 and DCT444-494 (Figs. 1 and 6). As shown in Fig. 2, *a* and *b*, both fusion proteins were purified without any degradation fragments and were reasonably recognized with the region-specific anti-dystrophin antibodies including PEX73 which was newly generated using DCT444-494 as an antigen.

Fig. 4 *a* shows the results of the overlay assay with these fusion proteins. As expected, DCT444-535 strongly reacted with A0 and β1-syntrophin, which is compatible with the above results that the residues between 3444-3535 contain the essential binding site for A0 and β1-syntrophin. Furthermore, DCT444-535 also reacted weakly but distinctly with an additional band below the broad staining on β1-syntrophin (lane *3* in Fig. 4 *a*). This band corresponds to α1-syntrophin (α-A1, syntrophin-1 or 59DAP-1), which exhibits 50% homology with β1-syntrophin but is the product of a different gene (Yamamoto et al., 1993; Ahn et al., 1994; Yang et al., 1994). On the other hand, when the shorter fusion protein

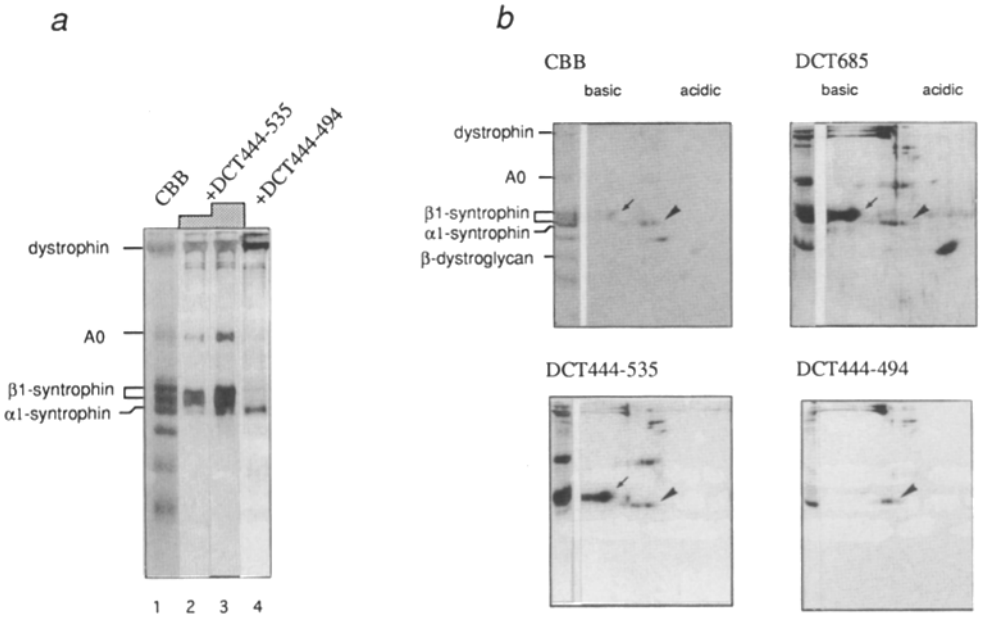


*Figure 4.* Both α1- and β1-syntrophin bind to dystrophin COOH-terminal region. (*a*) The results of overlay assays using DCT444-535 and DCT444-494. The protein concentrations of the overlaid samples are 5 and 25 μg/ml for DCT444-535 (lanes *2* and *3*, respectively), and 20 μg/ml for DCT444-494 (lane *4*). The antibody PEX73 was used for detection. (*b*) The overlay assay onto two dimensionally separated DAPs. The fusion proteins used are indicated at the top of each panel, except for CBB, which represents the Coomasie blue-stained blot membrane. On the left side of each panel is the result of the overlay assays performed on dystrophin-DAP complex separated by one-dimensional SDS-PAGE. An arrow and an arrowhead in each panel indicates the positions of β1- and α1-syntrophin, respectively. The used antibody is PEX73.

DCT444-494, which lacks the COOH-terminal half of the sequence contained in DCT444-535, was overlaid, A0 and β1-syntrophin were not stained, whereas α1-syntrophin was well stained.

Previously, we reported that DCT685 binds to A0 and β1-syntrophin (β-A1), but not to α1-syntrophin (α-A1) (Suzuki et al., 1994). This conclusion was based on the result of the overlay assay onto two dimensionally separated DAPs. In the case of DCT685, the staining on syntrophins was too strong and broad to analyze one dimensionally. We again failed to detect the staining on α1-syntrophin using the fusion proteins DCT444-535 and DCT444-494 under the same conditions as the previous experiment (data not shown). This suggests that the experimental conditions using an automated electrophoresis system with a precast minigel were not suitable to detect the weak binding of dystrophin onto α1-syntrophin. Therefore, in the present study, we improved the experiments by using manually cast gels (see Materials and Methods) and obtained results consistent with those obtained by one-dimensional SDS-PAGE analysis (Fig. 4 *b*): DCT444-535 bound to not only A0 and β1-syntrophin (*arrow*) but also α1-syntrophin (*arrowhead*), whereas DCT444-494 bound only to α1-syntrophin. We also confirmed that DCT685 bound to α1-syntrophin as well as A0, β1-syntrophin, and β-dystroglycan under these conditions.

### *Binding Sites for α1- and β1-Syntrophin Are Separated and Located Tandemly Around Alternative Splice-prone Exons*

As shown in Fig. 5 *a*, P34 peptide (Tanaka et al., 1989), which covers the non-overlap region between DCT444-535 and DCT444-494 (Figs. 1 and 6), bound to A0 and β1-syntrophin but not to α1-syntrophin. In addition, an excess amount of the peptide inhibited the binding of DCT444-535 to A0 and β1-syntrophin significantly, but not the binding to α1-syntrophin (Fig. 5 *b*). Hence, we concluded that (*a*) α1- and β1-syntrophin separately bind to the dystrophin COOH terminus, and (*b*) the binding site for α1-syntrophin resides within the amino acid residues 3444-3494, while that for A0/β1-syntrophin resides within the amino acid residues 3495-3535 (Fig. 6).

Secondary structure analysis (Chou and Fasman, 1978) predicted that the binding site for A0/β1-syntrophin forms a long α-helix flanked by the proline-rich turn structures (Fig. 6). Interestingly, this α-helix takes a leucine-zipper motif and is rich in acidic residues. Because β1-syntrophin is a basic protein (Yamamoto et al., 1993), the latter feature of the binding site suggested that the electrostatic interaction is important for the binding of dystrophin to β1-syntrophin. However, the increase of the ionic strength up to 1 M NaCl scarcely affected the interactions of DCT444-535 to A0/β1-syntrophin as well as to α1-syntrophin (Fig. 5 *c*). This may indicate that syntrophin binding of dystrophin COOH terminus involves a predominantly hydrophobic interaction.

### *Other Regions May Contribute to the Dystrophin-Syntrophin Interaction*

By performing competition assays between the constructed fusion proteins, we examined the syntrophin-binding affinity of each dystrophin COOH-terminal sequence. As shown in lanes *1* and *4* of Fig. 7, DCT444-535, which showed substantial binding activity to syntrophin (Fig. 4 *a*), could not inhibit

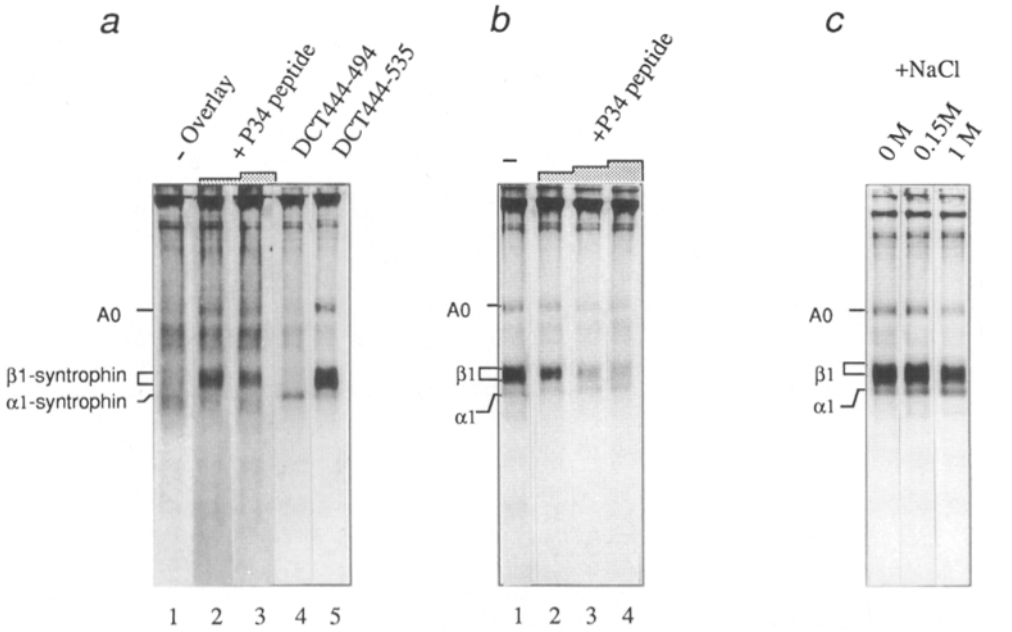


*Figure 5.* Detailed analysis of the binding between dystrophin and two forms of syntrophin. (*a*) An overlay assay was performed using P34 peptide corresponding to the amino acid residues 3495-3544 of human dystrophin as a probe (lanes *2* and *3*). Detection of bound peptide was performed with the antibody, P34a. The concentrations of the overlaid peptide are 170 μg/ml (lane 2) and 500 μg/ml (lane *3*). In lanes *4* and *5*, the results using DCT444-494 and DCT444-535 were shown, respectively, for comparison (the used antibody for detection was PEX73). (*b*) A competition assay between DCT444-535 and P34 peptide. 20 μg/ml of DCT444-535 was overlaid with (lanes *2–4*) or without (lane *1*) an excess amount of P34 peptide. The concentrations of p34 peptide are 57 μg/ml (lane *2*), 280 μg/ml (lane *3*), and 1,100 μg/ml (lane *4*). The binding of DCT444-535 was selectively detected by the antibody, PEX73. (*c*) The effect of salt concentration on the interaction between DCT444-535 and syntrophins. The concentrations of sodium chloride in the reconstituting buffer were changed from 0-1 M as indicated.

syntrophin binding of the entire COOH-terminal sequence of dystrophin (DCT685) even at 20-fold molar excess. This indicated that the flanking region of the essential binding site for syntrophin defined above must contribute to the secure binding to syntrophin. In fact, in contrast with DCT444-535, DCT444-685, which contains the distal full sequence of COOH-terminal region, was found to compete in the syntrophin binding with DCT685 to some extent (lane *3*). Therefore, the amino acid residues 3536-3685 may be involved in syntrophin binding. This idea was also supported by the result of another competition assay (lanes *5* and *6*): DCT535, which lacks this distal COOH-terminal sequence down-

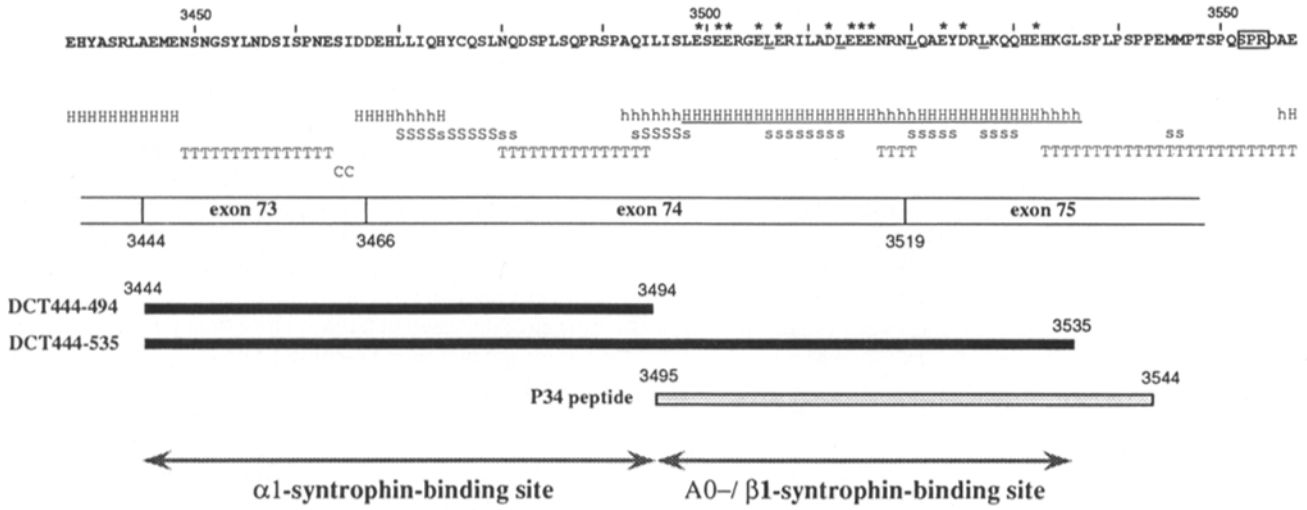


*Figure 6.* Secondary structure analysis of the essential syntrophin-binding region. The amino acid sequence (3438-3557) around the essential syntrophin-binding site is shown at the top with the predicted secondary structure (Chou and Fasman, 1978) and the exon boundaries (Roberts et al., 1993). In the Chou and Fasman analysis, the letters *h*, *s*, *t*, and *c* represent α-helix, β-sheet, turn, and coil structures, respectively. Upper case of each letter suggests the most favorable structure for the residue. Note that the β1-syntrophin–binding site (amino acid residues 3495-3535) is rich in acidic residues (marked with asterisks) and is predicted to take a long α-helix structure (*underlined*). This α-helix contains a stretch of four leucines in the heptad repeat (*underlined*), suggesting that it takes leucine-zipper motif. These leucine residues are well conserved even in utrophin (Tinsley et al., 1992). This leucine-zipper motif precedes the other one which has been suggested to locate within the amino acid residues 3558-3594 (Wagner et al., 1993). One of the potential phosphorylation site by $p34^{cdc2}$ is boxed (Milner et al., 1993). The bars below indicate the positions of DCT444-535, DCT444-494 (*black bars*), and P34 peptide (*lightly shaded bars*).

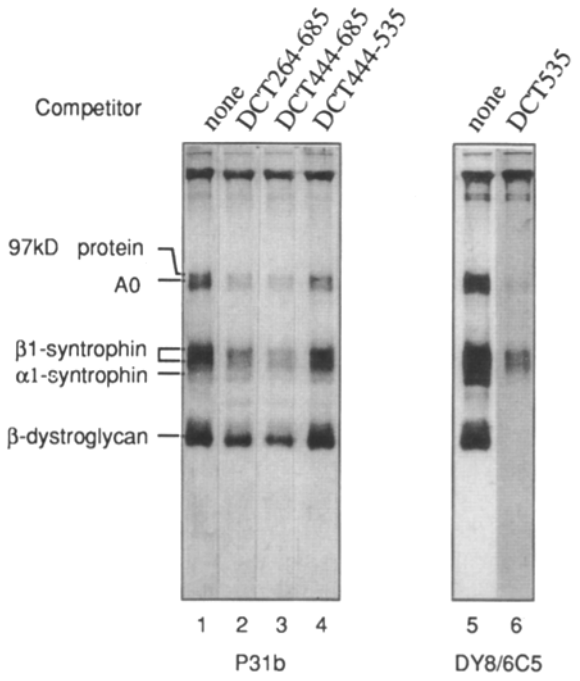


*Figure 7.* Competition assays between DCT685 and its deletion mutants. 20 μg/ml of DCT685 was overlaid with or without an excess amount of the deletion mutant indicated. The antibodies used, which were chosen to selectively detect the binding of DCT685, are indicated at the bottom. The protein concentration of the coexisting fusion proteins (competitors) was 250 μg/ml for all cases.

stream from residue 3536, inhibited syntrophin binding of DCT685 greatly but incompletely, although it completely abolished β-dystroglycan binding of DCT685. These results are consistent with the observation in Fig. 3 which suggested that this sequence may contain another low affinity-binding site for A0/β1-syntrophin.

On the other hand, the GPC-binding region may also affect the syntrophin binding of dystrophin, because the inhibition of DCT444-685 against A0/β1-syntrophin binding of DCT685 was rather incomplete (lane *3*). This was the case when DCT264-685 was used as a competitor in place of DCT444-685 (lane *2*). These results excluded the possibility that the steric hindrance arising from fused GST was the cause of the incompleteness of the inhibition, and suggested that the lack or disruption of the GPC-binding region may weaken the binding affinity of dystrophin to A0/β1-syntrophin.

## *Discussion*

In the present work, by using the overlay assay method, we showed that two forms of syntrophin, α1- and β1-syntrophin (Adams et al., 1993; Ahn et al., 1994; Yang et al., 1994), which are the membrane extrinsic proteins (Froehner, 1984; Ervasti and Campbell, 1991), directly bind to dystrophin at the vicinity of the GPC-binding site (Fig. 1). Their essential binding sites are confined to the region between amino acid residues 3444-3535. In addition, a low affinity-binding site is also suggested to exist at the more COOH-terminal region.

In Fig. 8, we propose a new idea on the domain organization of the dystrophin COOH-terminal region from the viewpoint of protein–protein interactions: the cysteine-rich domain and the first half of the COOH-terminal domain are together renamed "the dystroglycan-binding domain" (amino acid residues 3080-3408), since this region binds to the dystroglycan complex as a single functional unit (Suzuki et al., 1994). It should be noted that this domain is a genuine "cysteine-rich region" in which the well-conserved cysteine residues are concentrated (see Figs. 1 and 8). As we demonstrated previously, these cysteine residues are important for the binding to β-dystroglycan (Suzuki et al., 1994).

On the other hand, since the last half of the COOH-terminal domain, which is not essential for GPC binding (Suzuki et al., 1992, 1994), forms a domain to interact with syntrophins, we propose to call this region "the syntrophin-binding domain" (amino acid residues 3409-3685). This domain contains two leucine-zipper motifs possibly important

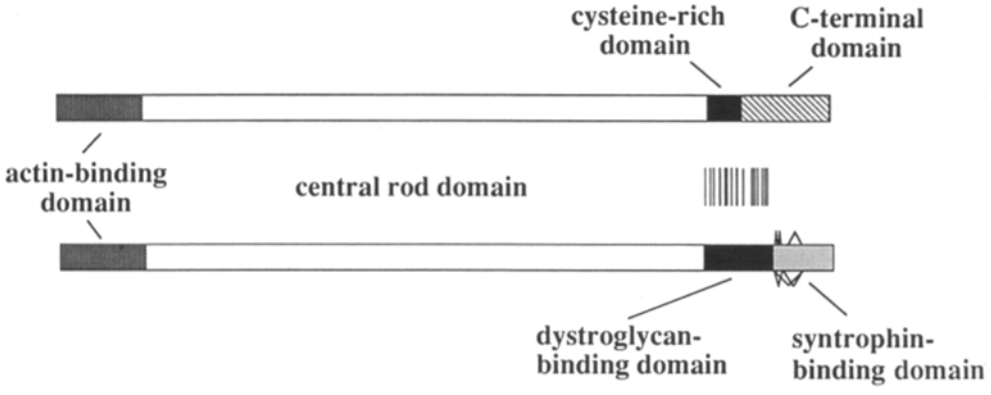


*Figure 8.* A new model of the domain organization of the dystrophin COOH terminus from the viewpoint of protein–protein interactions. The original model (*top*) based on the amino acid sequence analysis (Koenig et al., 1988; Hoffman and Kunkel, 1989) and a new model (*bottom*) based on the results of DAP-binding activities and the limited digestion of dystrophin (Suzuki et al., 1992; Yoshida et al., 1992; Suzuki et al., 1994; and the present work) are illustrated. The difference between the two lies at the COOH-terminal end of dystrophin, where the cysteine-rich domain and the first half of the COOH-terminal domain in the original model are combined to form "the dystroglycan-binding domain" (amino acid residues 3080-3408) in the new model (Suzuki et al., 1994). The remaining distal part of the dystrophin COOH terminus (amino acid residues 3409-3685), which corresponds to the last half of the COOH-terminal domain in the original model, is suggested to interact with syntrophin, thus designated as "syntrophin-binding domain." A0, a minor dystrophin-associated protein with a molecular mass of 97 kD which is immunochemically related to syntrophin, also binds to this domain. The vertical lines represent the positions of the cysteine residues. Alternative splicing patterns so far reported are also indicated (Feener et al., 1989; Bies et al., 1992).

for protein–protein interactions (one of which corresponds to the amino acid residues 3498-3533 [first suggested in this paper ; see Fig. 6] and the other corresponds to the residues 3558-3594 [Wagner et al., 1993]). We identified here that the proximal motif coincides with the essential binding site for β1-syntrophin. The syntrophin-binding domain also contains two predicted phosphorylation sites by $p34^{cdc2}$ (Milner et al., 1993), one of which is located within the intermediate region of the two leucine zipper motifs (Fig. 6). Together with the fact that this domain contains the alternative splice-prone region (Fig. 8; Feener et al., 1989; Bies et al., 1992), these results imply that this is the locus of various regulatory activities for dystrophin function. Because this domain is very susceptible to proteinase digestion, it is considered not to have a tightly packed structure (Suzuki et al., 1992; Yoshida et al., 1992).

It may be of biological importance that the essential syntrophin-binding site defined here (amino acid residues 3444-3535) coincides with the region which is subjected to the tissue-type specific alternative splicing (Fenner et al., 1989; Bies et al., 1992). This is consistent with the results of Ahn et al. (1994) that the sequence encoded by exon 74 is the site for β1-syntrophin binding (the accompanied paper). This means that the function of syntrophin is tightly linked to the isoform-specific function of dystrophin, and some dystrophin isoforms may function without interacting with syntrophins. Most tissues express multiple isoforms of dystrophin, suggesting that multiple functions of dystrophin are required even in a single cell (Bies et al., 1992). As for the isoforms lacking the region encoded by exon 74, they are predominantly detected in brain, heart and smooth muscles, but very rarely in skeletal muscles (Bies et al., 1992). These results indicate that the interaction with syntrophins is indispensable for skeletal muscle dystrophin. This represents a keen coincidence with the components of the sarcoglycan complex (Adhalin, A3b and 35DAG) which are solely expressed in the striated muscles (Roberds et al., 1993; Mizuno et al., 1993; Yamamoto et al., 1994) and the loss or great reduction of which is commonly observed in both SCARMD and DMD (Matsumura et al., 1992; Mizuno et al., 1994; Yamanouchi et al., 1994). This might also suggest that syntrophins work cooperatively with sarcoglycan complex in the modulation of the symptoms. Indeed, the binding site for α1-syntrophin, which is predominantly expressed in skeletal muscle (Adams et al., 1993; Yang et al., 1994), precisely matches amino acid residues 3444-3485, the deletion of which causes a phenotype showing severe muscle wasting (Roberts et al., 1992). In this sense, it is a challenging subject to clarify whether the direct interaction between syntrophin and the components of GPC exists or not.

Recent works on cDNA cloning established the heterogeneity of the mammalian syntrophin (Adams et al., 1993; Ahn et al., 1994; Yang et al., 1994): there are three forms of syntrophin (α1-, β1-, and β2-syntrophin), which originate from different genes (their nomenclature was given in Peters et al. [1994]). In the present work, we have shown that α1- and β1-syntrophin bind to very close but discrete sites on dystrophin. Considering the overall homology (50%) between the two forms of syntrophin (Ahn et al., 1994), it is interesting that they bind to completely different amino acid sequences on the dystrophin molecule (Fig. 6): the binding site for α1-syntrophin (amino acid residues 3444-3494) did not show affinity to β1-syntrophin, and vice versa (Figs. 4 and 5). This finding leads to the suggestion that the amino acid sequence of each syntrophin involved in the interaction with dystrophin should be different from the other. It is tempting to consider that the functional diversity of syntrophins is mostly localized in their respective interaction regions with dystrophin. One might consider the possibility that the bindings of α1- and β1-syntrophins are mutually exclusive, because the binding loci for both syntrophins are too close (Fig. 6). However, this possibility may be excluded by our preliminary observation that α1- and β1-syntrophin associate with each other (Yoshida and Ozawa, 1990). Therefore, it is likely that α1- and β1-syntrophin bind to a single dystrophin molecule in the fixed arrangement simultaneously.

Finally, we observed that A0 binds to dystrophin at the same region as β1-syntrophin. This protein, with a molecular mass of 94 kD, is a minor component of dystrophin-DAP complex purified from sarcolemma. Because A0 shows similar biochemical nature to syntrophin and cross-reacts with an anti-syntrophin antibody (Yamamoto et al., 1993), A0 may also be a member of the syntrophin family.

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