SKELETAL MUSCLE SYNTROPHIN INTERACTORS REVEALED BY YEAST TWO-HYBRID ASSAY

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ABSTRACT

Syntrophins are the cytoplasmic peripheral proteins of dystrophin glycoprotein complex, of which five (α1, β1, β2, γ1 and γ2) isoforms have been identified so far. Respective syntrophin isoforms are encoded by different genes but have similar domain structures. At the sarcolemma of skeletal muscle, the most abundant α1-syntrophin was shown to interact at its PDZ domain with many membrane proteins. Among them, the AQP4 interaction with α1-syntrophin PDZ domain was demonstrated by a Tg mouse study, prompting us to investigate the interaction between mouse α1-syntrophin (BC018546: nt.267–492, PDZ domain) pEXP-AD502 as prey vector and mouse AQP4 (NM009700: nt.805–969) pDBLeu as bait vector by the yeast two-hybrid assay, resulting in a negative study. We further studied the binding partner of another sarcolemma located β1-syntrophin, and performed a yeast two-hybrid experiment. With human β1-syntrophin as bait and human skeletal muscle cDNA library as prey, we obtained one positive clone which turned out to be α-dystrobrevin. Although the interaction of human β1-syntrophin with α-dystrobrevin has already been shown by immunoprecipitation assay, we have here confirmed this interaction by a yeast two-hybrid experiment.

Key Words: Syntrophins, Aquaporins, Dystrobrevin, Interactors, Yeast two-hybrid assay

INTRODUCTION

Syntrophin is a cytoplasmic protein of 58–60 kDa that was first identified in the postsynaptic membrane of a Torpedo electric organ;3) five isoforms including α1, β1, β2, γ1 and γ2 have been identified so far.4-7) One acidic (α1) and two basic (β1 and β2) proteins of 58–60 kDa were first found followed by two remaining isoforms (γ1 and γ2).7) Among them α1-syntrophin is present at its highest levels in skeletal muscle,1,4-6,8) where it is located close to the inner surface of muscle plasma membrane together with β1-syntrophin. In contrast, β2-syntrophin is mainly concentrated at the neuromuscular junction.7) The five syntrophins show unique tissue expression patterns. α1-Syntrophin mRNA was present at its highest levels in skeletal muscle, while also existing at moderate levels in cardiac muscle, kidney and brain.4) Intestine, liver and testis contained low but detectable levels of α1-syntrophin message.4) β1-Syntrophin message is found primarily in liver with moderate levels in kidney, skeletal muscle and lung, but at very low levels in brain and cardiac muscle.5) β2-Syntrophin message is expressed at the highest levels in testis,
at moderate levels in brain, cardiac muscle, kidney and lung, but only at low levels in intestine, liver and skeletal muscles. The human γ1-syntrophin gene encodes a brain-specific protein; while the transcript of the human γ2-syntrophin gene is expressed in brain and, to a lesser degree, in other tissues including skeletal muscle where γ2-syntrophin protein is localized at the sarcolemma of skeletal myofibers. The human genes for α1-, β1-, β2-, γ1- and γ2-syntrophins are located at chromosomes 20q11, 8q23–24, 16q23, 8q11 and 2p25, respectively.5-7)

The syntrophins are a family of five modular adapter proteins composed of at least one pleckstrin homology domain, a PDZ domain and a C-terminal syntrophin unique region. The name PDZ comes from the first three proteins which contain PSD-95, Drosophila discs large protein, and zona occludens protein 1. PDZ domains in other proteins bind to the C-termini of ion channels and neurotransmitter receptors containing the consensus sequence (S/T) XV-COOH and mediate the clustering or synaptic localization of these proteins. The α1-Syntrophin binds at its PDZ domain to various plasma membrane proteins, such as aquaporin (AQP) and neuronal nitric oxide synthase, and PDZ-domain-disrupted α1-syntrophin transgenic mice lack membrane localization of AQP4, which is absent at the sarcolemma and perivascular astrocyte endfeet in α1-syntrophin knockout mice. PDZ domains of β1- and β2-syntrophins interact with SCN4A, a skeletal muscle Na+-channel, and SCN5A, a cardiac muscle and intestinal smooth muscle Na+-channel, by the intermediary of the C-terminal sequence motif; however, whether β1- and β2-syntrophins interact with the AQP4 molecule remains unknown. β2-Syntrophin in skeletal muscle is concentrated at the neuromuscular junction rather than in an extrajunctional sarcolemma. β1-Syntrophin, however, is expressed in the entire sarcolemma, especially in fast-twitch myofiber, as is AQP4 molecule. If there is an interaction between AQP4 and basic syntrophins, the more likely candidate for a possible binding partner of AQP4 molecule would be β1-syntrophin.

Having been studying the expression of AQPs in skeletal myofibers, we became very interested in the presence of possible interactions of syntrophins with AQP4 molecule. Therefore, using this method, we carried out investigations into the interaction of α1-syntrophin and AQP4 by yeast two-hybrid assay as well as the search for β1-syntrophin binding partner(s) in human skeletal muscle.

MATERIALS AND METHODS

PCR cloning of syntrophins and AQP4

For the PCR amplification of the mouse α1-syntrophin (mSnt a1) cDNA (BC018546: nt.267–492, PDZ domain), mouse liver was used as a template, and the following primers were designed: forward, 5′-CAGGCTTTGTCCAGCCGCGGCGCAGCCATCAGCATC-3′ (mSnt a1 PDZ SalI Fw); reverse, 5′-GTACGCGTGCGGCCGAGAATACCTTACCTCCAAC-3′ (mSnt a1 PDZ NotI Rv). For the PCR of the mouse AQP4 cDNA (NM009700: nt.805–969), mouse lung was used as a template, and the following primers were designed: forward, 5′-GTACGCGTGCGGCCGAGAATACCTTACCTCCAAC-3′ (mSnt a1 PDZ NotI Rv). For the PCR of the mouse AQP4 cDNA (NM009700: nt.805–969), mouse lung was used as a template, and the following primers were designed: forward, 5′-GTACGCGTGCGGCCGAGAATACCTTACCTCCAAC-3′ (mSnt a1 PDZ NotI Rv). For the PCR of the mouse AQP4 cDNA (NM009700: nt.805–969), mouse lung was used as a template, and the following primers were designed: forward, 5′-GTACGCGTGCGGCCGAGAATACCTTACCTCCAAC-3′ (mSnt a1 PDZ NotI Rv). For the PCR of the mouse AQP4 cDNA (NM009700: nt.805–969), mouse lung was used as a template, and the following primers were designed: forward, 5′-GTACGCGTGCGGCCGAGAATACCTTACCTCCAAC-3′ (mSnt a1 PDZ NotI Rv). For the PCR of the mouse AQP4 cDNA (NM009700: nt.805–969), mouse lung was used as a template, and the following primers were designed: forward, 5′-GTACGCGTGCGGCCGAGAATACCTTACCTCCAAC-3′ (mSnt a1 PDZ NotI Rv). For the PCR of the mouse AQP4 cDNA (NM009700: nt.805–969), mouse lung was used as a template, and the following primers were designed: forward, 5′-GTACGCGTGCGGCCGAGAATACCTTACCTCCAAC-3′ (mSnt a1 PDZ NotI Rv). For the PCR of the mouse AQP4 cDNA (NM009700: nt.805–969), mouse lung was used as a template, and the following primers were designed: forward, 5′-GTACGCGTGCGGCCGAGAATACCTTACCTCCAAC-3′ (mSnt a1 PDZ NotI Rv).
dNTPs, 0.02 unit KOD-Plus-(1 unit/μl), and template DNA without or with 2× or 4× enhancer solution. The cycling protocol was as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 68°C for 3 min, and finally at 68°C for 5 min. PCR products were analyzed on 0.8% agarose gels and purified from gels by QIA quick gel extraction kit (Qiagen).

Construction of expression vector for yeast two-hybrid experiment, its sequence analysis and plasmid purification

The obtained PCR products were treated by restriction enzymes Sal I and Not I and incorporated by ligation reaction into bait vector pDBLeu and prey vector pEXP-AD502, which were also treated by the same two restriction enzymes. Thus the PCR product was subcloned into the Sal I/Not I sites of the vector pDBLeu and vector pEXP-AD502 to generate the constructs mAQP4 pDBLeu and mSnt a1-PDZ pEXP-AD502, or hSnt b1 pDBLeu. The bait and prey strains were created by incorporating mAQP4 pDBLeu and mSnt a1-PDZ pEXP-AD502 into Escherichia coli (E. coli) Mach-T1, which was incubated in LB buffer at 37°C overnight. A bait strain was created by incorporating hSnt b1 pDBLeu into E. coli TOP10 which was also incubated in LB buffer at 37°C overnight. The insert size of the clones obtained by the incorporation was analyzed using the PCR products, and then a sequence analysis of the insert ends was performed by restriction enzymes Sal I and Not I. The PCR primers were as follows: forward, 5’-GAATAAGTGCGACATCATCATC-3’ (pDBLeu-5’); reverse, 5’-GTAAATTTCTGGCAAGGTA-GAC-3’ (pDBLeu-3’) and forward, 5’-TATAACGCCTTGGGAATCAGC-3’ (pEXP-AD502 seqFw); reverse, 5’-GTAAATTTCTGGCAAGGTA-GAC-3’ (pEXP-AD502 seqRv). The reaction mixture contained 11.25 μl Platinum PCR Super Mix, 0.25 μl each of 10 μM primer pairs, colony, and 0.75 μl distilled water. The cycling protocol was as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 68°C for 3 min, and finally at 68°C for 5 min. To confirm whether or not the inserted sequence was hSnt b1 cDNA, using the extracted PCR product, a total DNA sequence analysis of the inserted full-length hSnt b1 cDNA was performed by the following primer: 5’-GTATGTGTGAACTGGGCTGAACCAG-3’ (seqRv2). The bait construct that incorporated hSnt b1 was then extracted at the transfection grade from the incubated E. coli TOP10 using the Invitrogen PureLink HiPure Plasmid Midiprep Kit.

Screening of syntrophin interactors by yeast two-hybrid experiment

With regard to the interaction of mSnt a1-PDZ and mAQP4, screening was performed in the SD-LTH medium plate containing 10 mM of 3 amino-1, 2, 4-triazole, using bait vector mAQP4 pDBLeu and prey vector mSnt a1-PDZ pEXP-AD502.

To search for hSnt b1 interactors, large-scale library screening was performed in the SD-LTH medium plate containing 50 mM of 3 amino-1, 2, 4-triazole, using bait vector hSnt b1 pDBLeu and prey vector ProQuest Two-Hybrid cDNA Library Human Skeletal Muscle (Invitrogen Cat No. 11367-018). When the variety of colony sizes became evident after incubation at 30°C for 3 days, 90 large colonies were picked up from the plate and incubated at 30°C overnight in the SD medium with 3 amino-1, 2, 4-triazole lacking leucin, tryptophan and histidine. The individually incubated clones were spotted on the nylon filter (Biodyne A 0.45 μ; Pall Corporation) which was put on the SD medium plate lacking leucin and tryptophan, the SD medium plate with 3 amino-1, 2, 4-triazole lacking leucin, tryptophan and histidine, and the YPD medium plate, and further incubated at 30°C during 3 days for the former 2 medium plates and overnight for the YPD medium plate. The growing colonies on the nylon filters were assayed with β-galactosidase. When the results of a β-galactosidase assay showed false-positive results, an additional experiment was performed with a SD medium plate without Ura and lacking leucin and tryptophan in order
Evaluation of potential interactors

The DNA including prey plasmid was extracted from the potentially positive clones obtained from second screening of the yeast colony with hSnt b1 pDBLeu and ProQuest Two-Hybrid cDNA Library Human Skeletal Muscle. The extracted DNA was introduced into *E. coli* DH10B by electroporation that was then incubated at 37°C for 3 days in the LB medium agarose plate containing 100 ng/μl ampicillin. Finally, 8 colonies were selected and PCR amplification was performed using the following primers: forward, 5’-TATAACGCGTTTGGAAATCAGCT-3’ (pEXP-AD502 seqFw); reverse, 5’-GTAAATTTCTGGGAAAGGTAGAC-3’ (pEXP-AD502 seqRv which is the same as pDBLeu-3’). The reaction mixture and the PCR cycling protocol were the same as those of primers pDBLeu-5’ and pDBLeu-3’. Furthermore the PCR products were treated with the restriction enzymes *Sal I* and *Not I*, and their insert size was estimated by 0.8% agarose gel electrophoresis. The resulting PCR products were purified from gels using the QIA quick gel extraction kit (Qiagen). The sequence was established by the DNA sequencing unit with a one-pass sequence using the previously described primer pEXP-AD502 seqFw.

**RESULTS**

**PCR cloning of syntrophins and AQP4**

The mAQP4 cDNA (NM009700: nt.805–969), mSnt a1 cDNA (BC018546: nt.268–492, PDZ domain) and hSnt b1 cDNA (NM021021) were successfully cloned by PCR, and their sequences completely coincided with those of the respective molecules.

**Confirmation of sequence and direction of inserted total DNA of bait and prey vectors and purification of vectors**

The analyses of the mAQP4, mSnt a1, and hSnt b1 cDNA insert sizes of PCR products of the constructs mAQP4 pDBLeu, mSnt a1-PDZ pEXP-AD502, and hSnt b1 pDBLeu revealed the estimated size, confirming that these cDNAs were shown to be incorporated correctly in the bait and prey constructs. Furthermore, the sequence analyses of the mAQP4, mSnt a1-PDZ, and hSnt b1 cDNA inserts of the constructs confirmed that complete agreement was achieved with the estimated total cDNA sequences, respectively. Therefore, these constructs were available in the transfection experiments.

**Yeast two-hybrid interaction**

No interaction between bait mAQP4 pDBLeu and prey mSnt a1-PDZ pEXP-AD502 was observed in this study. The large-scale library screening using bait hSnt b1 pDBLeu and prey of ProQuest Two-Hybrid cDNA Library Human Skeletal Muscle in the SD-LT medium plate (Fig. 1A, E) and the SD-LTH medium plate containing a 50 mM concentration of 3 amino-1, 2, 4-triazole (Fig. 1B, E) yielded numerous small and large colonies. Among the distinctly large colonies, 90 were selected and used for further incubation with the methods described in Materials and Methods. Almost all the 90 colonies in this medium plate revealed blue β-galactosidase activity (Fig. 1C, E). In order to exclude false positive reactions, an additional experiment was performed in order to detect stronger interactions using an SD medium plate without leucin, tryptophan, and Ura, which resulted in obtaining one clone (clone D1) (Fig. 1D, E).
Evaluation of potential interactor (clone D1)

E. coli DH10B with cDNA including prey plasmid were cultivated on the LB medium agarose plate containing 100 ng/μl ampicillin. From the growing colonies, 8 were selected, and the PCR was performed for the inserted cDNA. Among the 8 colonies selected, there were 4 in which the PCR product of one prey plasmid insert showed a band with approximately 2 kilobase pairs (D1-No. 01) while that of the remaining 3 prey plasmid inserts revealed bands with several hundred kilobase pairs (D1-No. 03, D1-No. 07, D1-No. 08) (Fig. 2). In the SD-LT medium plate (Fig. 3A, F), bait yeast with and without hSnt b1 plasmid and prey yeast from 4 colonies (No. 01, No. 03, No. 07, No. 08) showed a positive interaction. Even in the SD-LTH medium plate containing 50 mM 3 amino-1, 2, 4-triazole (Fig. 3B, F), a positive interaction was revealed.
Fig. 2  Agarose gel electrophoresis of the PCR products of inserted cDNA in prey plasmid. Each lane from 1 to 8 corresponds to each clone from No. 1 to No. 8. Lanes 1, 2, 4, 5 and 6 contained about 2 kilobase pair bands, while lanes 3, 7 and 8 contained several hundred base pair bands. Lane M: 1 kbPlusDNA Ladder.

Fig. 3  A, B, and F show interacting colonies in the SD-LT medium plate (A, F), the SD-LTH medium plate (B, F) containing 50 mM 3 amino-1, 2, 4-triazole, and clone D1-No. 01 that revealed a definite interaction as well as several other clones that showed weak interactions (B, F). A stronger interaction with bait and prey plasmids is detectable in the SD-LT-Ura medium plate where the bait hSnt b1 pDBLeu and prey D1-No. 01 showed positive interactions (C, F). In the SD-LT+5FOA medium plate, the non-interacting colonies are shown as white dots among which the D1-No. 01 clone is not included (D, F). The results of β-galactosidase assay (E) were the same as those of SD-LT medium plate (A, F) and SD-LTH medium plate with 50 mM 3AT (B, F).
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between the bait yeast with hSnt b1 plasmid and the prey yeast from 4 colonies (D1-No. 01, D1-No. 03, D1-No. 07, D1-No. 08), although the interaction was strongest between bait hSnt b1 yeast and prey D1-No. 01 yeast. The β-galactosidase activity (Fig. 3E, F) showed a similar finding. In the SD-LT+5FOA plate (Fig. 3D, F), negatively interacting yeasts showed white dots, in which the bait yeast did not have hSnt b1 cDNA. Finally, the bait yeast with hSnt b1 and prey yeast from the D1-No. 01 colony demonstrated a definite interaction in the SD-LTH medium plate containing 50 mM 3 amino-1, 2, 4-triazole (Fig. 3B, F). This prey plasmid contained an approximately 2 kilobase pair band that was extracted. The domain sequenced was amino acid sequence 396 to 601, and the result corresponded to the sequence of α-dystrobrevin (Fig. 4).

DISCUSSION

We have been studying the expression of AQPs in normal skeletal muscle together with their altered expression in myofibers of muscular dystrophies. Among AQPs, the expression and distribution of AQP4 in normal myofibers, and its altered expression in dystrophic myofibers have been most intensively investigated. AQP4 expression in skeletal muscle is severely reduced in human muscular dystrophies such as Duchenne and Fukuyama muscular dystrophies and in animal models with muscular dystrophies. AQP4 expression was also found to be absent in the skeletal muscle of α1-syntrophin knockout mice. Adams et al. demonstrated that α1-syntrophin interacted with AQP4 in the PDZ domain of α1-syntrophin. We performed the yeast two-hybrid investigation to determine whether any interaction was present between mouse AQP4 and the PDZ domain of mouse α1-syntrophin in the first step. Although the interaction of mouse AQP4 with the PDZ domain of mouse α1-syntrophin was observed using other methods, the interaction could not be detected in the present yeast two-hybrid assay. However, efforts to search the binding partners of α1-syntrophin using a variety of approaches have been made, and the resulting partners included α-dystrobrevin, AQP4, voltage gated Na+ channel, and kinases. In contrast, the search for the binding partner of β1-syntrophin seem to have fallen short, which is why we performed the yeast two-hybrid experiments with β1-syntrophin as bait and a human skeletal muscle cDNA library as prey. Although we again could not find any interaction between β1-syntrophin and AQPs, we did confirm the interaction of human β1-syntrophin with α-dystrobrevin by the yeast two-hybrid method; this interaction was originally reported by Sadoulet-Puccio et al. using immunoprecipitation assay. They also showed

Fig. 4 Human β1-syntrophin interaction site of prey vector. Human skeletal muscle cDNA library inserted downstream of the GAL4 activating domain (AD) contained an interaction site that turned out to be α-dystrobrevin amino acid sequence 396-601.
the interaction of human α1-syntrophin with α-dystrobrevin by the yeast two-hybrid assay.\(^2\) Using mouse muscle and the syntrophin isoform-specific antibodies, Peters et al.\(^21\) confirmed the association of α1- and β1-syntrophins with dystrobrevin.

The β1-syntrophin interactor α-dystrobrevin is a member of the dystrophin protein complex (DPC) and a key component of the DPC that binds directly to dystrophin and syntrophin.\(^2,29-33\) In mammalian muscle, there are at least three isoforms, i.e., α-dystrobrevin-1 (94 kDa), α-dystrobrevin-2 (62 kDa), and α-dystrobrevin-3 (42 kDa), all of which are generated by alternative splicing of a single α-dystrobrevin gene.\(^33-35\) α-Dystrobrevin-1 and 2 contain dystrophin- and syntrophin-binding sites, while α-dystrobrevin-3 lacks both of these sites.\(^36\) The functional significance of the individual isoform remains unclear. α-Dystrobrevin-deficient mice are reported to suffer abnormalities of the neuromuscular junction\(^37\) and also to develop mild muscular dystrophy.\(^38\) Nevertheless, the loss of α-dystrobrevin results neither in perturbation of the DPC nor in abnormalities of the structural integrity of the sarcolemma.\(^38\) This implies that a mechanism such as aberrant cell signal transduction is also likely to contribute to the pathogenesis of muscular dystrophy.\(^38,39\) Therefore, the α-dystrobrevins have been proposed to play roles both in forming structure at the neuromuscular junction (NMJ) of myofiber and in signaling at the muscle plasma membrane. On the other hand, β1-syntrophin, which is expressed both in the extrajunctional and junctional sarcolemma, is also important for the maintenance of a normal NMJ structure. Shiao et al.\(^40\) found that neuronal nitric oxide synthase (nNOS) null mutants, dystrophin-deficient mdx mice, and α-syntrophin null mutants showed reductions in their concentration of acetylcholine receptors (AchRs) at the postsynaptic membrane. nNOS transgene increased both AchR concentration and the size of NMJs, and partially corrected the defects of NMJs in mdx mice and α-syntrophin null muscles. However, the partial rescue of NMJ structure by the expression of a NOS transgene required the expression of α- or β1-syntrophin at the NMJ, since a partial NMJ rescue was seen in the muscles of α-syntrophin mutants that expressed β1-syntrophin, whereas no rescue was noted in the muscles of α-syntrophin mutants that also lacked β1-syntrophin. These observations confirm that β1-syntrophin in collaboration with α-syntrophin plays important roles in the rescue of altered NMJ integrity. Thus, β1-syntrophin may also contribute to signaling at the myofiber surface, especially at the NMJs. Finally, the perturbed expression of both β1-syntrophin and α-dystrobrevin in the muscles of dystrophin-deficient muscular dystrophy patients may lead to signaling defects at the myofiber surfaces of dystrophic muscles.

Although the interaction of human β1-syntrophin with AQPs was not detected by the present assay system, this does not necessarily exclude an interaction between these two molecules. Further studies with variety of approaches including immunoprecipitation assay may succeed in revealing the interaction of β1-syntrophin with AQPs.

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