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Characterization of the dystrophin-syntrophin interaction using the two-hybrid system in yeast

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Abstract The carboxy-terminal region of dystrophin has previously been shown to interact directly with α1 syntrophin, a cytoplasmic component of the dystrophin-glycoprotein complex, by in vitro biochemical studies such as overlay assay or immunoprecipitation. Using the two-hybrid system, we have isolated from a human heart cDNA library the entire coding sequence of human α1 syntrophin, therefore confirming for the first time this interaction via an in vivo approach. In addition, we have reduced the interaction domain to the distal half of α1 syntrophin.

Key words: Protein-protein interaction; 59 DAP; Duchenne muscular dystrophy

1. Introduction

Dystrophin, the protein product of the Duchenne muscular dystrophy gene, is thought to link the cytoskeleton to the extracellular matrix in skeletal muscle through a glycoprotein complex (DGC). This DGC is composed of at least five sarcolemmal glycoproteins (α and β dystroglycans, α, β, and γ sarcoglycans) and intracellular proteins belonging to the same family: syntrophins [1–4]. Syntrophins are a heterogeneous group of phosphorylated 58–59 kDa proteins encoded by at least three different genes which are classified in acidic (α) and basic (β) components and show a different expression pattern [5]. Alpha-1 syntrophin, which has been cloned in mouse [6] and rabbit [7], is abundantly expressed in skeletal muscle. Beta-1 syntrophin, which has been isolated in human, is ubiquitously expressed but at lower levels in brain and heart than in other tissues [8]. Finally, β2 syntrophin, cloned in mouse and only partially in human [5,8], is expressed at low levels in numerous tissues but seems to be especially concentrated at the neuromuscular junction in muscle [9].

Among DGC proteins, only syntrophins and β dystroglycan have been involved in direct interactions [10,11] with the carboxy-terminus of dystrophin or with an isoform of dystrophin encoding only the last two domains from exon 63 to 79, namely apodystrophin 1 [10]. Binding sites have been localized within residues 3445–3494 of human dystrophin for mouse α1 syntrophin and within residues 3495–3544 for β1 syntrophin by overlay assays with fusion proteins [12] or by co-immunoprecipitation with in vitro translated proteins [13]. However, no in vivo assay has ever demonstrated this interaction. As dystrophin shows a distinct subcellular localization in heart and skeletal muscle, it has been suggested that it could play different roles in these two tissues, potentially by different protein-protein interactions. We have therefore screened a human heart cDNA library using the two-hybrid system in order to look for new partners of dystrophin or of its more distal isoform, apodystrophin 1. Using a bait covering exon 64 to 75 (but deleted of exon 71), we have identified among positive clones several ones coding for a human α syntrophin, very homologous to mouse and rabbit α1 syntrophins.

2. Material and methods

2.1. Two-hybrid constructs

Human dystrophin carboxy-terminus encoding residues 3103–3549 was generated by polymerase chain reaction on the full length dystrophin cDNA deleted of exon 71 and kindly provided by M. Koenig (Illkirch, France), using the following forward and reverse oligonucleotides with incorporated restriction sites: 64 ID *Eco*RI: 5′ CGG AAT TCT CAG CTT ATA GG 3′ and 75 CT *Xho*I: 5′ CCG CTC GAG AGG TGG GCA TCA 3′. The amplified product was cloned into the *Eco*RI/*Sal*I sites of pGBT9 plasmid (Clontech) containing Gal4 binding domain downstream from the *Saccharomyces cerevisiae* ADH1 constitutive promoter and the resulting construct was called pGBT9-3. The insertion was entirely sequenced using dideoxyNTPs and sequenase 2.0 according to the manufacturer's specification (U.S. Biochemical). The background of this construct was appreciated by co-transformation with control plasmids containing the Gal4 transcriptional activation domain (Gal4AD) alone as pGAD424 (Clontech), or with an in phase cDNA such as those for Snif4 [14] or Raf [15] in HF7c yeast strain: *Mat a, ura 3-52, his 3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4-542, gal 80-538, ura3::* Gal4 binding sites-CYC1-LacZ, *lys2::Gal1-His3*. No background for β-galactosidase activity was obtained after overnight incubation at 30°C or for histidine expression after 5 days at 30°C on selective medium lacking tryptophan, leucine and histidine and supplemented with 15 mM 3-amino-1,2,4-triazole (3AT) for all double-transformants.

2.2. Library screening

HF7c yeast strain already transformed with pGBT9-3 plasmid, which contains a tryptophan (TRP1) marker, was transformed with 250 μg DNA of a commercial human heart cDNA library cloned into plasmid pGAD10 (Clontech), which contains the Gal4 activation domain and a leucine (LEU) auxotrophic marker using the already described lithium acetate method [16].

350 000 transformants were plated on selection plates lacking tryptophan, leucine and histidine and supplemented with 15 mM 3AT. After 12 days, colonies grown on this selective medium were replicated on Whatman paper (no. 40). After overnight incubation at 30°C, these filters were immersed in liquid nitrogen for a few seconds and layered on Whatman paper (no. 3) humidified with 3 ml of Z buffer and incubated at 30°C. Z buffer contains 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 30 mM β-mercaptoethanol and 5-bromo-4 chloroindolyl-β-D-galactoside (XGal) at a final concentration of 0.2 mg/ml.

2.3. Rescue of positive plasmids

To rescue Gal4AD fusion plasmids, total yeast DNA was isolated according to the method of Hoffman and Winston [17]. To avoid waste of time in this step, 96 different DNA preparations were placed in 96 well microplates and analyzed by pooling DNAs from each of the 8 rows and 12 columns. Each of these 20 pools was then used to directly electroporate *E. coli* HB101 as recommended in the Bio-Rad user's guide. This step allows one to eliminate the Gal4 DNA-binding

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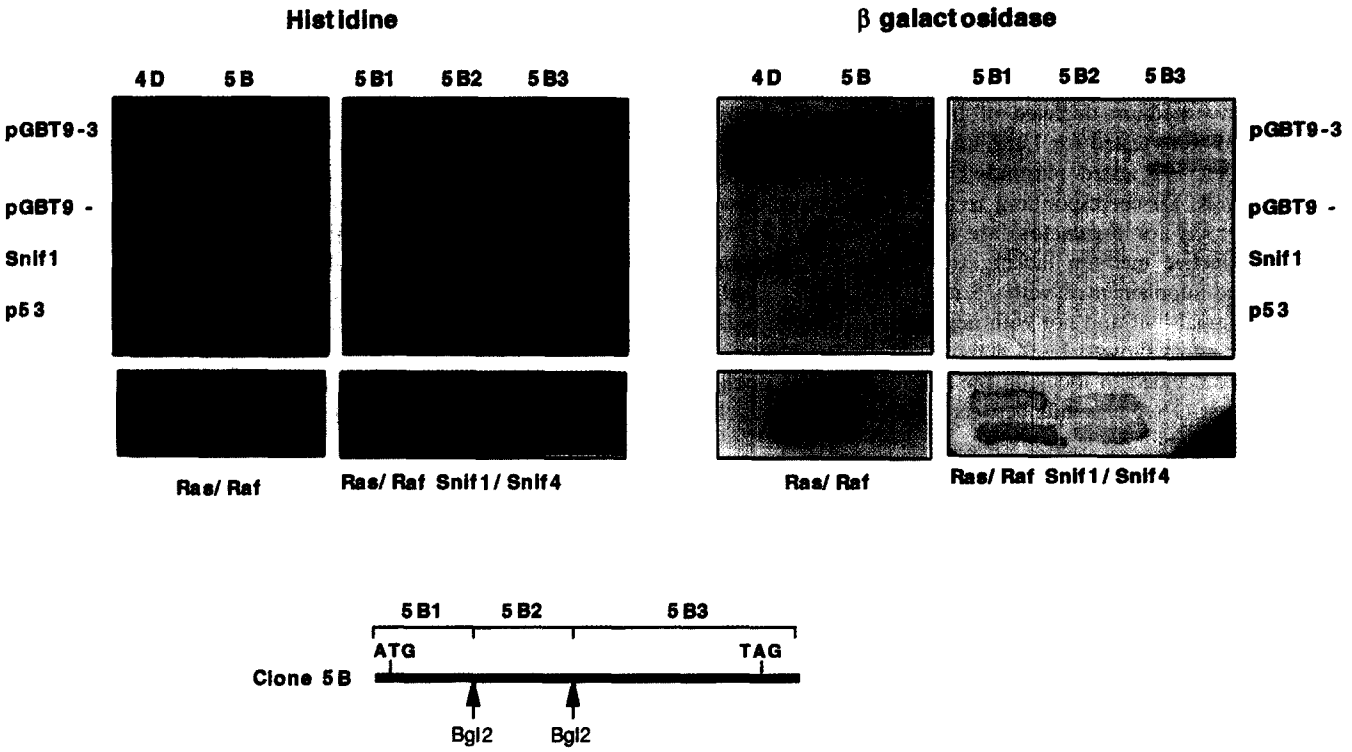


Fig. 1. Histidine and β-galactosidase expression of interacting dystrophin and α1 syntrophin clones. Two independant clones were patched and allowed to grow on selective medium (depleted in tryptophane and leucine) for each co-transformation and replicated either on a filter for LacZ activity or on a medium lacking also histidine and containing 15 mM 3AT for histidine expression. The co-transformed plasmids are indicated above and on the side for each co-transformation. Snif1/Snif4 and Ras/Raf are positive controls of strong interactive proteins. Only the co-transformants pGBT9-3/5B, pGBT9-3/4D and pGBT9-3/5B3 grow on selective medium and show LacZ activity. A schematic representation of clones 5B, 5B1, 5B2 and 5B3 is indicated below with the location of *Bgl*II sites and the initiation and stop codons.

plasmid in M9 medium supplemented with ampicillin because HB101 strain has a defect in the *leuB* gene, which can be complemented by *LEU2* from yeast harbored by the Gal4AD plasmid. M9 medium contains M9 minimal salts 1% (w/v), glucose 0.4% (w/v), NaCl 0.5‰ (w/v), proline 0.1‰ (w/v), Bacto-agar 1.5% (w/v), thiamine 1 mM, $MgSO_4$ 2 mM.

2.4. Subcloning of syntrophin plasmids

The *Eco*RI/*Bgl*II 0.45 kb fragment of clone 5B was subcloned in pGAD424 plasmid, which has been digested with *Eco*RI and *Bam*HI, so that the syntrophin fragment was in phase with Gal4AD, giving the 5B1 construct. After a *Bgl*II digestion of clone 5B, fragments of 0.4 and 0.85 kb were subcloned in pGAD424 digested with *Bam*HI, giving clone 5B2 and 5B3, respectively. These 3 plasmids were co-transformed in Hf7c with the same specific (pGBT9-3) and non-specific plasmids (Gal4 DNA-binding domain fused to non-specific cDNAs) and colonies were allowed to grow on selective medium and tested for LacZ activity.

3. Results

To search for cDNA clones encoding proteins that interact with the carboxy-terminal part of dystrophin, we used the two-hybrid system for detection of protein-protein interaction in yeast [18]. The HF7c yeast strain used for the screen harbors integrated LacZ and HIS3 gene under the control of Gal4 multimerized sites and Gal1 promoter, respectively. These marker genes become transcriptionally active only if a functional trans-activator binds on these promoter sites.

As bait, we created a chimeric plasmid, pGBT9-3, which contains amino acids 3103–3549 but deleted of amino acids 3409–3421 of the dystrophin sequence, fused in frame to the Gal4 DNA-binding domain (Gal4DBD).

We first verified that pGBT9-3 lacked transcriptional activity on its own by co-transforming the HF7c yeast strain with this vector and control vectors containing the Gal4AD alone (pGAD424, Clontech) or fused in phase with different cDNAs as Raf or Snif4 cDNAs.

To identify cDNA clones encoding proteins that interacted with dystrophin, we transformed the above yeast strain with pGBT9-3 and a human heart cDNA library in which the oligo(dT) and random primed cDNAs (mean size 1.7 kb) were fused to the Gal4AD.

Screening 350 000 independent transformants for activation of the LacZ and histidine markers yielded 640 colonies that stained for β-galactosidase activity between 2 h and an overnight 30°C incubation.

Two frequent problems encountered in two-hybrid assays are to isolate positive clones in the original cDNA screen that turn out not to be confirmed after purification of the isolated plasmid, or not to be specific. To determine whether activation of the marker genes reflected a specific interaction, and in order to accelerate this time-consuming step when numerous clones have been isolated in the first screening, each cDNA clone was rescued from yeast and 96 DNAs were pooled in series of 12 and 8 DNAs (see section 2). Each pool of DNAs was used to electroporate *E. coli* HB101, allowing us to perform only 20 electroporations instead of 96. A minipreparation of plasmid DNA was performed after a 2 day liquid culture in M9 medium at 37°C (Wizard kit, Promega) and the DNA obtained was retransformed into the same HF7c yeast strain, either with the original bait pGBT9-3, or with different control plasmid in which the Gal4 DBD is alone

(pGBT9-) or fused to a non-specific cDNA such as those for p53 [19] of Snif 1 [14] which are not expected to interact with dystrophin. These transformed yeasts were directly spotted onto selective medium depleted of tryptophan and leucine. Grown spots were tested for LacZ and histidine activity.

On the first 192 tested plasmids collected in 40 pools, 4 pools (4, 5, B, D) corresponding to 2 columns and 2 rows stained intensely for β-galactosidase activity within 1 h and grew on selective medium lacking tryptophan, leucine and histidine and supplemented with 15 mM 3AT in 48 h, only with the original bait and not with negative control plasmids. Three other pools showed LacZ activity after more than 4 h. We therefore decided to focus on the 4 earlier positive pools. Crossing yeast DNA plasmids between positive rows and columns (clones 5B, 4D) were retransformed into HF7c with the different negative controls. Both showed the same histidine and LacZ expression pattern as the corresponding pools (Fig. 1).

To determine whether clones 5B and 4D might represent previously identified proteins, we sequenced the 5′-and 3′-ends of the cDNA and searched with fasta and blast algorithms in the database for amino acid and nucleotide homologies. Both clones showed a strong homology with rabbit and mouse α1 syntrophins (Fig. 2). 5B and 4D clones were entirely sequenced and showed differences only in their 5′-non-coding extremity (after 14 common 5′-nucleotides, clone 4D shows a 72 nucleotide long DNA stretch, homologous to rabbit syntrophin, which is not present in clone 5B; afterwards both

A

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4D GCGGCCGCTCGACGGGTAGGGTGGCGGCTGGCCCAGCCGGGCCCCGCTGCCCTCTGCCCTGGGCGCTTGGTCGGAGCGGGCTGGGCA -46

AAGCGGGACAGGGCGAGCGCGGCGCCCGGGGGCTCGGAGGCGAAG ATG GCG TCC GGC AGG CCG GCC CCG CGC ACC    30
                                                M  A  S  G  R  P  A  P  R  T
GGG CTG CTG GAG CTG CGC GCC GGG GCG GGC TCG GGG GCC GGC GCG GAG CGA TGG CAG CGG GTG CCT  96
 G  L  L  E  L  R  A  G  A  G  S  G  A  G  A  E  R  W  Q  R  V  P
GTG AGT CTG GCG GAG GAC GTG CTG ACC GTG AGC CCC GCC GAC GGC GAC CCT GGT CCC GAG CCC GGC 162
 V  S  L  A  E  D  V  L  T  V  S  P  A  D  G  D  P  G  P  E  P  G
GCT CCG CGG GAG CAG GAG CCC GCG CAG CTC AAC GAC GCC GCG GAG CCG GGC GCC GGG CCC CCG CAG 228
 A  P  R  E  Q  E  P  A  Q  L  N  D  A  A  E  P  G  A  G  P  P  Q
CTG CCA GAG GCG CTA CTG CTC CAG CGG CGC CGC GTG ACG GTG CGC AAG GCC GAC GCC GGT`GGG CTG 294
 L  P  E  A  L  L  L  Q  R  R  R  V  T  V  R  K  A  D  A  G  G  L
GGC ATC AGC ATC AAA GGC GGC CGG GAG AAC AAG ATG CCT ATT CTC ATT TCC AAG ATC TTC AAG GGA 360
 G  I  S  I  K  G  G  R  E  N  K  M  P  I  L  I  S  K  I  F  K  G
CTG GCA GCT GAC CAG ACA GAG GCC CTT TTT GTG GGG GAT GCC ATC CTG TCT GTG AAT GGG GAA GAC 426
 L  A  A  D  Q  T  E  A  L  F  V  G  D  A  I  L  S  V  N  G  E  D
TTG TCC TCT GCT ACC CAT GAT GAG GCG GTG CAG GTC CTC AAG AAG ACA GGC AAG GAG GTG GTG CTG 492
 L  S  S  A  T  H  D  E  A  V  Q  V  L  K  K  T  G  K  E  V  V  L
GAG GTC AAG TAT ATG AAG GAC GTC TCA CCG TAT TTC AAG AAC TCT ACT GGT GGG ACC TCG GTC GGC 558
 E  V  K  Y  M  K  D  V  S  P  Y  F  K  N  S  T  G  G  T  S  V  G
TGG GAC TCA CCT CCT GCC TCA CCC CTT CAG CGG CAG CCT TCC TCC CCT GGC CCC ACA CCC CGG AAC 624
 W  D  S  P  P  A  S  P  L  Q  R  Q  P  S  S  P  G  P  T  P  R  N
TTC AGC GAG GCC AAA CAC ATG TCC TTG AAG ATG GCA TAT GTC TCG AAG AGG TGC ACC CCC AAT GAC 690
 F  S  E  A  K  H  M  S  L  K  M  A  Y  V  S  K  R  C  T  P  N  D
CCG GAG CCC AGG TAT CTG GAG ATC TGC TCG GCA GAT GGT CAA GAC ACC CTC TTC CTG AGG GCC AAG 756
 P  E  P  R  Y  L  E  I  C  S  A  D  G  Q  D  T  L  F  L  R  A  K
GAT GAG GCT AGT GCG AGG TCG TGG GCG ACT GCC ATC CAA GCC CAG GTC AAT ACT CTG ACG CCG CGG 822
 D  E  A  S  A  R  S  W  A  T  A  I  Q  A  Q  V  N  T  L  T  P  R
GTC AAG GAT GAG CTG CAG GCA CTG TTG GCA GCC ACC AGC ACA GCT GGG AGC CAG GAC ATC AAG CAG 888
 V  K  D  E  L  Q  A  L  L  A  A  T  S  T  A  G  S  Q  D  I  K  Q
ATT GGC TGG CTA ACT GAG CAG CTG CCC AGT GGG GGC ACA GCC CCC ACC CTG GCC CTG CTA ACT GAA 954
 I  G  W  L  T  E  Q  L  P  S  G  G  T  A  P  T  L  A  L  L  T  E
AAG GAA CTG CTC CTC TAC TTG TCT CTC CCC GAG ACC CGC GAG GCC CTG AGC CGG CCA GCC CGT ACT 1020
 K  E  L  L  L  Y  L  S  L  P  E  T  R  E  A  L  S  R  P  A  R  T
GCC CCA CTC ATC GCC ACC AGA CTG GTG CAC TCA GGC CCC TCC AAG GGC TCA GTG CCC TAC GAT GCA 1086
 A  P  L  I  A  T  R  L  V  H  S  G  P  S  K  G  S  V  P  Y  D  A
GAG CTC TCT TTT GCC CTG CGC ACG GGC ACG CGT CAC GGT GTG GAC ACT CAC CTG TTC AGC GTG GAG 1152
 E  L  S  F  A  L  R  T  G  T  R  H  G  V  D  T  H  L  F  S  V  E
TCA CCG CAG GAG CTG GCT GCC TGG ACC CGC CAG CTT GTG GAT GGC TGT CAC CGG GCC GCC GAG GGT 1218
 S  P  Q  E  L  A  A  W  T  R  Q  L  V  D  G  C  H  R  A  A  E  G
GTG CAG GAG GTG TCT ACA GCC TGC ACG TGG AAT GGG CGT CCC TGC AGC CTG TCT GTG CAC ATC GAC 1284
 V  Q  E  V  S  T  A  C  T  W  N  G  R  P  C  S  L  S  V  H  I  D
AAG GGC TTC ACA CTG TGG GCG GCT GAG CCA GGT GCA GCC CGA GCT GTG CTC CTG CGA CAG CCC TTC 1350
 K  G  F  T  L  W  A  A  E  P  G  A  A  R  A  V  L  L  R  Q  P  F
GAG AAG CTG CAG ATG TCT TCA GAT GAC GGT GCC AGT CTC CTT TTC CTG GAT TTT GGA GGT GCT GAA 1416
 E  K  L  Q  M  S  S  D  D  G  A  S  L  L  F  L  D  F  G  G  A  E
GGC GAG ATC CAG CTG GAC CTG CAC TCG TGT CCC AAA ACC ATA GTC TTC ATC ATC CAC TCC TTC CTG 1482
 G  E  I  Q  L  D  L  H  S  C  P  K  T  I  V  F  I  I  H  S  F  L
TCG GCC AAA GTC ACC CGC CTC GGG CTG TTG GCC TAG AAGTCGCCGGATGCACTAGCCCTGAAGAGGGGGTGTCCA 1557
 S  A  K  V  T  R  L  G  L  L  A
TGACATGGCCTGAGCTGGGCCTCCACCGACTGCCTGCTCACCCCTGGGCTGAGGGAAGGGAGTCGAGCGGCCGCGAATTCCA      1642
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Fig. 2. (A) Nucleotide sequence and predicted open reading frame of human α1 syntrophin. Nucleotide sequence of clone 4D. The positions of the nucleotides are indicated on the right from the first translated nucleotide. *Bgl*II sites corresponding respectively to the end of clone 5B1 and beginning of clone 5B2 for the first one and the end of clone 5B2 and beginning of clone 5B3 are in bold. The predicted amino acid sequence is indicated in single-letter amino acid code. (B) Alignment of the amino acid sequence of human, rabbit and mouse α1 syntrophins. The amino acid sequence of the corresponding proteins indicated on the right is shown in single letter amino acid code. Identical residues between two consecutive sequences are indicated by a bar, while similarity is indicated by two points.

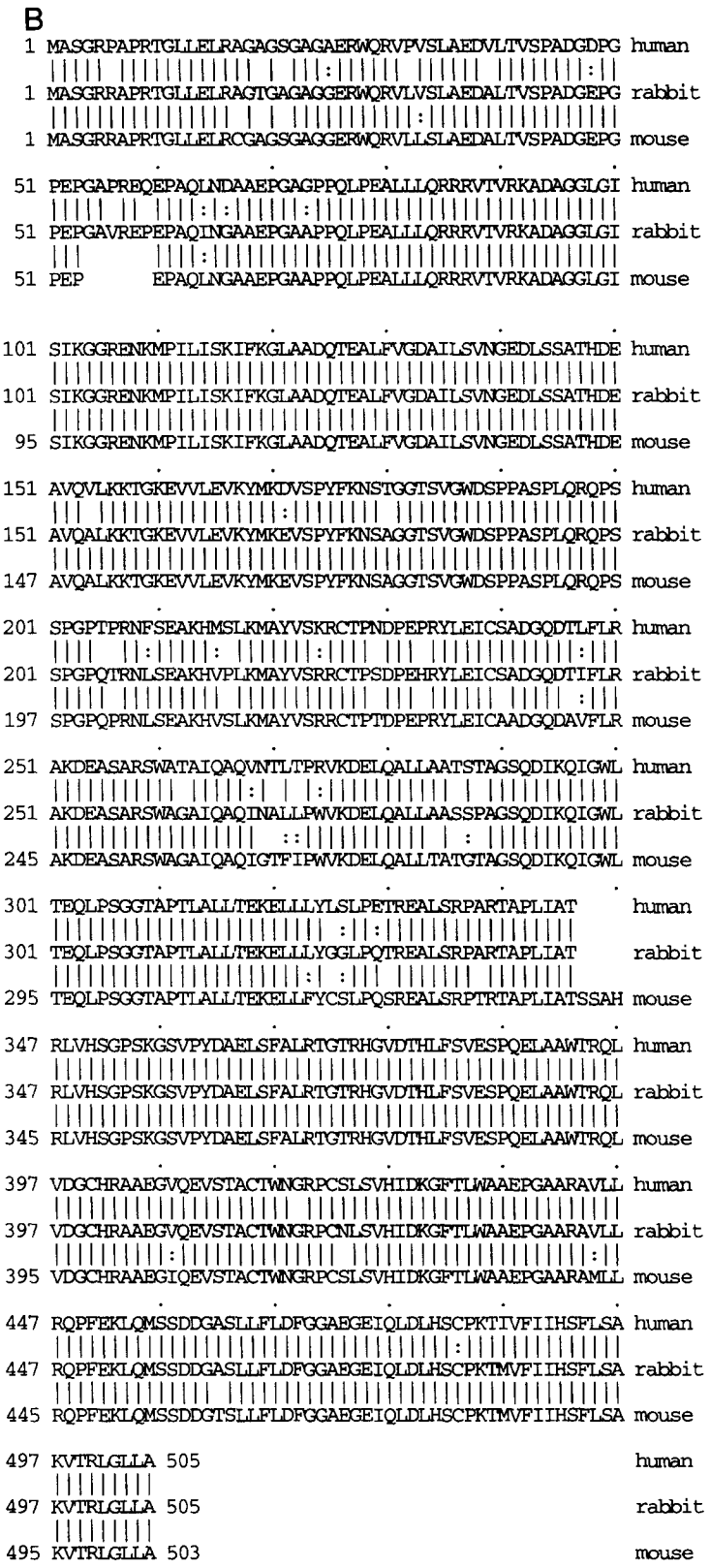


Fig. 2. Continued.

sequences are identical). Human syntrophin sequence shows 95.84% similarity and 92.87% identity with rabbit α1 syntrophin at the amino acid level and 89.28% identity at the nucleotide level.

To investigate further the interaction between human dystrophin and human α1 syntrophin, we subcloned clone 5B into three parts. Before having the entire sequence of clones 5B and 4D, we first verified that some restriction sites were

conserved between rabbit and human. Among *Bgl*II, *Pst*I and *Pvu*II enzymes, used to digest clone 5B, only *Bgl*II gave the expected profile, according to the rabbit sequence. We therefore subcloned the 3 restriction fragments obtained with *Bgl*II in pGAD424 plasmid in the same phase as mouse and rabbit α1 syntrophins. These 3 subclones (5B1, 5B2, 5B3) were tested for their interaction with dystrophin in the two-hybrid system. Only clone 5B3, corresponding to the last 268 amino acids out of the 505 residues of human syntrophin, allows for activation of the reporter histidine and LacZ genes (Fig. 1).

4. Discussion

The syntrophin family is a heterogeneous group of at least three isoforms encoded on three distinct genes. They are all expressed in a wide variety of tissues, but α1 and β1 syntrophins for example show, in the same tissue, a segregated subcellular distribution. Immunoaffinity isolation of syntrophin from a variety of rat tissues has shown that dystrophin, Dp71 or apodystrophin1 and utrophin are able to copurify with syntrophins [10]. Most of the experiments showing direct interaction between dystrophin or its distal isoforms and the different members of the syntrophin family have used immunoprecipitation of in vitro translated proteins, or overlay assays with skeletal muscle extracts [6,12,13]. We have confirmed this interaction between dystrophin and α1 syntrophin in heart by demonstrating an in vivo interaction in yeast using the two-hybrid system. Moreover, using a truncated carboxy-terminal sequence of dystrophin as bait, we have isolated the syntrophin partner among a human heart cDNA library.

Previous studies have located the syntrophin binding sites on the dystrophin sequence to amino acids 3445–3494 for α1 syntrophin and amino acids 3495–3535 for β1 syntrophin and have suggested that the flanking region (amino acids 3536 to 3685) also contributes to the stable binding to syntrophin [12]. The dystrophin bait we used ends at residue 3549 and shows strong activation of the reporter gene, suggesting it is, however, sufficient to interact with syntrophin. By immunoprecipitation with in vitro translated fusion proteins, Ahn and Kunkel [13] showed that the interacting region of β1 syntrophin was carried by its carboxy-terminal half (last 335 amino acids of the 537 residues of β1 syntrophin). Accordingly, we demonstrate in this paper that the carboxy-terminal half of α1 syntrophin, 53% homologous with β1 syntrophin, also interacts with dystrophin.

The human coding sequence of α1 syntrophin was not known previously. As expected, it exhibits a strong sequence conservation with rabbit and mouse α syntrophins. The syntrophin α1 gene has been recently located on human chromosome 20 by Froehner's group [20], who also describes four conserved domains in the syntrophin family members: two pleckstrin homology domains, found in many proteins involved in intracellular signalling, a PDZ domain (for post synaptic density protein-95, *Drosophila* disc large tumor suppressor protein, zonula occludens-1 protein) and a carboxy-terminal 56 amino acid domain, highly conserved among species and isoforms and showing no homology with other proteins [20]. The PDZ domain contains a GLG(F/I) sequence, which is present in a heterogeneous family of signalling proteins localized to specialized cell-cell junctions; the GLG(F/I) motif has also been found in nitric oxide synthase protein (NOS), a protein recently shown to be complexed with dystrophin and abnormally localized in mdx mouse and Duchenne patients' sarcolemma [21]. The PDZ domain of human α1 syntrophin is entirely contained in the 5B1 and 5B2 constructs, with the GLGI sequence present in the 5B1 construct; this domain, therefore, is not involved in the interaction with dystrophin. Instead, the syntrophin/dystrophin binding could involve the conserved 56 carboxy-terminal residues specific to the syntrophin family; we are currently testing this hypothesis.

In conclusion, we have confirmed via an in vivo approach the direct interaction between dystrophin and α1 syntrophin and report the coding sequence of the human syntrophin gene, very well conserved with respect to the mouse and rabbit sequences. By pooling yeast DNA, we also propose a time-winning step to demonstrate the specificity of numerous positive clones after a two-hybrid screen in yeast. The domain involved in the dystrophin/α1 syntrophin interaction is located at the distal half of syntrophin, a region particularly conserved among species and members of this family.

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