The survival motor neuron protein interacts with the transactivator FUSE binding protein from human fetal brain

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Abstract To identify interacting proteins of survival motor neuron (SMN) in neurons, a fetal human brain cDNA library was screened using the yeast two-hybrid system. One identified group of SMN interacting clones encoded the DNA transactivator FUSE binding protein (FBP). FBP overexpressed in HEK293 cells or endogenously expressed in fetal and adult mouse brain bound specifically in vitro to recombinant SMN protein. Furthermore, an anti-FBP antibody specifically coimmunoprecipitated SMN when both proteins were overexpressed in HEK293 cells. These results demonstrate that FBP is a novel interacting partner of SMN and suggests a possible role for SMN in neuronal gene expression.

Key words: Spinal muscular atrophy; Survival motor neuron; FUSE binding protein; Yeast two-hybrid

1. Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disease that is the second most common genetic cause of childhood death [1,2]. The pathological hallmark of SMA is the degeneration of the anterior motor neurons in the spinal cord [3]. SMA is classified into three subtypes (type I or Werdnig–Hoffman disease, type II, and type III or Kugelberg–Welander disease) based on the age of onset and severity of the disease [4]. All three subtypes of SMA map to chromosome 5q11.2–13.3 [5,6] and the determining gene has been identified as the survival motor neuron (SMN) gene [7].

The SMN gene encodes a putative 294 amino acid protein [7] that shares no sequence homology with any known protein. The SMN protein is expressed fairly ubiquitously in a variety of tissues [7,8]. In SMA patients compared to control patients, the relative expression level of the SMN protein is reduced significantly in the spinal cord and liver [8,9] and modestly in fibroblasts, lymphocytes, and skeletal muscle [8], which makes it intriguing why alterations in SMN expression primarily and specifically affect the function of motor neurons in the spinal cord. We have shown that the SMN protein is differentially localized in spinal cord and skeletal muscle [10], two of the target tissues of SMA, which suggests that the specificity of SMN could be due to its association with tissue specific factors or imply tissue specific functions.

Currently, there are several functions proposed for SMN including a role in RNA processing [11–13], a role in antiapoptotic effects [14], and a role in regulating gene expression [15]. However, these studies were performed in various cell types (*Xenopus oocytes*, HeLa, or NT-2) and not in the primary SMA target tissue, the neuron. To date, the only reported study using spinal cord motor neurons suggests that SMN's role in RNA processing is conserved in this tissue [16]. In addition, ultrastructural studies of the spinal cord anterior horn suggest that the SMN protein may have neuronal specific functions in nucleocytoplasmic and dendritic transport [16].

Both SMN mRNA and protein have been detected in fetal tissue [17,18]. Since early embryonic SMN deficient mice undergo massive cell death [19], SMN must play a critical role in development. Determination of SMN's role and identifying the proteins with which it interacts in developing neurons should help to reveal the mechanism by which mutations in SMN lead specifically to the loss of motor neurons. To approach this problem, we used SMN as a bait to screen a fetal human brain library in the yeast two-hybrid interaction trap system and have identified the FUSE binding protein (FBP) as a novel interacting partner of SMN.

2. Materials and methods

2.1. Materials

The *Saccharomyces cerevisiae* EGY48 strain, the human fetal brain acid fusion cDNA library, and the plasmids pEG202, pSH18-34, pJK101, pSH17-4, and pRFHM1 [20] were generously provided by Dr. R. Brent (Massachusetts General Hospital, Boston, MA). The FBP expression plasmid, pcDNA1.1-FBP, and anti-FBP antibody were generously provided by Dr. D. Levens (NIH, Bethesda, MD).

2.2. Yeast two-hybrid interaction trap screening

The manipulation of yeast and the screening of the fetal brain interaction trap cDNA library were performed according to Finley and Brent [21]. Briefly, the SMN cDNA encoding amino acids 1–294 [10] was cloned into pEG202. The resulting bait plasmid, pEG202-SMN, and plasmids of the human fetal brain acid fusion cDNA library were cotransformed into the EGY48 yeast strain. The transformants ($\sim 1.2\times10^6$) were tested for galactose dependent growth on Leu^- medium and for galactose dependent β-galactosidase activity, resulting in 103 positive clones. Plasmid miniprep DNA from each positive yeast clone was isolated and the cDNA inserts were categorized into 16 groups by restriction enzyme analysis [21]. Subsequently, library plasmids from each group were rescued [21] by transforming KC8 *Escherichia coli* (Clontech, Inc., Palo Alto, CA). The interaction with SMN in the EGY48 yeast strain was verified as previously described [21].

2.3. DNA sequencing

The pEG202-SMN bait plasmid and the library plasmid inserts were sequenced as previously described [10].

2.4. Construction of the SMN expression plasmid

To express SMN in cultured mammalian cells, we created a plasmid

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containing a hemagglutinin (HA) SMN chimera by replacing the Rab3 coding region of the pCI-HA-Rab3 plasmid (generously provided by Dr. B. Dickey, Baylor College of Medicine) with the coding region of the SMN cDNA. The resulting plasmid, pCI-HA-SMN, encodes the ~46 kDa HA tagged SMN protein.

2.5. Transient transfection of HEK293 cells and preparation of cell lysates

Monolayer cultures of HEK293 cells (~3×10^6 per 100 mm plate) were transfected with the indicated expression plasmids using the TransFast reagent (Promega, Madison, WI). Forty-eight hours after transfection, the cells were scraped into lysis buffer (50 mM Tris, pH 7.5, 2 mM EGTA, 0.1% NP-40, 2 mM aminobenzamidine, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin). After brief sonication, the cell lysates were clarified by centrifugation at 15 000×*g* for 15 min at 4°C.

2.6. Preparation of brain lysates

At the time they were killed, the whole brains from adult and fetal (E18.5 day) mice were removed and then immediately placed into liquid nitrogen. The frozen brains were weighed, minced, and resuspended in 10 volumes (w/v) of lysis buffer. After homogenization and brief sonication, the brain lysates were clarified by centrifugation at 15 000×*g* for 15 min at 4°C.

2.7. In vitro protein binding assay

Bacterially expressed, purified *E. coli* maltose binding protein (MBP) and MBP–SMN fusion protein [10] were prebound to amylose beads (New England Biolabs, Beverly, MA) for 1 h at 4°C in TE buffer (50 mM Tris, pH 7.5, 2 mM EGTA). Lysates from cells transfected with pcDNA1.1-FBP (~100 μg/450 μl) or from brain tissue (~2 mg/450 μl) were incubated with MBP or MBP–SMN beads overnight at 4°C. After extensive washing, the bound proteins were solubilized in 2×Laemmli SDS sample buffer [22] and separated on a 12% SDS–polyacrylamide gel. The separated proteins were analyzed by Western blot [10] using a 1:1000 dilution of rabbit polyclonal anti-FBP antibody and detected by chemiluminescence using the Super-Signal® substrate (Pierce, Rockford, IL).

2.8. Co-immunoprecipitation assay

Lysates prepared from HEK293 cells transiently transfected with the control vector pCI-HA-Rab3 (containing an unrelated cDNA), pCI-HA-SMN, and/or pcDNA1.1-FBP were precleared by incubation with a 1:500 dilution of normal rabbit IgG for 1 h at 4°C, followed by precipitation with UltraLink™ Immobilized Protein A/G Plus (Pierce, Rockford, IL). The precleared samples were incubated with a 1:500 dilution of the anti-FBP antibody for 2 h at 4°C and then precipitated with Protein A/G Plus. The immunoprecipitates were separated on a 12% SDS–polyacrylamide gel as described above and immunoblotted using a 1:1000 dilution of the mouse monoclonal anti-HA antibody (clone 12CA5, Boehringer Mannheim, Indianapolis, IN) to detect the SMN protein.

3. Results

3.1. Identification of SMN interacting cDNA clones

We screened a human fetal brain cDNA library by the yeast two-hybrid interaction trap method using SMN as the bait, which resulted in 103 positive clones. These positive clones were classified into 16 groups based on restriction enzyme analysis and partial DNA sequencing. One of these groups consisted of 40 independent partial cDNA clones with a ~1.6 kb insert. BLAST search of GenBank revealed that this insert encoded amino acid residues 7–280 of the previously identified SMN interacting protein 1 or SIP1 [12]. A second group consisted of 27 independent partial cDNA clones with an insert size of 0.8 or 1.2 kb. This second group of cDNA clones contained an open reading frame encoding the FBP [23] with the 0.8 kb insert encoding amino acids 571–644 and the 1.2 kb insert encoding amino acids 521–644. Since the number of independent clones in each of the other 14 groups was significantly lower, we have not fully characterized these putative SMN interacting clones.

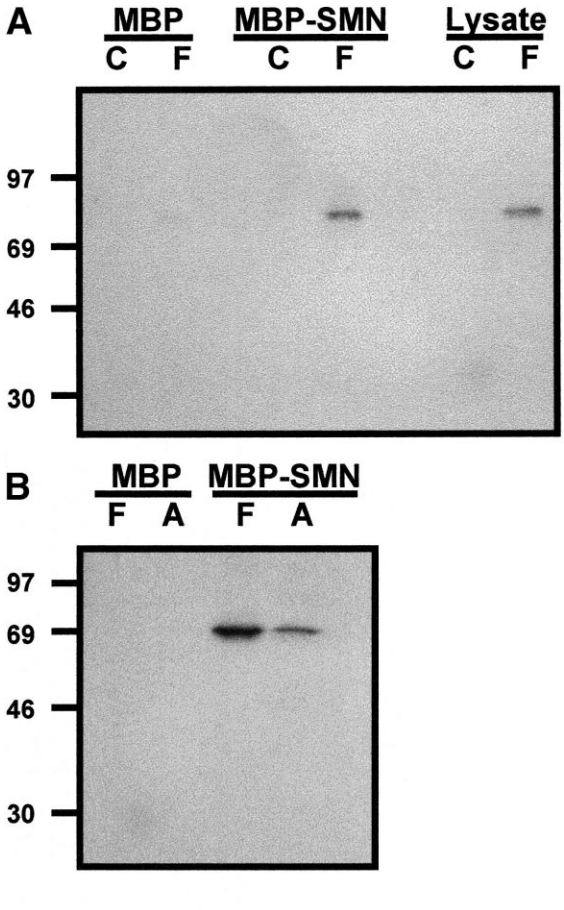

Fig. 1. SMN interacts with FBP in vitro. Cell or tissue lysates were subjected to an in vitro protein binding assay using MBP or MBP–SMN immobilized to amylose beads and then Western blotted with anti-FBP antibody as described in Section 2. A: Western blot of lysates from HEK293 cells transiently transfected with control vector (C) or pcDNA1.1-FBP plasmid (F). B: Western blot of tissue lysates from fetal (F) or adult (A) mouse brain.

To quantify the interactions with SMN, the EGY48 yeast strain was co-transformed with the SMN bait plasmid and one of the library plasmids containing the partial SIP1 or FBP cDNA. In two independent assays, galactose induced β-galactosidase activity in each transformant was quantified using ONPG as the substrate (Table 1). In the presence of glucose, the β-galactosidase levels in the yeast cotransformed with the SMN bait and a library plasmid were similar to those found in the yeast transfected with the negative control plasmid alone. However, in the presence of galactose, yeast co-transformed with the plasmids containing the SMN bait and the SIP1 or FBP partial cDNA displayed high levels of β-galactosidase activity. Additionally, interaction of the expressed SMN bait with the longer fragment of FBP (amino acids 521–644) resulted in ~7.9-fold higher β-galactosidase activity than the shorter fragment of FBP (amino acids 571–644). The β-galactosidase activity resulting from SMN's interaction with the longer FBP fragment was quantitatively similar to that resulting from SMN's interaction with SIP1. These results suggest that in the yeast two-hybrid system, interaction

of SMN with the FBP is as strong as its interaction with the previously characterized SIP1 protein.

3.2. Interaction of SMN with FBP in vitro and in vivo

To verify the yeast two-hybrid results, we tested whether FBP could bind to SMN in vitro. Purified MBP or MBP–SMN bound to amylose beads was separately incubated with HEK293 cell lysates or brain tissue lysates. The bound proteins were resolved by SDS–PAGE and FBP was detected by Western blot analysis. Fig. 1 shows the results of one representative experiment ($n=4$). As shown in Fig. 1A, the recombinant full-length FBP is expressed as a ~74 kDa protein in HEK293 cell lysates and is captured from the lysate by the MBP–SMN fusion protein bound beads, but not by the MBP protein bound beads. Similar results were obtained with the endogenously expressed FBP in fetal and adult mouse brain extracts (Fig. 1B). These results indicate that SMN and FBP interact in vitro.

To demonstrate that SMN and FBP interact in vivo, we performed co-immunoprecipitation experiments ($n=4$). HEK293 cells were transiently transfected with pCI-HA-Rab3 (control plasmid), pCI-HA-SMN (HA-SMN expression plasmid), and both pCI-HA-SMN and pcDNA1.1-FBP (FBP expression plasmid). An anti-FBP antibody was used for immunoprecipitation from cell lysates prepared from each transfected plate. The immunoprecipitates were analyzed by immunoblotting with the 12CA5 monoclonal antibody. The HA-SMN protein was detected as a ~46 kDa protein in the cell lysate prepared from cells transfected with the SMN expression plasmid (Fig. 2A,B). After immunoprecipitation with the anti-FBP antibody, the HA-SMN protein was detected in the lysate of cells overexpressing both HA-SMN and FBP (Fig. 2A,B), but neither in the control lysate (Fig. 2A) nor in the cell lysate expressing HA-SMN alone (Fig. 2B). The prominent band detected in the control lysate at ~32 kDa is the HA tagged Rab3 protein. Similar size but less intense non-specific bands also appear in the other lanes. Taken together, these results indicate that SMN interacts with FBP in vivo.

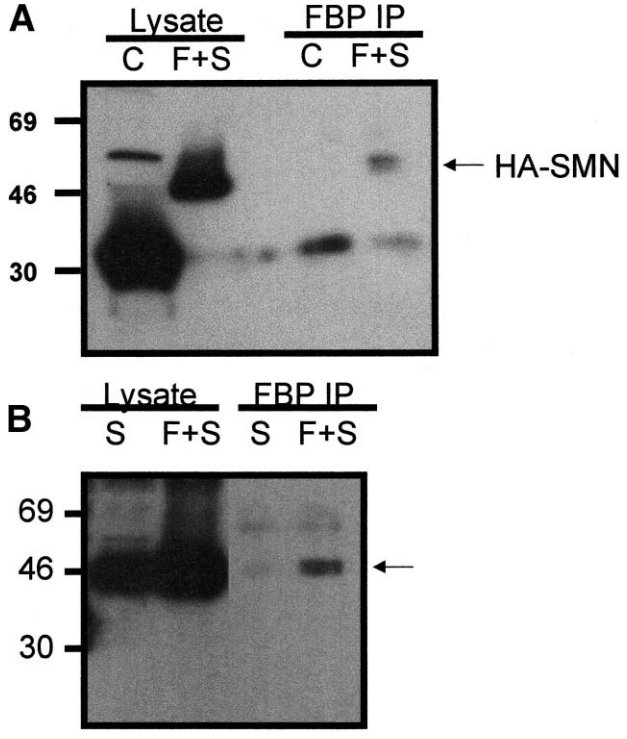

Fig. 2. SMN interacts with FBP in vivo. Cell lysates were prepared from transiently transfected HEK293 cells. The anti-FBP antibody was used for immunoprecipitation (FBP IP) and the anti-HA tag antibody was used for Western blotting as described in Section 2. A: Western blot of lysates and immunoprecipitates from HEK293 cells transfected with control plasmid (C) or with both pcDNA1.1-FBP and pCI-HA-SMN plasmids (F+S). B: Western blot of lysates and immunoprecipitates from HEK293 cells transfected with pCI-HA-SMN plasmid (S) or with both pcDNA1.1-FBP and pCI-HA-SMN plasmids (F+S).

Table 1
Verification of SMN interactions

Expressed proteins	β-Galactosidase activity (AU)	
	glucose	galactose
Lex A/Gal4 fusion protein (positive control)	7935 ± 347	ND
LexA/biconoid fusion protein (negative control)	9 ± 3	ND
SMN+SIP1 (amino acids 7–280)	5 ± 1	1030 ± 88
SMN+FBP (amino acids 521–644)	5 ± 2	912 ± 17
SMN+FBP (amino acids 571–644)	5 ± 1	116 ± 2

EGY48 yeast were transformed with control plasmids or with the SMN bait plasmid and a library plasmid encoding the indicated proteins. The library cDNA inserts are under the control of the GAL1 promoter. The control proteins are under the control of the constitutive ADH promoter. Three independent colonies from each yeast transformation were grown overnight at 30°C in synthetic medium containing either glucose or galactose and then assayed for β-galactosidase activity using *o*-nitrophenyl-β-D-galactoside as the substrate [21]. β-Galactosidase activity is expressed in arbitrary units, AU (mean ± S.E.M.). ND = not determined.

4. Discussion

Previously, SMN was shown to interact with the RNA binding protein fibrillarin [11], a novel protein named SIP1 [12], an anti-apoptotic protein Bcl-2 [14], and the nuclear transcription activator E2 of papillomavirus [15]. Additionally, SMN was demonstrated to interact with itself [11,14]. These SMN interacting proteins were identified either from a HeLa cell cDNA library [11,12,15] or from a human thymocyte cDNA library [14], suggesting that SMN could play a universal role in all cells. However, none of these interactions provide any clue as to the relationship between an SMN gene defect and specifically, degeneration of motor neurons. Using the yeast two-hybrid interaction trap system, we isolated FBP as an SMN interacting protein from a fetal brain library. The SMN–FBP interaction was biochemically verified by in vitro binding of SMN fusion protein to the full-length recombinant FBP expressed in cultured mammalian cells and to the endogenously expressed FBP in fetal and adult mouse brain. Furthermore, the in vivo interaction between SMN and FBP was confirmed by a co-immunoprecipitation assay. Collectively, the results obtained from the yeast two-hybrid system, in vitro protein binding assay, and co-immunoprecipitation assay strongly supports an in vivo interaction of FBP and SMN.

FBP was originally cloned by Duncan et al. [23] and belongs to a family of single stranded DNA binding transactivators [24]. FBP contains two functional domains: a DNA binding domain and a transactivating domain [23,24]. The DNA binding domain is located in the central part of FBP

and is comprised of four 'FBP repeat' sequences [23]. The 'FBP repeat' has high homology with the RNA binding KH domain of hnRNP protein K [25]. The transactivating domain of FBP is located in the C-terminus and contains three tyrosine motifs with the conserved sequence of AW(A/E)(A/E)YY at amino acids 531–547, 584–592, and 609–626 [26]. Interestingly, all the partial cDNA clones of FBP that interacted with the SMN bait in the yeast two-hybrid system encoded at least the last two tyrosine motifs. The FBP clone that elicited the highest β-galactosidase activity when cotransfected with the SMN bait in the yeast EGY48 encodes all three tyrosine motifs. These results suggest that SMN binds within the transactivating domain of FBP and the affinity of this interaction is dependent on the presence of the tyrosine motifs.

Two functions for FBP have been identified: one as a transcriptional regulator of the growth promoting c-*myc* gene [23] and the second as an mRNA stability regulator of GAP-43 [27], which plays a key role in axonal growth and in the formation of neural connections [28]. FBP is expressed only in proliferating and undifferentiated cells and not in quiescent, differentiated cells [23,29]. FBP expression is also developmentally regulated [30]. The highest levels of FBP mRNA and protein were detected in both the mouse and chicken embryonic brain [30]. In adult, however, the FBP protein was detected only in the brain and testis [30]. Furthermore, the relative expression level of FBP protein was decreased in adult mouse brain as compared to that in the embryonic brain [30]. In agreement with the lower expression of FBP in the adult brain, we found relatively less SMN bound FBP in the extract of adult mouse brain than in the extract of fetal mouse brain (Fig. 1B).

The expression of SMN mRNA and protein is also developmentally regulated. The SMN mRNA was detected in neural tissue as early as 8 weeks of human fetal life and was found to be present throughout postnatal and adult life, which suggested that SMN might play a role in neuronal development [17]. More specifically, during the second fetal trimester, SMN mRNA was detected in neuroblast cells and expression in the motor neurons was maintained from neonatal to adulthood [17]. The SMN protein is expressed at 16 fetal weeks with relatively high expression levels detected in the brain and the kidney [18]. In contrast, the relative expression level of SMN was significantly lower in most postnatal tissues examined compared to their respective fetal tissues [18]. Based on the expression pattern in various tissues examined so far, the developmental expression pattern of the SMN protein in brain seems to parallel that of FBP. Since FBP and SMN both are highly expressed in developing neuronal tissue, it is conceivable that an interaction between the SMN and FBP might be critical for motor neuron development and that a disruption of this interaction might contribute to the onset of the SMA disease (especially in type I, which begins in utero and is the most severe).

In summary, we have demonstrated that the DNA transactivator FBP protein is a novel interacting partner of SMN. Since this interaction with SMN was originally identified from a human fetal brain library, this may have relevance to SMN's specific role in the neuron.

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