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The Survival Motor Neuron Protein of *Schizosacharomyces pombe*

CONSERVATION OF SURVIVAL MOTOR NEURON INTERACTION DOMAINS IN DIVERGENT ORGANISMS*

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Spinal muscular atrophy is a common often lethal neurodegenerative disease resulting from deletions or mutations in the survival motor neuron gene (*SMN*). SMN is ubiquitously expressed in metazoan cells and plays a role in small nuclear ribonucleoprotein assembly and pre-mRNA splicing. Here we characterize the *Schizosacharomyces pombe* orthologue of SMN (yeast SMN (ySMN)). We report that the ySMN protein is essential for viability and localizes in both the cytoplasm and the nucleus. Like human SMN, we show that ySMN can oligomerize. Remarkably, ySMN interacts directly with human SMN and Sm proteins. The highly conserved carboxyl-terminal domain of ySMN is necessary for the evolutionarily conserved interactions of SMN and required for cell viability. We also demonstrate that the conserved amino-terminal region of ySMN is not required for SMN and Sm binding but is critical for the housekeeping function of SMN.

Spinal muscular atrophy (SMA)[1] is a common autosomal recessive neurodegenerative disease that is one of the leading hereditary causes of infant mortality (1). SMA is characterized by degeneration of motor neurons of the anterior horn of the spinal cord resulting in muscle weakness and atrophy (2). SMA results from reduced levels of expression or mutations in the survival motor neuron protein (SMN). The SMN gene is duplicated as an inverted repeat on human chromosome 5 at 5q13 (3, 4), and the telomeric copy of *SMN* (*SMN1*) is deleted or mutated in over 98% of SMA patients, although they retain at least one copy of centromeric *SMN* (*SMN2*), which produces mostly carboxyl-terminally truncated SMN protein (5). The SMN protein is expressed in all tissues of metazoan organisms, but particularly high levels are expressed in motor neurons. In contrast, individuals affected by the most severe form of SMA, Werdnig-Hoffman syndrome or SMA type I, have barely detectable levels of SMN in motor neurons (6, 7).

The *SMN* gene encodes a 294-amino acid protein, which is part of a multiprotein complex. Gemin2 (formerly SIP1) associates with SMN directly (8). In addition, SMN interacts with Gemin3 and with several Sm proteins, and it is capable of homotypic interactions (8–12). SMN, Gemin2, Gemin3, and Gemin4, another component of the complex that does not interact with SMN directly (13), are located in the cytoplasm and the nucleus of somatic cells. In the nucleus, these proteins are highly concentrated in gems, nuclear bodies that are similar in size, number, and are often found associated with coiled bodies (8, 9, 12, 13). In higher eukaryotes both SMN and Gemin2 play a crucial role in the assembly of snRNPs (14, 15, 16). This process takes place in the cytoplasm, where the snRNAs combine with Sm proteins, and the assembled snRNPs are subsequently imported to the nucleus where pre-mRNA splicing occurs (17–19). SMN also plays a role in the nucleus, where it is required for maintaining an active pre-mRNA splicing machinery likely by regenerating functional snRNPs after splicing (14). The essential roles of SMN were further demonstrated using mouse and *Caenorhabditis elegans* models (20–24). Mice with homozygous *SMN* disruption display massive cell death during early embryonic development (20). Reduction of SMN protein levels in the spinal cord of SMN heterozygous mice leads to motor neuron degeneration resembling SMA type III (22). Using a mouse model it was also shown that SMA is caused by insufficient SMN production by the *SMN2* gene (24).

A candidate orthologue of SMN was identified by sequence analysis of the *Schizosacharomyces pombe* genome (hypothetical protein Yab8) (25). The genetic approaches to which *S. pombe* is amenable makes it an attractive system for the study of SMN function. Here we characterize the *S. pombe* orthologue of SMN (ySMN). We show that smn^+ is essential for viability of *S. pombe*. We demonstrate that ySMN can interact with SMN and Sm proteins indicating the remarkable conservation of SMN functional domains in *S. pombe*.

EXPERIMENTAL PROCEDURES

Strains and Media—To construct a diploid strain for use in the disruption of smn^+, the two haploid strains FY261 (ATCC; *h⁻ ura4-D18 leu1–32 ade6-M210 can1–1*) and KGY554 (ATCC; h^+ *ura4-D18 leu1–32 ade6-M216 his3-D1*) were crossed to produce the diploid C2. *S. pombe* strains were grown at 30 °C in YES or EMM medium lacking the appropriate auxotrophic supplements for marker selection (26). For preparing plates, the media were solidified by adding 2% agar. Medium containing 5-fluoro-orotic acid (5-FOA) was prepared as described previously (27). *Escherichia coli* cells were grown in LB broth or LB agar (28) and supplemented with ampicillin or kanamycin at 100 μg/ml.

Genetic Manipulations—To generate a null allele of smn^+, most of the coding region was replaced by the *hisG-ura4+-hisG* cassette, which can be used repeatedly for constructing gene disruptions and/or for sequentially introducing a $ura4^+$ marker plasmid (29). The starting plasmid was pDM291 (a gift from Leonard Guarente, Massachusetts Institute of Technology). The 5′-flanking region was obtained by genomic PCR using the following oligonucleotides: 5′-CCGTGAAGAT-

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[1] The abbreviations used are: SMA, spinal muscular atrophy; SMN, survival motor neuron; snRNP, small nuclear ribonucleoprotein; 5-FOA, 5-fluoro-orotic acid; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; hSMN, human SMN; ySMN, yeast SMN.

CTCGTCGTAATTACGTGGAGCTACC-3′ and 5′-TACGAAGATCTCA-TCTAGCCTAGAATCCGGGT-3′.

The obtained fragment was inserted into pDM291, which was then used to clone the 3′-flanking region. The 3′-flanking region was obtained by genomic PCR using the following oligonucleotides: 5′-ATAC-GGATCCCTTAGCGACGAAAAGCCTGA-3′ and 5′-ATACGGATCCAG-GTAATCGGTATACGTCGC-3′.

To replace one chromosomal copy of *smn*$^+$, the final plasmid was digested with *Bgl*I and transformed into the C2 diploid strain using the lithium acetate protocol (30). Transformed cells were spread on EMM medium -Ura (26) and incubated for 5 days. Individual colonies were streaked on the same media again, and 20 single colony isolates were checked by genome PCR to determine the correct integration of the inserted cassette, as described previously (31). A diploid strain with one chromosomal copy of *smn*$^+$ replaced with the knockout cassette was then transformed with pSP1/ySMN, which had *smn*$^+$ as hemagglutinin epitope amino-terminal fusion under the control *nmt1* promoter of different strengths (pSP1/ySMN173, pSP1/ySMN273, or pSP1/ySMN373). Random spore analysis was performed again to get the haploid strain Δsmn (*h*$^{--}$ *ura4-D18 leu1–32 ade6-M210 can1–1 smn::ura4*$^+$ (pSP1/ySMN, *smn*$^+$, *LEU2*)). The Δsmn1 (*h*$^{--}$ *ura4-D18 leu1–32 ade6-M210 can1–1 smn* (pSLF/ySMN, *smn*$^+$, *ura4*$^+$)) strain was produced by transforming Δsmn with pSLF/ySMN plasmids (pSLF173/ySMN, pSLF273/ySMN, or pSLF373/ySMN) expressing ySMN at different levels. The *ura4*$^+$ selectable marker was excised from the disruption allele by selecting the Δsmn strain on 5-FOA medium. Transformants were streaked on nonselective YES medium, and individual colonies were plated on EMM-Ura and -Leu plates. The clones, which could grow on -Ura medium but could not grow on -Leu media, were referred to as Δsmn1.

To check the ability of *hSMN* to substitute for *smn*$^+$ we used the following method. The tester strain Δsmn was maintained with *smn*$^+$ on pSP1/ySMN plasmids (pSP1/ySMN173, pSP1/ySMN273, or pSP1/ySMN373) carrying *LEU2* selectable marker. The *ura4*$^+$ selectable marker was excised by plating cells on medium containing 5-FOA so that we could later transform them with pSLF/ySMN plasmids (pSLF173/ySMN, pSLF273/ySMN, or pSLF373/ySMN) carrying *ura4*$^+$. Transformants were streaked on nonselective YES medium, and 500 individual colonies were plated on EMM-Ura plates selective for *smn*$^+$ or -Leu plates selective for *hSMN*.

The plasmid shuffle technique was used to test the ability of deletion mutants of *smn*$^+$ to substitute for wild type *smn*$^+$ as described previously (32). The tester strain Δsmn1 carried *smn*$^+$ on pSLF/ySMN plasmids (pSLF173/ySMN, pSLF273/ySMN, or pSLF373/ySMN). The wild type ySMN and the deletion mutants, ySMNΔN47 and ySMNΔYG, expressed from the different *nmt1* promoters, were transformed into the tester strain on a second plasmid bearing the *LEU2* selectable marker (pSP1/ΔN47/173, pSP1/ΔN47/273, pSP1/ΔN47/373, pSP1/ΔYG173, pSP1/ΔYG273, pSP1/ΔYG373, pSP1/ySMN173, pSP1/ySMN273, and pSP1/ySMN373). The LEU$^+$ transformants were plated to medium containing 5-FOA. Only cells that lost the pSLF/ySMN plasmid expressing wild type *smn*$^+$ can grow on 5-FOA plates.

Plasmid Construction—Standard recombinant DNA techniques were performed as described previously (28). DNA fragments corresponding to open reading frames of ySMN, deletion mutants of ySMN, and human SMN (hSMN) were generated by PCR amplification using specific primers. The obtained PCR fragments carrying *smn*$^+$ or deletion mutants of *smn*$^+$ were cloned into pSLF173, pSLF273, and pSLF373 vectors (33). The obtained plasmid (pSLF173/ySMN, pSLF273/ySMN, pSLF373/ySMN, pSLF173/ΔN47, pSLF273/ΔN47, pSLF373/ΔN47, pSLF173/ΔYG, pSLF273/ΔYG, and pSLF373/ΔYG) carry *HA-smn*$^+$ or *HA-smn* under three different versions of *nmt1* promoter (173, full-strength; 273, medium strength; 373, low strength). The plasmids were used to produce PCR fragments carrying *smn*$^+$ or *smn* under the control of the same promoters. The obtained fragment was cloned into the pSP1 vector (34) to produce pSP1/ySMN173, pSP1/ySMN273, pSP1/ySMN373, pSP1/ΔN47/173, pSP1/ΔN47/273, pSP1/ΔN47/373, pSP1/ΔYG173, pSP1/ΔYG273, and pSP1/ΔYG373.

Plasmids carrying Myc-tagged *hSMN* and *hSMNΔN27* were previously described (14). Myc-tagged hSMNΔYG lacking the last 26-carboxyl-terminal amino acids was generated by PCR amplification using specific primers. All the 6His-tagged constructs were generated by cloning the PCR inserts into the pET28 vector (Novagene). Green fluorescent protein·ySMN fusion protein was produced by cloning the entire open reading frame of ySMN into the pSGP573 vector (a gift from Susan Forsburg, The Salk Institute). The green fluorescent protein·ySMN protein was expressed under the control of the strong *nmt1* promoter. All plasmids were checked by DNA sequencing.

Production of Proteins in Vitro—The [^{35}S]methionine-labeled proteins were produced by *in vitro* coupled transcription-translation reactions (Promega Biotech) in the presence of [^{35}S]methionine (Amersham Pharmacia Biotech). 6His-tagged fusion proteins were produced from a pET28 bacterial expression system in the *E. coli* strain BL21(DE3) and purified using nickel chelation chromatography (Novagene) according to the manufacturer's protocol. The glutathione *S*-transferase (GST)·ySMN, GST·hSMN, and GST·hSmB fusion proteins were expressed from a GST expression vector, pGEX-5X-3 (Amersham Pharmacia Biotech), in the *E. coli* strain BL21 and purified on glutathione-Sepharose (Amersham Pharmacia Biotech) according the manufacturer's protocol.

In Vitro Protein Binding Assay—Purified GST or GST fusion proteins (2 μg) bound to 25 μl of glutathione-Sepharose beads were incubated with *in vitro* translated protein in 1 ml of binding buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM EDTA, 0.1% Nonidet P-40, 2 μg/ml leupeptin and pepstatin A, and 0.5% aprotinin). After incubation for 1 h at 4 °C, the resin was washed five times with 1 ml of binding buffer. The bound fraction was eluted by boiling in SDS-PAGE sample buffer and resolved by SDS-PAGE. The gels were fixed for 30 min, and the signal was enhanced by treatment with Amplify solution (Amersham Pharmacia Biotech). For direct *in vitro* binding, purified GST or GST·ySMN proteins (2 μg) bound to 25 μl of glutathione-Sepharose beads were incubated with 5 μg of purified 6His-tagged ySMN or 6His-tagged human SmB in 1 ml of binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.05% Nonidet P-40, 2 μg/ml leupeptin and pepstatin A, and 0.5% aprotinin). After incubation for 1 h at 4 °C, the resin was washed five times with 1 ml of binding buffer. The bound fraction was eluted by boiling in SDS-PAGE sample buffer, resolved by SDS-PAGE, and immunoblotted using a rabbit polyclonal anti-His-tag antibody (Santa Cruz Biotechnology).

Production of Anti-ySMN Monoclonal Antibody—Anti-ySMN antibody 1G6 was produced by immunizing Balb/C mice with ySMN protein fused to GST. Hybridoma production, screening, and ascites fluid production were performed as described previously (35) using 6His-tagged ySMN protein.

Gel Filtration Chromatography—Purified recombinant His-tagged ySMN or ySMNDYG (40 mg) proteins were applied to a TSK-GEL G3000-SW glass column (Tosohaas, Montgomeryville, PA) equilibrated in a buffer containing 50 mM Hepes, pH 7.9, 100 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol. Fractions were collected at a 0.25 ml/min flow rate, pooled as indicated, and analyzed by SDS-PAGE and Western blotting with an anti-T7 tag monoclonal antibody (Novagen).

Immunofluorescence on S. pombe—Immunofluorescence on *S. pombe* was performed as described by Hagan and Hyams (36). Laser confocal fluorescence microscopy was performed with a *Leica* TCS 4D (Germany) confocal microscope, and images from each channel were recorded separately.

RESULTS

The SMN Gene Is Essential for Viability of S. pombe—We began the analysis of ySMN by determining whether *smn*$^+$ is essential for *S. pombe*. We constructed a cassette for disruption of a chromosomal copy of the *smn*$^+$ gene. In this cassette, most of the coding sequence of *smn*$^+$ was replaced with *hisG-ura4*$^+$-*hisG*. After transformation of the cassette and *ura4*$^+$ selection, random spore analysis was performed to assess the haploid phenotype. None of the examined individual haploid clones was able to grow on EMM-Ura medium selective for the knockout allele (data not shown). This demonstrates that the knockout of the *SMN* gene is lethal. To obtain a knockout of the *SMN* gene in a haploid strain, it was necessary to express ySMN from a plasmid. To do so, we transformed diploid cells heterozygous for *smn::ura4*$^+$ with a *LEU2* plasmid carrying a wild type copy of the *smn*$^+$ gene. To obtain haploid clones with a knocked out allele of *smn* and a plasmid carrying normal *smn*$^+$, we performed random spore analysis. To confirm that the *smn*$^+$ gene is essential we checked the stability of the plasmid carrying *smn*$^+$. All 500 of the tested clones retained the plasmid expressing *smn*$^+$ demonstrating that the *smn*$^+$ gene is essential for *S. pombe*. The importance of ySMN for survival of *S. pombe* demonstrates that ySMN plays a fundamental role in cell physiology and suggests that the functions performed by SMN are evolutionarily conserved from *S. pombe* to mammals.

hSMN Cannot Replace smn$^+$—We tested whether hSMN

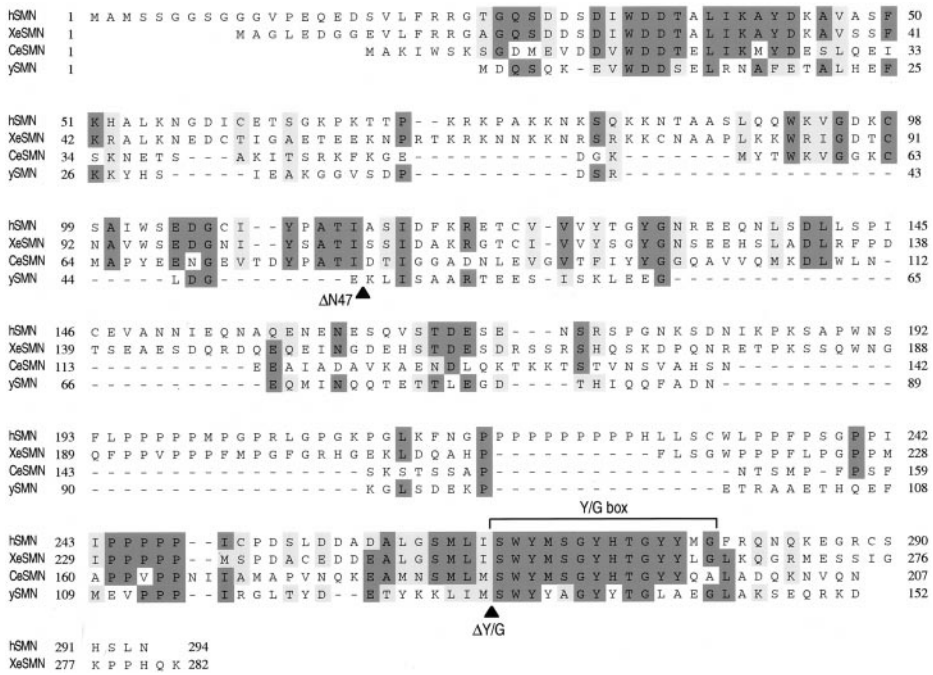

FIG. 1. **Amino acid sequence alignment of human SMN (hSMN, GenBank™ Q16637), *X. laevis* SMN (XeSMN(8)), *C. elegans* SMN (CeSMN, GenBank™ AF156887.1), and *S. pombe* SMN (ySMN, GenBank™ 09808).** *Light gray boxes* indicate similar amino acids, and *dark gray boxes* indicate identical amino acids. The conserved Y/G box is indicated with a *bracket*. *Black arrowheads* indicate the amino and carboxyl termini of the ySMNΔN47 and ySMNΔYG mutants, respectively.

could functionally substitute for the yeast protein *in vivo*. Because the smn^+ gene is essential for viability, our tester Δsmn strain was maintained with smn^+ under the control of *nmt1* promoters of different strengths (strong, medium, or weak) on a *LEU2* plasmid. The tester strain was transformed with $ura4^+$ plasmids, which express *hSMN* at different levels using the same promoters. Transformants were streaked on nonselective YES medium, and 500 individual clones were plated on EMM-Ura plates selective for smn^+ or -Leu plates selective for *hSMN*. None of the different combinations of plasmids expressing smn^+ and *hSMN* lost the plasmids carrying the smn^+ gene demonstrating that *hSMN* can not replace smn^+ *in vivo* (data not shown). The inability of hSMN to functionally substitute for ySMN is perhaps not surprising, because the overall primary sequence similarity between these proteins is only 24% and is restricted almost entirely to the amino-terminal and carboxyl-terminal regions of the protein (Fig. 1).

Intracellular Localization of the ySMN Protein—To further study the ySMN protein we produced an anti-ySMN monoclonal antibody (1G6). Western blot analysis shows that the 1G6 monoclonal antibody does not cross-react with *Xenopus laevis* and human SMNs but specifically recognizes the purified ySMN recombinant protein and a single band of similar size in total yeast extract (Fig. 2*A*). Endogenous ySMN migrates at a size corresponding to about 32 kDa instead of the predicted size of 18 kDa. We note that hSMN also migrates at higher molecular weight than is predicted from its molecular mass (9). To further confirm that antibody 1G6 reacts specifically with the ySMN protein, we performed immunoprecipitation of ySMN produced by transcription and translation *in vitro*. IG6 antibody, but not the nonspecific SP2/0 antibody, efficiently immunoprecipitates ySMN (Fig. 2*B*). The 1G6 antibody was further used to localize ySMN by confocal immunofluorescence microscopy. ySMN is found in both the nucleus and the cytoplasm (Fig. 3, *A* and *B*). A similar distribution was also observed upon expression of a green fluorescent protein·ySMN fusion protein (Fig. 3, *C* and *D*). This indicates that the subcellular localization of SMN is evolutionary conserved. In the nucleus of mammalian cells, SMN is highly concentrated in gems (9). However, we were unable to discern whether ySMN is concentrated in similar subnuclear structures because of the much smaller size of *S. pombe* nuclei.

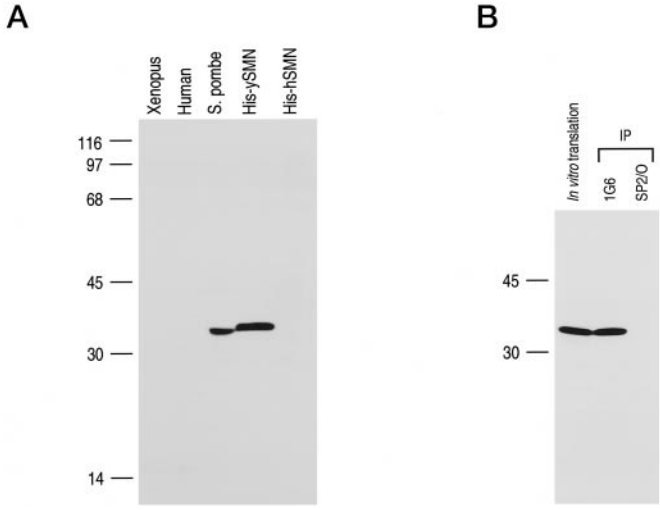

FIG. 2. *A,* Western blot analysis using the anti-ySMN monoclonal antibody 1G6 on total cell lysates of *X. laevis* XL177, human HeLa and *S. pombe*, and on 6His-tagged yeast- (ySMN) or hSMN-purified recombinant proteins (10 ng each). The position of the molecular mass markers in kDa is indicated on the *left*. *B,* 6His-tagged ySMN was *in vitro* translated in rabbit reticulocyte lysate in the presence of [^{35}S]methionine, immunoprecipitated using either 1G6 or nonspecific SP2/0 antibodies, and resolved by SDS-PAGE and autoradiography. The *left panel* shows 10% of the input. *IP*, immunoprecipitate.

ySMN Oligomerizes and Interacts Directly with Human SMN and Sm Proteins—Human SMN interacts with itself, Gemin2, Gemin3, and the spliceosomal core Sm proteins (8–12). If, in *S. pombe*, ySMN performs similar functions to its human orthologue, it would be expected to display similar interactions. To test this possibility, we performed *in vitro* protein binding assays with ySMN as well as with hSMN, hGemin2, and the human Sm proteins B, D1, D2, D3, E, F, and G (37–39). We used human Sm proteins for these experiments because they are similar to their yeast counterparts (45–85% similarity), readily available, and because we were interested in determining the degree of conservation of the interactions between SMN and Sm proteins. ySMN was first produced as a fusion protein with GST. Purified GST or GST·ySMN immobi-

lized on glutathione-Sepharose were incubated with *in vitro* translated [^{35}S]methionine-labeled ySMN, hSMN, hGemin2, and Sm proteins. Full-length ySMN, hSMN, and human Sm B, D2, and D3 bound specifically to immobilized GST·ySMN but not to GST alone (Fig. 4 and data not shown). ySMN interacts considerably better with itself than with both human SMN and human SmB (see "Discussion"). To exclude the possibility that these interactions are mediated by some components present in the rabbit reticulocyte lysate, wild-type full-length ySMN, and human SmB were produced as recombinant 6His-tagged proteins and incubated with GST or GST·ySMN. As shown in Fig. 5, ySMN and human SmB bind specifically and directly to ySMN but not to GST alone. These findings demonstrate the evolutionary conservation of SMN interactions; ySMN can oligomerize and bind Sm proteins as well as hSMN. As an additional control we used GST·Gemin2 fusion and detected no interaction (data not shown).

An Evolutionarily Conserved Domain of ySMN Mediates Its Interactions with SMN and with Sm Proteins—The evolutionary conservation of SMN interactions despite the low overall sequence similarity between the hSMN and ySMN proteins prompted us to analyze the role of the conserved domains of ySMN in oligomerization and binding to the human Sm proteins. Two short regions of SMN appear to be particularly highly conserved through evolution (Fig. 1). In hSMN the conserved amino terminus is involved in binding Gemin2 and nucleic acids, whereas the conserved Y/G box near the carboxyl terminus (25) plays a crucial role in oligomerization and Sm and Gemin3 binding (8, 10, 11, 12, 15). We therefore generated two deletion mutants of ySMN, ySMNΔN47 and ySMNΔYG, which lack the amino-terminal or carboxyl-terminal conserved domains, respectively (Fig. 1). Immobilized GST, GST·ySMN, or GST·hSmB fusion proteins were incubated with in *vitro* translated [^{35}S]methionine-labeled full-length ySMN, ySMNΔN47, or ySMNΔYG. Fig. 6*A* shows that full-length ySMN and ySMNΔN47, but not ySMNΔYG, bound specifically to GST·ySMN and GST·hSmB. No binding to GST alone was observed. Similarly, the deletion of the conserved Y/G box of hSMN completely abolished binding to GST·hSMN and GST·SmB (Fig. 6*B*). We conclude that the Y/G box in ySMN and hSMN is necessary for both SMN oligomerization and for its binding to Sm proteins.

To examine directly the state of oligomerization of ySMN and the ySMNΔYG deletion mutant, we performed gel filtration experiments using purified recombinant ySMN or ySMNΔYG. Fig. 6*C* shows that ySMN forms large oligomers of up to a molecular mass corresponding to ~440 kDa. We note that no monomeric ySMN is detectable. In contrast, the deletion mutant ySMNΔYG is unable to oligomerize. Therefore, ySMN like its human counterpart efficiently oligomerizes, and the Y/G box is required for oligomerization.

The Conserved Domains of ySMN Are Essential for Viability—In light of the findings described above we asked whether the SMNΔN47 and SMNΔYG deletion mutants could functionally substitute for full-length ySMN protein *in vivo*. To do so, we used the plasmid shuffle technique (32). Because the *smn*$^+$ gene is essential for viability, our tester Δsmn1 strain carried the *smn*$^+$ gene on a *ura4* plasmid. The wild type ySMN and the deletion mutants, SMNΔN47 and SMNΔYG, expressed from the *nmt1* promoters of different strengths, were transformed into the tester strain on a second plasmid bearing the *LEU2* selectable marker. The LEU$^+$ transformants were plated on medium containing 5-FOA. Only cells that have lost the *ura4*$^+$ plasmid expressing *smn*$^+$ can grow on 5-FOA plates. Thus, the transformants will not grow unless the deletion mutants of ySMN can functionally substitute for ySMN. As shown in Fig. 7, the test strain transformed with a *LEU2* plasmid expressing ySMN lacking either the carboxyl- or amino-terminal domains failed to grow on 5-FOA-containing medium. This demonstrates that these conserved domains of SMN are essential for ySMN function and cell viability.

Effect of Overexpression of Wild Type and Mutant SMN on Cell Growth—Interestingly, we found that smn cells containing a plasmid carrying *smn*$^+$ under the control of a strong *nmt1* promoter formed small colonies and grew significantly slower than cells containing *smn*$^+$ expressed under the control of medium or weak promoters (Fig. 8). This effect did not depend on strain background, because overexpression of ySMN in wild type strain FY261 also slowed down growth (data not shown).

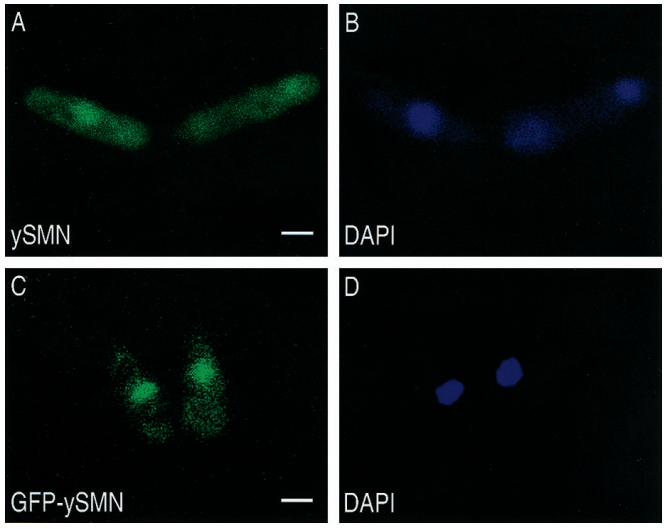

FIG. 3. **Intracellular localization of ySMN.** *A,* laser confocal image of indirect immunofluorescence on *S. pombe* cells using the anti-ySMN monoclonal antibody 1G6. *B,* DAPI-4′,6-diamidino-2-phenylindole staining of the field shown in *A*. *C,* laser confocal image of *S. pombe* cells expressing the green fluorescent protein·ySMN fusion protein. *D,* DAPI-4′,6-diamidino-2-phenylindole staining of the field shown in *C*. Scale bars indicate 2 μm.

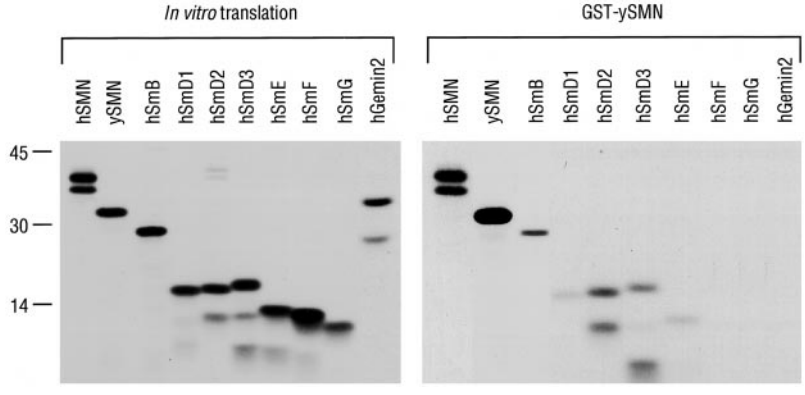

FIG. 4. **ySMN interacts *in vitro* with human Sm proteins as well as with yeast and human SMN proteins.** *In vitro* translated [^{35}S]methionine-labeled 6His-ySMN, Myc-hSMN, Myc-hGemin2, and Myc-hSm proteins B, D1, D2, D3, E, F, and G were incubated with purified GST·ySMN or GST alone as described under "Experimental Procedures." Bound proteins were analyzed by SDS-PAGE and fluorography. The *in vitro* translation panel shows 10% of the input. The position of the molecular mass markers in kDa is indicated on the left.

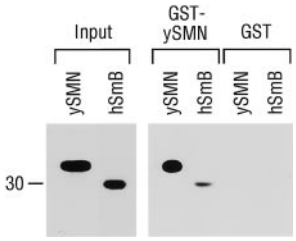

FIG. 5. **ySMN interacts directly with itself and human SmB (hSmB) *in vitro*.** Purified 6His-tagged ySMN or hSmB recombinant proteins were incubated with purified GST·ySMN or GST alone. The input lane shows 10% of 6His-tagged proteins. Bound proteins were analyzed by SDS-PAGE and Western blotting with anti-His-tag antibody.

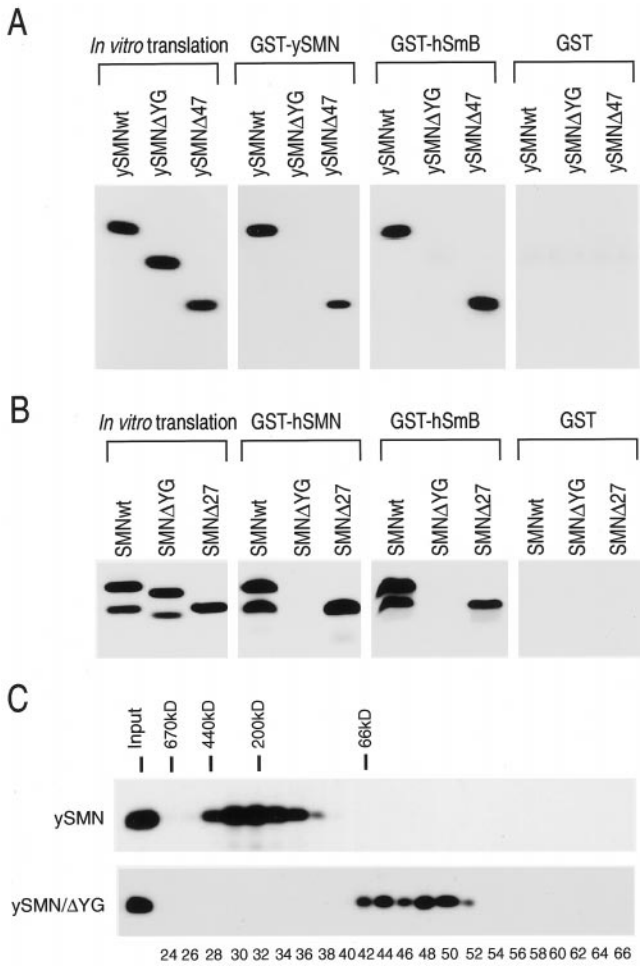

FIG. 6. **The conserved Y/G box of ySMN is necessary for oligomerization and interaction with Sm proteins.** *A, in vitro* translated [^{35}S]methionine-labeled wild type and mutant ySMN proteins were incubated with purified GST·ySMN, GST·hSmB, or GST as indicated. *B, in vitro* translated [^{35}S]methionine-labeled wild type and mutant Myc-tagged hSMN proteins were incubated with purified GST·hSMN, GST·hSmB, or GST as indicated. Bound proteins were analyzed by SDS-PAGE and fluorography. The *in vitro* translation panel shows 20% of the input. The interaction of SMN with SmB is weaker than with itself and the exposure time was 3-fold longer in Sm binding experiments. The shorter forms of hSMN and hSMNΔYG correspond to products translated from the endogenous AUG codon of the hSMN open reading frame. *C,* purified recombinant His-tagged ySMN and ySMNΔYG proteins were analyzed individually by high pressure liquid chromatography gel filtration. Fractions were pooled as indicated and analyzed by SDS-PAGE and Western blotting with anti-T7 tag monoclonal antibody.

This indicates that overexpression of ySMN is disadvantageous to cells. Cells expressing SMNΔYG under a strong promoter did not have the same phenotype (Fig. 8).

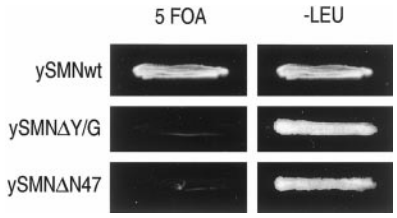

FIG. 7. **Ability of deletion mutants of *smn*$^+$ to complement a *smn* disruption.** Plasmids carrying the *LEU2* selectable marker and the indicated deletion of *smn* expressed from *nmt1*** (weak) promoter were introduced into the Δsmn1 tester strain. LEU+ transformants were patched onto plates lacking leucine (-LEU) and then replica-plated to both 5-FOA plates and -Leu plates. Plates were incubated at 30 °C for 2 days (-LEU) or 3 days (5-FOA).

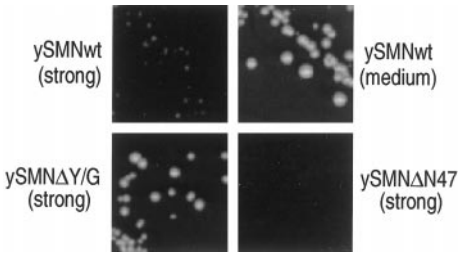

FIG. 8. **The Δsmn1 strain is maintained with pSLF373/ySMN, which carries *smn*$^+$ under the control of *nmt1*** (weak) promoter.** This strain was transformed with plasmids expressing wild type ySMN (*upper left panel*), ySMNΔYG (*lower left panel*), ySMNΔN47 (*lower right panel*), or under the strong *nmt1* promoter or wild type ySMN under medium *nmt1** promoter (*upper right panel*). Transformants were streaked on -Leu plates and incubated at 30 °C for 3 days.

We also found that cells overexpressing the ySMNΔN47 allele under control of the strong *nmt1* promoter did not grow (Fig. 8). This effect did not depend on the background level of *smn*$^+$ expression. We conclude that overexpression of ySMNΔN47 is particularly toxic to these cells and suggests that it has a dominant negative phenotype on cell growth. We have recently determined that hSMN deleted of the first 27 amino acids (hSMNΔN27) has a dominant negative phenotype (14). Fig. 6*B* shows that hSMNΔN27 has similar binding avidity for hSMN and SmB as wild type hSMN. Thus, both hSMNΔN27 and ySMNΔN47 maintain the ability to interact with SMN and Sm proteins and display a dominant negative phenotype.

DISCUSSION

A putative *S. pombe* orthologue of mammalian SMN, Yab8, was previously identified by sequence similarity (25). However, the extent of primary sequence similarity between Yab8 open reading frame and hSMN is only 24% and is restricted to the amino-terminal and carboxyl-terminal regions of these proteins. This raised a question as to whether the Yab8 is a genuine orthologue of hSMN. The findings reported here support the conclusion that Yab8 is indeed an orthologue of hSMN, and we therefore refer to it here as ySMN.

The knockout of the *smn*$^+$ gene is lethal. The importance of ySMN for survival of *S. pombe* demonstrates that ySMN plays a fundamental role in cell function. We showed that ySMN is found in both the nucleus and the cytoplasm. These results show that the subcellular localization of SMN is evolutionary conserved, because a generally similar localization of SMN in both cytoplasm and nucleus is also seen in mammalian cells and *C. elegans* (9, 21). Oligomerization of hSMN and its interaction with the Sm proteins are likely required for its function in snRNP assembly and splicing machinery regeneration (8, 11). If, in *S. pombe*, the ySMN protein performs similar functions to its human orthologue, it would be expected to display

similar interactions *i.e.* oligomerization and binding to Sm proteins. Indeed, we found that ySMN displayed similar interactions to the vertebrate SMN protein in that it can oligomerize and bind Sm proteins. These findings strongly suggest that Yab8p is the *S. pombe* orthologue of hSMN.

ySMN and hSMN interact with each other and ySMN interacts with human Sm proteins. Considering the evolutionary divergence between ySMN and hSMN proteins (24% similarity) and between *S. pombe* Sm proteins and human Sm proteins (45–85% similarity), these results indicate a remarkable conservation of SMN interactions. This prompted us to examine further the role of the conserved domains of ySMN in oligomerization and binding to Sm proteins. Our binding experiments demonstrate that the Y/G box of ySMN and hSMN is necessary for both SMN self-association and for interaction with Sm proteins.

Studies with SMN deletion constructs indicate that both the amino-terminal and carboxyl-terminal domains of ySMN are essential for cell viability. The fact that SMNΔN47 has similar avidity for ySMN and human Sm proteins as the wild type protein suggests that other interactions, in addition to SMN oligomerization and Sm binding, are likely essential for the housekeeping function of SMN. The most likely candidate for such interactions would be the *S. pombe* orthologue of human Gemin2, which interacts with the conserved amino-terminal region of hSMN (8, 14).

The central region of human SMN (amino acids 96–160) shows amino acid sequence similarity to a tudor domain (40). The specific function of this domain is still an open issue. It has been proposed that the tudor domain might represent a RNA- and/or protein binding domain, and it has recently been suggested that the tudor domain of SMN in higher eukaryotes is necessary and sufficient for binding of Sm proteins (16). The results we report here are in contrast to this conclusion. The ySMN protein does not contain a tudor domain (41), but nevertheless ySMN interacts with Sm proteins. We cannot entirely exclude the possibility that the region corresponding to the tudor domain in human SMN contributes to Sm binding; however, we demonstrate that the evolutionarily conserved carboxyl-terminal Y/G region of both hSMN and ySMN is necessary for SMN-Sm binding. Some of the discrepancy in the results may be accounted for by a difference in experimental conditions. Previous experiments in this laboratory have also shown that a partial deletion or a point mutation in the Y/G box of hSMN indeed severely compromise Sm protein binding, and SMN oligomerization is required for high affinity interaction with Sm proteins (11). Thus, we conclude that the tudor domain is not sufficient for Sm binding as previously suggested (16).

Overexpression of ySMN is deleterious to growth in *S. pombe*. Interestingly, cells overexpressing ySMNΔYG did not show this phenotype. This could be explained by the fact that the Y/G deletion mutant does not interact with SMN and Sm proteins and thus represents a loss of function protein, which therefore does not interfere with the function of the wild type protein. In contrast, overexpression of the ySMNΔN47 mutant allele is toxic in these cells. We conclude that overexpression of ySMNΔN47 has a dominant negative phenotype on cell growth. This is reminiscent of a recently described dominant negative mutant of hSMN, SMNΔN27 (14). Expression of SMNΔN27 causes a dramatic reorganization of snRNPs in the nucleus and inhibits pre-mRNA splicing (14). hSMNΔN27 has similar binding avidity for hSMN and SmB as wild type hSMN. Thus, both hSMNΔN27 and ySMNΔN47 maintain the ability to oligomerize SMN and to interact with Sm proteins. Although the molecular basis of this dominant negative effect of ySMNΔ47 overexpression is not yet known, the identification of such a dominant negative mutant in a distant organism such as *S. pombe* strengthens the conclusion that the amino-terminal region of SMN has a critical, though hitherto unknown, function. Finally, the availability of the *S. pombe* system and reagents we describe here should open the way for further advances on the structure and function of the SMN protein and help unveil the molecular pathogenesis of SMA.

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REFERENCES

1. Pearn, J. (1978) *J. Neurol. Sci.* **38,** 263–275
2. Melki, J. (1997) *Curr. Opin. Neurol.* **10,** 381–385
3. Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., Benichou, B., Cruaud, C., Millasseau, P., Zeviani, M., Le Pasiler, D., Frezal, J., Cohen, D., weissenbach, J., Munnich, A., and Melki, J. (1995) *Cell* **80,** 155–165
4. Lefebvre, S., Burglen, L., Frezal, J., Munnich, A., and Melki, J. (1998) *Hum. Mol. Genet.* **7,** 1531–1536
5. Burghes, A. H. (1997) *Am. J. Hum. Genet.* **61,** 9–15
6. Coovert, D. D., Le, T. T., McAndrew, P. E., Strasswimmer, J., Crawford, T. O., Mendell, J. R., Coulson, S. E., Androphy, E. J., Prior, T. W., and Burghes, A. H. (1997) *Hum. Mol. Genet.* **6,** 1205–1214
7. Lefebvre, S., Burlet, P., Liu, Q., Bertrandy, S., Clermont, O., Munnich, A., Dreyfuss, G., and Melki, J. (1997) *Nat. Genet.* **16,** 265–269
8. Liu, Q., Fischer, U., Wang, F., and Dreyfuss, G. (1997) *Cell* **90,** 1013–1021
9. Liu, Q., and Dreyfuss, G. (1996) *EMBO J.* **15,** 3555–3565
10. Lorson, C. L., Strasswimmer, J., Yao, J. M., Baleja, J. D., Hahnen, E., Wirth, B., Le, T., Burghes, A. H., and Androphy, E. J. (1998) *Nat. Genet.* **19,** 63–66
11. Pellizzoni, L., Charroux, B., and Dreyfuss, G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 11167–11172
12. Charroux, B., Pellizzoni, L., Perkinson, R. A., Shevchenko, A., Mann, M., and Dreyfuss, G. (1999) *J. Cell Biol.* **147,** 1181–1194
13. Charroux, B., Pellizzoni, L., Perkinson, R., Yong, J., Shevchenko, A., Mann, M., and Dreyfuss, G. (2000) *J. Cell Biol.* **148,** 1177–1186
14. Pellizzoni, L., Kataoka, N., Charroux, B., and Dreyfuss, G. (1998) *Cell* **95,** 615–624
15. Fischer, U., Liu, Q., and Dreyfuss, G. (1997) *Cell* **90,** 1023–1029
16. Buhler, D., Raker, V., Luhrmann, R., and Fischer, U. (1999) *Hum. Mol. Genet.* **8,** 2351–2357
17. Mattaj, I. W., and De Robertis, E. M. (1985) *Cell* **40,** 111–118
18. Mattaj, I. W. (1986) *Cell* **46,** 905–911
19. Luhrmann, R., Kastner, B., and Bach, M. (1990) *Biochim. Biophys. Acta* **1087,** 265–292
20. Schrank, B., Gotz, R., Gunnersen, J. M., Ure, J. M., Toyka, K. V., Smith, A. G., and Sendtner, M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 9920–9925
21. Miguel-Aliaga, I., Culetto, E., Walker, D. S., Baylis, H. A., Sattelle, D. B., and Davies, K. E. (1999) *Hum. Mol. Genet.* **8,** 2133–2143
22. Jablonka, S., Schrank, B., Kralewski, M., Rossoll, W., and Sendtner, M. (2000) *Hum. Mol. Genet.* **9,** 341–346
23. Hsieh-Li, H. M., Chang, J. G., Jong, Y. J., Wu, M. H., Wang, N. M., Tsai, C. H., and Li, H. (2000) *Nat. Genet.* **24,** 66–70
24. Monani, U. R., Sendtner, M., Coovert, D. D., Parsons, D. W., Andreassi, C., Le, T. T., Jablonka, S., Schrank, B., Rossol., W., Prior, T. W., Morris, G. E., and Burghes, A. H. M. (2000) *Hum. Mol. Genet.* **9,** 333–339
25. Talbot, K., Ponting, C. P., Theodosiou, A. M., Rodrigues, N. R., Surtees, R., Mountford, R., and Davies, K. E. (1997) *Hum. Mol. Genet.* **6,** 497–500
26. Moreno, S., Klar, A., and Nurse, P. (1991) *Methods Enzymol.* **194,** 795–823
27. Sikorski, R. S., and Boeke, J. D. (1991) *Methods Enzymol.* **194,** 302–318
28. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
29. McNabb, D. S., Pak, S. M., and Guarente, L. (1997) *BioTechniques* **22,** 1134–1139
30. Keeney, J. B., and Boeke, J. D. (1994) *Genetics* **136,** 849–856
31. Ling, M., Merante, F., and Robinson, B. H. (1995) *Nucleic Acids Res.* **23,** 4924–4925
32. Boeke, J. D., Trueheart, J., Natsoulis, G., and Fink, G. R. (1987) *Methods Enzymol.* **154,** 164–175
33. Forsburg, S. L., and Sherman, D. A. (1997) *Gene* (*Amst.*) **191,** 191–195
34. Cottarel, G., Beach, D., and Deuschle, U. (1993) *Curr. Genet.* **23,** 547–548
35. Choi, Y. D., and Dreyfuss, G. (1984) *J. Cell Biol.* **99,** 1997–2004
36. Hagan, I. M., and Hyams, J. S. (1988) *J. Cell Sci.* **89,** 343–357
37. Lehmeier, T., Raker, V., Hermann, H., and Luhrmann, R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 12317–12321
38. Hermann, H., Fabrizio, P., Raker, V. A., Foulaki, K., Hornig, H., Brahms, H., and Luhrmann, R. (1995) *EMBO J.* **14,** 2076–2088
39. Raker, V. A., Plessel, G., and Luhrmann, R. (1996) *EMBO J.* **15,** 2256–2269
40. Ponting, C. P. (1997) *Trends Biochem. Sci.* **22,** 51–52
41. Talbot, K., Miguel-Aliaga, I., Mohaghegh, P., Ponting, C. P., and Davies, K. E. (1998) *Hum. Mol. Genet.* **7,** 2149–2156