THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 9, Issue of March 1, pp. 7540–7545, 2002

*Printed in U.S.A.*

# Purification of Native Survival of Motor Neurons Complexes and Identification of Gemin6 as a Novel Component*

Received for publication, October 22, 2001, and in revised form, December 7, 2001
Published, JBC Papers in Press, December 17, 2001, DOI 10.1074/jbc.M110141200

**Livio Pellizzoni‡§, Jennifer Baccon‡§, Juri Rappsilber¶‖, Matthias Mann¶, and Gideon Dreyfuss‡****

*From the ‡Howard Hughes Medical Institute and Department of Biochemistry & Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6148 and the ¶Protein Interaction Laboratory, Center for Experimental Bioinformatics and Department of Biochemistry & Molecular Biology, University of Southern Denmark, DK-5230 Odense M, Denmark*

**The survival of motor neurons (SMN) protein, the product of the gene responsible for the motor neuron degenerative disease spinal muscular atrophy (SMA), is part of a large macromolecular complex. The SMN complex is localized in both the cytoplasm and the nucleus and contains SMN, Gemin2, Gemin3, Gemin4, Gemin5, and a few not yet identified proteins. The SMN complex plays a key role in the biogenesis of spliceosomal small nuclear ribonucleoproteins (snRNPs) and other ribonucleoprotein particles. As a step toward the complete characterization of the components of the SMN complex, we generated stable cell lines that express FLAG-tagged SMN or Gemin2 under the control of a tetracycline-inducible promoter. Native SMN complexes of identical protein composition to those isolated by immunoprecipitation with anti-SMN antibodies were purified by affinity chromatography from extracts of both cell lines. Here we report the identification by mass spectrometry of a novel protein component of the SMN complex termed Gemin6. Co-immunoprecipitation, immunolocalization, and *in vitro* binding experiments demonstrate that Gemin6 is a component of the SMN complex that localizes to gems and interacts with several Sm proteins of the spliceosomal snRNPs.**

Spinal muscular atrophy (SMA)[1] is an autosomal recessive disease characterized by degeneration of motor neurons of the spinal cord resulting in muscular atrophy (1). SMA is the most common genetic cause of infant mortality and the second most frequent lethal autosomal recessive disease after cystic fibrosis. Deletions or mutations in the *survival of motor neurons* (*SMN1*) gene, the SMA-causing gene, are detected in over 95% of SMA patients (2). SMN is essential for viability in all organisms and cell types tested, including humans, mice, chicken DT40 cells, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Schizosaccharomyces pombe* (3–9).

SMN associates with Gemin2 (10), Gemin3 (11), Gemin4 (12), and Gemin5 (13) and a few not yet identified components to form a large multiprotein complex. The SMN complex is found both in the cytoplasm and in the nucleus, where it is concentrated in gems, nuclear bodies similar in size and number to Cajal (coiled) bodies and often associated with them (14). In addition to the integral components of the SMN complex described above, several proteins have been identified that directly interact with SMN and/or the Gemins. These include the spliceosomal snRNP Sm and Lsm proteins (10, 15–17), the snoRNP proteins fibrillarin and GAR1 (18, 19), RNA helicase A (20), hnRNP Q (21), and coilin (22). A strong correlation has emerged between a defect of SMN interaction with these proteins and the SMA phenotype, because SMN, but not SMN mutants found in SMA patients, interact directly with these proteins (15–22). A common feature of these proteins is the presence of arginine- and glycine-rich (RG) domains that are necessary for their direct interaction with SMN (17–22). Specific arginine residues of these domains are di-methylated *in vivo*, and this enhances the affinity for SMN, indicating that SMN preferentially interacts with post-translationally modified RG-rich protein targets (23). The known targets of the SMN complex are all components of RNPs and have diverse roles in several aspects of cellular RNA metabolism. These functions include transcription and processing of most cellular pre-mRNAs and rRNAs. This observation suggests that the SMN complex likely functions in some essential steps of RNA metabolism. Previous studies have provided evidence that the SMN complex functions in the assembly of spliceosomal snRNPs, pre-mRNA splicing, and transcription (16, 20, 24–26). The SMN complex may also play a role in the metabolism of snoRNPs involved in processing and modification of ribosomal RNA (18, 19).

Although the molecular mechanisms of SMN function are not yet known, these findings have led to the proposal that the SMN complex is a macromolecular machine, which functions in the assembly and function of several RNP complexes (15–26). Understanding the function of the SMN complex at the molecular level is necessary to uncover the defect responsible for SMA. To identify all the components of the SMN complex and to characterize its biochemical, structural, and functional properties, we developed a system for the purification of native SMN complexes. We generated stably transfected HeLa cell

* This work was supported in part by a grant from the National Institute of Health (to G. D.) and by a grant from the Danish National Research Foundation to the Center of Experimental Bioinformatics. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF453443.*

§ These authors have contributed equally to this work.

‖ A Marie Curie Fellow.

** An Investigator of the Howard Hughes Medical Institute. To whom correspondence should be addressed: Howard Hughes Medical Institute and Department of Biochemistry & Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6148. Tel.: 215-898-0398; Fax: 215-573-2000; E-mail: gdreyfuss@hhmi.upenn.edu.

[1] The abbreviations used are: SMA, spinal muscular atrophy; SMN, survival of motor neurons protein; snoRNP, small nucleolar ribonucleoprotein; hnRNP, heterogeneous nuclear ribonucleoprotein; snRNP, small nuclear ribonucleoprotein; EST, expressed sequence tag; CMV, cytomegalovirus; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; snRNA, small nuclear RNA.

lines expressing FLAG-tagged SMN or Gemin2 fusion proteins and used these cell lines to affinity-purify endogenous SMN complexes. Unlike complexes isolated by immunoprecipitation with currently available antibodies to SMN or Gemin2, these complexes can be eluted from the antibodies with the FLAG peptide and thus are obtained in native form. The purified SMN complexes likely contain all the core components in their native conformation, correct stoichiometry, and proper post-translational modifications. From these experiments, novel proteins were identified by mass spectrometry and peptide microsequencing. Here, we characterized one of these proteins, Gemin6, as a novel component of the SMN complex.

## EXPERIMENTAL PROCEDURES

*DNA Constructs*—The open reading frames of SMN and Gemin2 fused at their 5′-end to the sequence encoding the epitope (FLAG-SMN and FLAG-Gemin2) were generated by PCR amplification using specific primers. The inserts were cloned into the pTRE vector (CLONTECH). The cDNA corresponding to Gemin6 was generated by PCR amplification using the EST clone N. 4764576 (IMAGE) as a template. The inserts were cloned downstream of the CMV promoter into modified pcDNA3 vectors (Invitrogen) containing either the myc-tag or the FLAG-tag sequences at the amino terminus. Gemin6 cDNA was cloned into the pGEX-5X vector (Amersham Biosciences, Inc.) to obtain the amino-terminal GST fusion. DNA constructs encoding myc-tagged Sm proteins were described previously (11). All constructs were analyzed by automated DNA sequencing. Human Gemin6 sequence has been deposited in the GenBank™ data base under accession number AF453443.

*Cell Culture and Treatments*—HeLa PV, HeLa Tet-ON, or 293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Cells were transfected by the standard calcium phosphate method. Following overnight incubation with DNA, cells were washed and fresh medium added.

*Generation of Stable Cell Lines*—HeLa Tet-ON cells (CLONTECH) that constitutively express the tetracycline transactivator were co-transfected with the pTRE plasmids encoding FLAG-SMN or FLAG-Gemin2 (25 $\mu$g) and the pTK-Hyg plasmid (1.25 $\mu$g) carrying the hygromycin resistance gene. Stable clones were obtained by double selection in the presence of G418 (100 $\mu$g/ml) and hygromycin (200 $\mu$g/ml). Individual clones were isolated and analyzed by Western blot for the expression of FLAG-SMN and FLAG-Gemin2 following induction by doxycycline (5 $\mu$g/ml) for 24 h.

*Affinity Purification of SMN Complexes*—FLAG-SMN and FLAG-Gemin2 cell lines were grown in the presence of G418 (100 $\mu$g/ml), hygromycin (100 $\mu$g/ml), and doxycycline (5 $\mu$g/ml). HeLa Tet-ON (control) cells were grown in the presence of G418 and doxycycline. Total cell extracts were prepared by resuspending cell pellets in RSB-100 (10 mM Tris HCl, pH 7.4, 2.5 mM $MgCl_2$ 100 mM NaCl) buffer containing 0.1% Nonidet P-40 and protease inhibitors. Cells were passed five times through a 27-gauge needle and sonicated briefly. Following centrifugation at 10,000 rpm for 15 min, supernatants were passed through a 0.2-$\mu$m filter and added to anti-FLAG beads (Sigma) pre-washed with the same buffer. Extracts were incubated with anti-FLAG beads for 2 h at 4 °C. Supernatants were discarded, and beads were extensively washed with RSB-100 containing 0.02% Nonidet P-40. Three high salt washes were performed with ten bed volumes of RSB-500 (500 mM NaCl) buffer containing 0.02% Nonidet P-40 for 15 min at 4 °C. Following three washes with RSB-100 containing 0.02% Nonidet P-40, bound complexes were eluted with 10 bed volumes of the same buffer containing 0.25–0.5 mg/ml 3X-FLAG peptide (Sigma Chemical Co.) for 1 h at 4 °C.

*Protein Micro-sequencing by Mass Spectrometry*—Bands were excised from a single one-dimensional or two-dimensional silver-stained polyacrylamide gel and in gel digested with trypsin as described previously (27). Tryptic peptides in the supernatant were analyzed by nano-electrospray tandem mass spectrometry as described previously (28) using a QSTAR (MDS Sciex, Toronto, Canada) equipped with a nano-electrospray ion source (MDS Proteomics, Odense, Denmark). Comprehensive protein and expressed sequence tag (EST) data bases were searched using the Protein and Peptide Software Suite (MDS Proteomics). No limitations on protein molecular weight and species of origin were imposed.

*Antibodies*—Antibodies used in these experiments were as follows: 2B1, mouse IgG1 monoclonal anti-SMN (14); 2E17, mouse IgG1 monoclonal anti-Gemin2 (10); 11G9, mouse IgG1 monoclonal anti-Gemin3 (11); 22C10, mouse IgG1 monoclonal anti-Gemin4 (12); 4B10, mouse IgG2a monoclonal anti-hnRNPA1 (29); R288, rabbit polyserum anti-p80 coilin (30); 9E10, mouse IgG1 monoclonal anti-myc; A-14, affinity-purified rabbit polyserum anti-myc 9E10 epitope (Santa Cruz Biotechnologies); M2, mouse IgG1 monoclonal anti-Flag (Sigma).

*Immunoprecipitation Experiments*—Immunoprecipitation experiments were performed from 293T total cell extracts as previously described (29). Western blot and two-dimensional gel electrophoresis were performed as previously described (10).

*Immunofluorescence Microscopy*—HeLa cells, plated on glass coverslips, were briefly washed with PBS, fixed in 2% formaldehyde/PBS for 20 min at room temperature, permeabilized in 0.5% Triton X-100/PBS for 5 min at room temperature. Cells were blocked in 3% bovine serum albumin for 1 h at room temperature. Double-label immunofluorescence experiments were performed by separate sequential incubations of each primary antibody, diluted in PBS containing 3% bovine serum albumin, followed by the specific secondary coupled to fluorescein isothiocyanate or TXRD. All incubations were at room temperature for 1 h. Indirect epifluorescence microscopy was performed with a Nikon Eclipse E800 microscope. Digital images were collected with a Cook Sensicam high performance camera and processed with the IP laboratory software.

*In Vitro Binding Experiments*—Recombinant GST and GST-Gemin6 proteins were expressed in *Escherichia coli* BL21 and purified by affinity chromatography on glutathione-Sepharose beads (Amersham Biosciences, Inc.) according to the manufacturer's instructions. *In vitro* binding experiments were carried out using purified recombinant GST or GST-Gemin6 (5 $\mu$g) proteins bound to glutathione beads and Sm proteins translated *in vitro* in the presence of [$^{35}$S]methionine (Amersham Biosciences, Inc.) in 1 ml of binding buffer containing 0.02% Nonidet P-40 as previously described (15).

## RESULTS AND DISCUSSION

*Generation of Stable Cell Lines That Express FLAG-tagged SMN and Gemin2*—We wished to establish a cell system that would allow isolation of the native endogenous SMN complex for the characterization of its composition, structure, and function. To do so, we generated stable cell lines that express epitope-tagged SMN and Gemin2. The open reading frames encoding SMN or Gemin2 fused at the amino terminus to the FLAG epitope (FLAG-SMN and FLAG-Gemin2) were generated by PCR amplification and were cloned into the pTRE vector downstream of a promoter containing the tetracycline-responsive element (pTRE-FLAG-SMN and pTRE-FLAG-Gemin2). HeLa Tet-ON cells were transfected either with pTRE-FLAG-SMN or with pTRE-FLAG-Gemin2, and stably integrated clones were selected with antibiotics (see "Experimental Procedures"). Over 50 clones were isolated and analyzed for the expression of the FLAG-tagged proteins in the presence of doxycycline (a tetracycline analogue) in the medium. To avoid possible side effects of SMN or Gemin2 overexpression, we selected those stable cell lines that express substoichiometric levels of the FLAG-tagged proteins (as determined by Western blotting using anti-SMN antibodies) and whose morphology, growth, and division rates are indistinguishable from those of the parental cell line (HeLa Tet-ON). Fig. 1 shows Western blot analysis of FLAG-SMN and FLAG-Gemin2 expression in representative clones after 48 h of incubation in the absence or in the presence of the indicated concentrations of doxycycline. The expression levels of FLAG-SMN and FLAG-Gemin2 directly correlate with the amount of doxycycline over a wide range of concentrations and do not exceed 20% of the corresponding endogenous proteins. We did not observe changes in the endogenous levels of any of the SMN complex components upon expression of FLAG-SMN or FLAG-Gemin2 (Fig. 1 and data not shown). Taken together, we conclude that the expression of the FLAG-tagged SMN or Gemin2 has no effects on the metabolism of the respective cell lines.

*Purification of Native SMN Complexes*—Next we carried out affinity purification of SMN complexes from these cell lines. In all the experiments described below, cells were cultured in the presence of doxycycline at a concentration of 5 $\mu$g/ml. Total cell extracts from the FLAG-SMN and FLAG-Gemin2 stable cell

lines, and the parental HeLa Tet-ON strain as a control, were analyzed by immunoprecipitation with anti-FLAG antibodies as described under "Experimental Procedures." Bound proteins were eluted by competition with an excess of FLAG peptide and analyzed by polyacrylamide gel electrophoresis and silver staining. Fig. 2*A* shows that several specific proteins are detected in the immunoprecipitate from the FLAG-SMN and FLAG-Gemin2 cell extracts as compared with the control extract. Silver staining and Western blot analysis indicate that high salt washes greatly improve the purity of the complexes without compromising the association of the SMN complex components (Fig. 2*A* and data not shown). Moreover, the protein composition of the complexes isolated via FLAG-SMN or FLAG-Gemin2 is identical and they likely contain all the integral components of the SMN complex (Fig. 2*B*). Among the specific proteins, all the known components of the complex, SMN, Gemin2, Gemin3, Gemin4 are present in similar relative stoichiometry to that observed in complexes purified with anti-SMN or anti-Gemin2 antibodies (Ref. 10 and see below). The presence of smaller amounts of Sm and U1A proteins, whose association is partially labile to high salt washes, was confirmed either by mass spectrometric peptide sequencing or by Western blot analysis with specific monoclonal antibodies (data not shown).

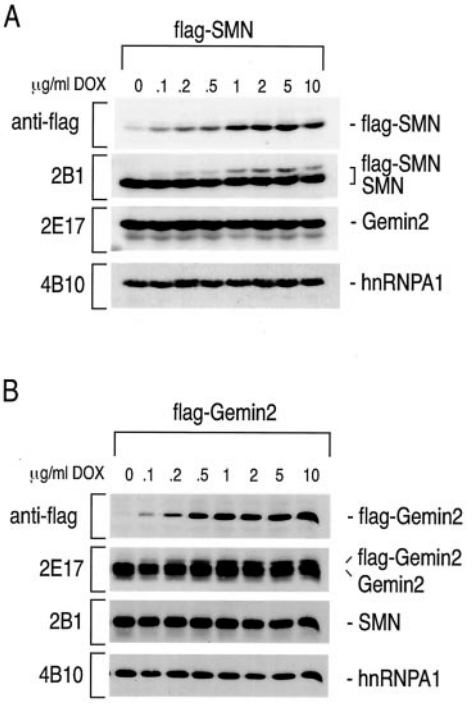


FIG. 1. **Generation of stable cell lines that express FLAG-tagged SMN and Gemin2 under the control of a tetracycline inducible promoter.** Western blot analysis of total cell lysates from the FLAG-SMN (*A*) and FLAG-Gemin2 (*B*) stable cell lines cultured for 24 h in the absence or in the presence of the indicated amounts of doxycycline (*DOX*). In each panel the monoclonal antibodies used are indicated on the *left* and the corresponding proteins on the *right*.

We conclude that this system is suitable for the purification of native SMN complexes containing all the core components of the complex with correct stoichiometry, native conformation, and putative post-translational modifications. Both cell lines grow in suspension allowing large-scale purification of the SMN complex. The availability of purified native SMN complexes will play an important role in future studies of the structure and molecular function of this macromolecular machine.

*Mass Spectrometry Analysis of Purified SMN Complexes*—Several not yet identified proteins that co-purified with complexes isolated via FLAG-tagged SMN or Gemin2 are very likely to be novel components of the SMN complex (Fig. 2). We analyzed several of the more prominent proteins by peptide sequencing by mass spectrometry. Additionally, sub-stoichiometric amounts of additional proteins with molecular masses in the 68 to 160 kDa range specifically co-purify with both FLAG-SMN and FLAG-Gemin2 (brackets in Fig. 2*A*). In particular, a 70-kDa protein co-purifies reproducibly with the SMN complex. Western blotting indicated that the 70-kDa protein is neither the U1 70K protein nor the heat shock cognate 70 protein (data not shown). The identification of this protein and whether it represents a genuine component of the SMN complex is currently under investigation. The 175-kDa

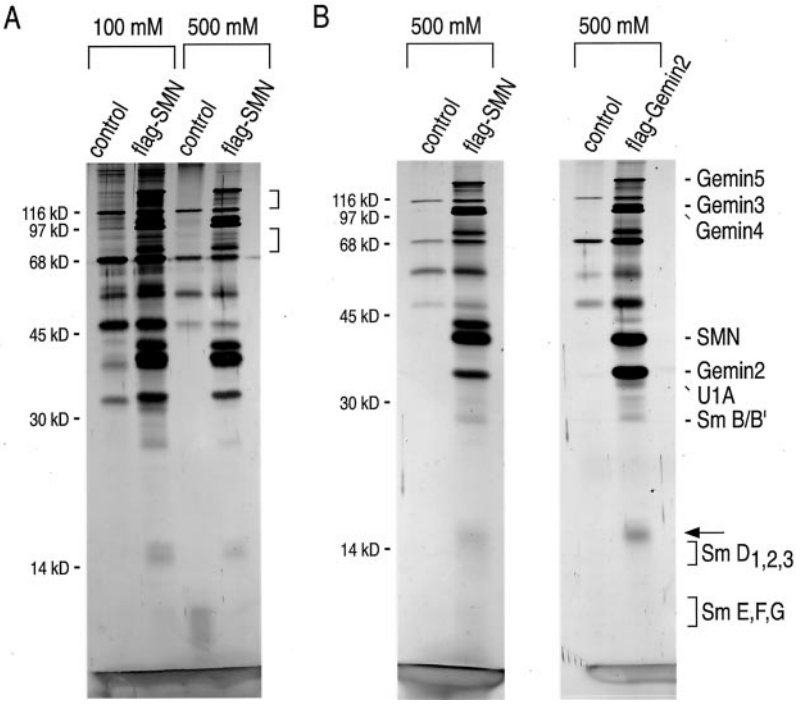


FIG. 2. **Affinity purification of native SMN complexes.** Total cell extracts from FLAG-SMN, FLAG-Gemin2, and HeLa Tet-ON (control) cells were prepared as described under "Experimental Procedures." SMN complexes were purified by affinity chromatography using anti-FLAG beads, washed extensively, and eluted with an excess of the FLAG peptide. *A*, SDS-PAGE analysis on a 12.5% polyacrylamide gel and silver staining of proteins eluted from FLAG-SMN and HeLa Tet-ON (control) total extracts using anti-FLAG beads following washes in the presence of 100 or 500 mM NaCl. *Brackets* indicate unidentified proteins specifically associated with the SMN complex. *B*, SDS-PAGE analysis on a 12.5% polyacrylamide gel and silver staining of proteins eluted from FLAG-SMN, FLAG Gemin2, and HeLa Tet-ON (control) total extracts using anti-FLAG beads following washes in the presence of 500 mM NaCl. Known components of the SMN complexes are indicated on the *right*. Molecular mass markers are indicated on the *left*.

polypeptide, also described by Meister *et al.* (31), is a novel protein component of the SMN complex, termed Gemin5, that binds to SMN and localizes to gems (13). Mass spectrometry analysis and peptide sequencing of the 16-kDa protein band, which is specifically purified via FLAG-SMN and FLAG-Gemin2, indicated the presence of SmD1, SmD2, and SmD3 proteins as well as of unrelated proteins (*arrow* in Fig. 2). In earlier work (10), two SMN complex components of ~16 and 15 kDa, named SIP2 and SIP3, respectively, were identified in anti-SMN immunoprecipitates (see also Fig. 3, *2B1 panel*). To demonstrate directly the presence of both these proteins, we compared the immunoprecipitates obtained from total HeLa cell extracts labeled with [$^{35}$S]methionine using anti-SMN (2B1) or control (SP2/0) antibodies and the complexes purified via FLAG-SMN by two-dimensional gel electrophoresis and autoradiography or silver staining. Fig. 3 shows that, in addition to SmF, specific proteins of ~16 and 15 kDa, previously referred to as SIP2 and SIP3, respectively (10), are present in both SMN immunoprecipitates. The spots corresponding to these proteins were excised from the gel and digested with trypsin. The resulting peptides were sequenced by nanoelectrospray mass spectrometry as described under "Experimental Procedures." Peptide sequences obtained from both proteins revealed that they are unknown proteins matching perfectly to two different EST sequences in the data base. Below we present the characterization of the 16-kDa protein (SIP2) as a novel component of the SMN complex that we name Gemin6.

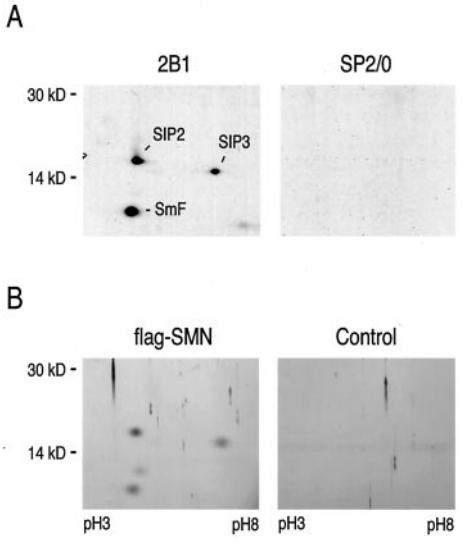


FIG. 3. **p16 (SIP2) and p15 (SIP3) are specific protein components of the SMN complex.** *A*, total extracts from HeLa cells labeled with [$^{35}$S]methionine were analyzed by immunoprecipitation using anti-SMN (*2B1*) or control (*SP2/0*) antibodies and analyzed by two-dimensional gel electrophoresis and autoradiography. *B*, total extracts from FLAG-SMN (*flag-SMN*) and HeLa Tet-ON (*Control*) cells were analyzed by immunoprecipitation using anti-FLAG antibodies, and bound proteins were analyzed by two-dimensional gel electrophoresis and silver staining. The positions corresponding to the molecular mass markers and the pH are indicated on the *left* and on the *bottom*, respectively. Proteins are labeled as reported previously (10).

*Gemin6 Is a Component of the SMN Complex*—The peptide (VQDLIEGHLTASQ) obtained from the 16-kDa protein (Gemin6) matched perfectly to several EST sequences in the data base, and the corresponding cDNA was obtained by PCR amplification. Gemin6 cDNA contains both start and stop codons, and an in-frame stop codon upstream of the initial methionine indicates that the cDNA represents the full-length transcript. Gemin6 cDNA encodes a protein of 167 amino acids of predicted molecular mass (18 kDa) and isoelectric point (pI = 4.84) consistent with the size and the pI of the protein eluted and analyzed by mass spectrometry. Fig. 4 shows the alignment of putative Gemin6 orthologues that are found in mouse (identity 86%, similarity 96%) and *C. elegans* (identity 22%, similarity 41%). SMN is found in several organisms, including *S. pombe* and *D. melanogaster* (5–8), whose entire genome sequence has been completed. We were unable to identify Gemin6 orthologues in these organisms possibly due to the evolutionary divergence of its amino acid sequence. Indeed, Gemin2 orthologue of *S. pombe* was identified based on its interaction with SMN and not based on its amino acid sequence conservation (7). Alternatively, it is possible that Gemin6 is not present in these organisms. Data base searches revealed that Gemin6 has no significant homology to other proteins and contains no known motifs that may suggest possible functions. The observation that Gemin6 co-purifies with SMN under highly stringent conditions indicates that it is a novel core component of the SMN complex. To confirm this, we transiently transfected 293T cells with a vector encoding a FLAG-tagged Gemin6 fusion protein (FLAG-Gemin6) or with an empty vector as a control (mock). Total extracts were analyzed by immunoprecipitation using anti-FLAG antibodies, and bound proteins were analyzed by SDS-PAGE and Western blotting. Fig. 5 shows that FLAG-Gemin6 is expressed and efficiently immunoprecipitated by anti-FLAG antibodies. All the components of the SMN complex, SMN, Gemin2, Gemin3, and Gemin4, are co-immunoprecipitated with FLAG-Gemin6. The immunoprecipitation is specific, because the abundant protein hnRNP A1 is not immunoprecipitated with FLAG-Gemin6 and none of the proteins are immunoprecipitated from a control extract that

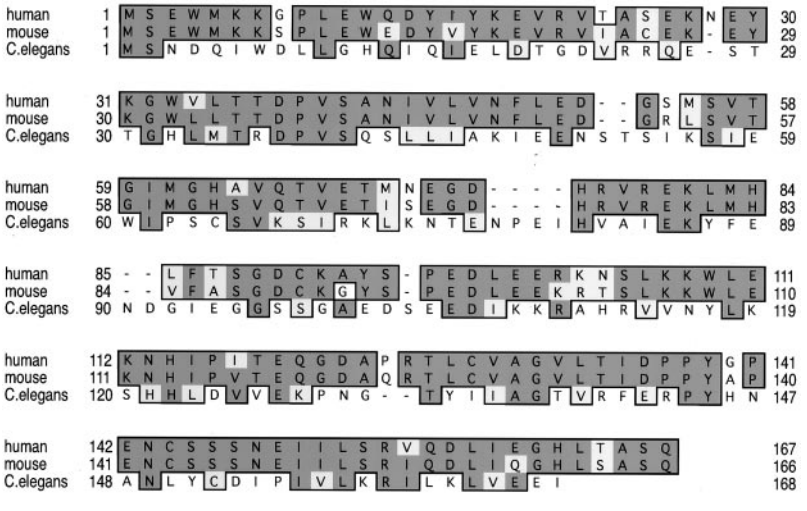


FIG. 4. **Alignment of Gemin6 protein sequences.** Sequences corresponding to human Gemin6 (GenBank™ AF453443) and its putative mouse (GenBank™ gi:12847586) and *C. elegans* (GenBank™ gi:7498072) orthologues were aligned using ClustalW alignment of the MacVector 6.5 program. *Dark gray boxes* indicate identical amino acids; *light gray boxes* indicate similar amino acids.

does not express Gemin6. These results demonstrate that Gemin6 is a novel component of the SMN complex.

*Gemin6 Co-localizes with SMN in Gems*—SMN localizes in both the cytoplasm and the nucleus, where it is highly concentrated in gems (14). Gems are nuclear bodies that are similar in size and number to Cajal bodies and can be found, depending on the cell type or tissue analyzed, either separated or associated with them (14, 32–34). All the components of the SMN complex, including Gemin2, Gemin3, and Gemin4, have an identical subcellular localization in gems (10–14). In contrast, most targets of SMN complex interaction such as snRNPs and snoRNPs localize to Cajal bodies but not to gems when these structures are found separated (14, 35). To further characterize Gemin6, we sought to analyze its subcellular localization. To do so, we transiently transfected myc-tagged Gemin6 in HeLa PV cells and carried out indirect immunofluorescence microscopy. Fig. 6*A* shows that Gemin6 localizes to discrete nuclear domains in addition to the cytoplasm and the nucleoplasm. These nuclear domains are gems, because they colocalize perfectly with SMN (Fig. 6, *A–C*) but not with coilin (Fig. 6, *D–F*), the marker of Cajal bodies (30). Similar to all previously identified components of the SMN complex (10–14), Gemin6 staining without coilin can be observed in transfected cells. In HeLa S3 cells that typically display fused gems and Cajal bodies, Gemin6 coincides with both SMN and coilin (data not shown). These experiments demonstrate that Gemin6 is a novel component of the SMN complex and is localized similarly in cells.

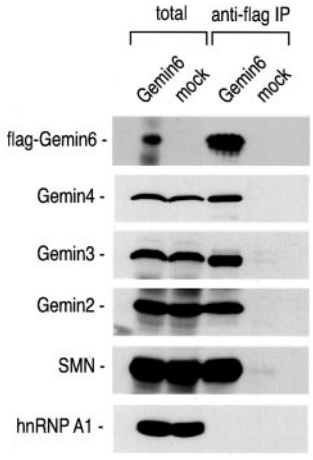


FIG. 5. **Gemin6 is a component of the SMN complex.** 293T cells were transiently transfected with a pcDNA3 vector encoding FLAG-tagged Gemin6 (*Gemin6*) or empty pcDNA3 vector (*mock*). Total extracts were prepared from these cells and analyzed by immunoprecipitation using anti-FLAG antibodies. Bound proteins were analyzed by SDS-PAGE and Western blotting with monoclonal antibodies against the proteins indicated on the *left*. *total* represents 10% of the extract used for the immunoprecipitation.

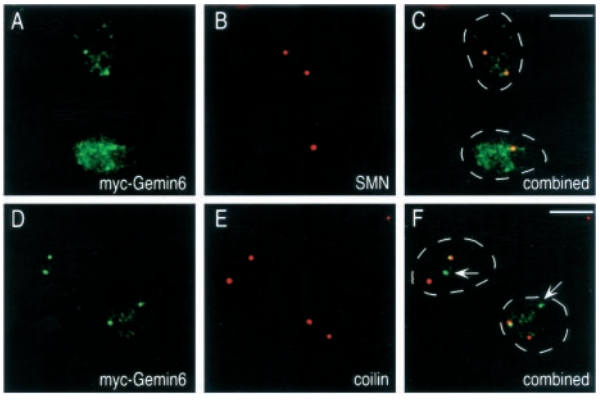


FIG. 6. **Gemin6 localizes to gems.** HeLa PV cells were transiently transfected with a pcDNA3 vector encoding myc-tagged Gemin6 (*myc-Gemin6*) and analyzed by indirect immunofluorescence and epifluorescence microscopy. *A–C*, double-labeling experiments of cells expressing myc-Gemin6 using anti-myc (*A*) or anti-SMN (*B*) antibodies. *D–F*, double-labeling experiments of cells expressing myc-Gemin6 using anti-myc (*D*) or anti-coilin (*E*) antibodies. Combined images are shown in *C* and *F*. *Arrows* indicate gems that are distinct from Cajal bodies. The intensity of the signal of the cytoplasm is underestimated compared with that of the nucleoplasm because of the focal plane that was chosen to show gems and Cajal bodies. *Dotted lines* demarcate the nucleus. *Scale bar*, 5 $\mu$m.

Next, we investigated the interactions of Gemin6 with the Sm proteins of spliceosomal snRNPs, the best characterized targets of SMN complex interaction and function. *In vitro* translated [$^{35}$S]methionine-labeled Sm proteins were incubated with purified recombinant GST-Gemin6 or GST, as a control. Fig. 7 shows that Gemin6 interacts specifically with SmD2 and SmE and, to a lesser extent, with SmB and SmD3. No interactions of Gemin6 with SmD1, SmF, and SmG or of any of the Sm proteins with GST were detected. These experiments demonstrate that, consistent with its presence in the SMN complex, Gemin6 interacts specifically with a subset of Sm proteins. Each of the core components of the SMN complex, except Gemin2, interacts with a distinct set of Sm proteins. Upon oligomerization, SMN interacts avidly with the symmetrically dimethylated arginines of the carboxyl-terminal RG-tails of SmD1 and SmD3 and with SmB (15, 17, 23). The DEAD box RNA helicase Gemin3 interacts with SmB and SmD3 (11), whereas Gemin4 and Gemin5 interact with SmB, SmD1, SmD2, SmD3, and SmE (12, 13). Gemin6 is the SMN complex component that interacts most avidly with SmD2 and SmE. Sm proteins form a heptameric ring, known as the Sm core, on the Sm site of all spliceosomal snRNAs (36). Co-immunoprecipitation experiments demonstrated that the SMN complex associ-

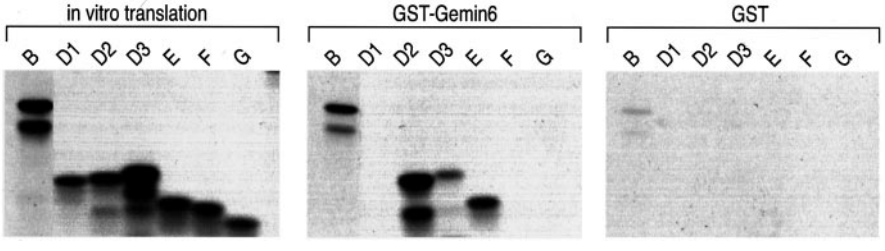


FIG. 7. **Gemin6 interacts *in vitro* with several Sm proteins of spliceosomal snRNPs.** Sm proteins were translated *in vitro* in the presence of [$^{35}$S]methionine and incubated with either GST-Gemin6 or GST purified from bacteria as described under "Experimental Procedures." Following extensive washes, bound proteins were analyzed by SDS-PAGE and autoradiography. The *in vitro translation panel* shows 5% of the input.

ates with all the Sm proteins *in vivo* and mediates their assembly possibly by delivering the Sm proteins to the snRNA (10, 24). Our previous and present studies indicate that individual components of the SMN complex interact with distinct sets of Sm proteins, providing direct evidence for multiple contacts between the Sm proteins and the SMN complex. It is possible that these multiple interactions may not take place simultaneously but that individual Sm proteins change binding partners within the SMN complex as a result of spatial rearrangements associated with the Sm core assembly on the snRNA.

With the identification of Gemin6 the inventory of the integral constituents of the SMN complex appears to be nearly complete. The system described here for purification of the native SMN complex will facilitate further studies of its interactions, structure, and function.

*Acknowledgments*—We thank Dr. Eng Tan for anti-p80 coilin antibodies. We are grateful to members of our laboratory for stimulating discussions and, in particular, Drs. Amelie Gubitz and Westley Friesen for critical comments on this manuscript.

REFERENCES

1. Melki, J. (1997) *Curr. Opin. Neurol.* **10,** 381–385
2. Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., Benichou, B., Cruaud, C., Millasseau, P., and Zeviani, M., *et al.* (1995) *Cell* **80,** 155–165
3. 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
4. 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
5. Owen, N., Doe, C. L., Mellor, J., and Davies, K. E. (2000) *Hum. Mol. Genet.* **9,** 675–684
6. Paushkin, S., Charroux, B., Abel, L., Perkinson, R. A., Pellizzoni, L., and Dreyfuss, G. (2000) *J. Biol. Chem.* **275,** 23841–23846
7. Hannus, S., Buhler, D., Romano, M., Seraphin, B., and Fischer, U. (2000) *Hum. Mol. Genet.* **9,** 663–674
8. Miguel-Aliaga, I., Chan, Y. B., Davies, K. E., and van den Heuvel, M. (2000) *FEBS Lett.* **486,** 99–102
9. Wang, J., and Dreyfuss, G. (2001) *J. Biol. Chem.* **276,** 9599–9605
10. Liu, Q., Fischer, U., Wang, F., and Dreyfuss, G. (1997) *Cell* **90,** 1013–1021
11. Charroux, B., Pellizzoni, L., Perkinson, R. A., Shevchenko, A., Mann, M., and Dreyfuss, G. (1999) *J. Cell Biol.* **147,** 1181–1194
12. Charroux, B., Pellizzoni, L., Perkinson, R. A., Yong, J., Shevchenko, A., Mann, M., and Dreyfuss, G. (2000) *J. Cell Biol.* **148,** 1177–1186
13. Gubitz, A. K., Mourelatos, Z., Abel, L., Rappsilber, J., Mann, M., and Dreyfuss, G. (November 19, 2001) *J. Biol. Chem.* 10174/jbc.M109448200
14. Liu, Q., and Dreyfuss, G. (1996) *EMBO J.* **15,** 3555–3565
15. Pellizzoni, L., Charroux, B., and Dreyfuss, G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 11167–11172
16. Buhler, D., Raker, V., Luhrmann, R., and Fischer, U. (1999) *Hum. Mol. Genet.* **8,** 2351–2357
17. Friesen, W. J., and Dreyfuss, G. (2000) *J. Biol. Chem.* **275,** 26370–26375
18. Pellizzoni, L., Baccon, J., Charroux, B., and Dreyfuss, G. (2001) *Curr. Biol.* **11,** 1079–1088
19. Jones, K. W., Gorzynski, K., Hales, C. M., Fischer, U., Badbanchi, F., Terns, R. M., and Terns, M. P. (2001) *J. Biol. Chem.* **276,** 38645–38651
20. Pellizzoni, L., Charroux, B., Rappsilber, J., Mann, M., and Dreyfuss, G. (2001) *J. Cell Biol.* **152,** 75–85
21. Mourelatos, Z., Abel, L., Yong, J., Kataoka, N., and Dreyfuss, G. (2001) *EMBO J.* **20,** 5443–5452
22. Hebert, M. D., Szymczyk, P. W., Shpargel, K. B., and Matera, A. G. (2001) *Genes Dev.* **15,** 2720–2729
23. Friesen, W. J., Massenet, S., Paushkin, S., Wyce, A., and Dreyfuss, G. (2001) *Mol. Cell* **7,** 1111–1117
24. Fischer, U., Liu, Q., and Dreyfuss, G. (1997) *Cell* **90,** 1023–1029
25. Pellizzoni, L., Kataoka, N., Charroux, B., and Dreyfuss, G. (1998) *Cell* **95,** 615–624
26. Meister, G., Buhler, D., Laggerbauer, B., Zobawa, M., Lottspeich, F., and Fischer, U. (2000) *Hum. Mol. Genet.* **9,** 1977–1986
27. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal. Chem.* **68,** 850–858
28. Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T., and Mann, M. (1996) *Nature* **379,** 466–469
29. Pinol-Roma, S., Choi, Y. D., Matunis, M. J., and Dreyfuss, G. (1988) *Genes Dev.* **2,** 215–227
30. Andrade, L. E., Tan, E. M., and Chan, E. K. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 1947–1951
31. Meister, G., Buhler, D., Pillai, R., Lottspeich, F., and Fischer, U. (2001) *Nat. Cell Biol.* **3,** 945–949
32. Matera, A. G., and Frey, M. R. (1998) *Am. J. Hum. Genet.* **63,** 317–321
33. Carvalho, T., Almeida, F., Calapez, A., Lafarga, M., Berciano, M. T., and Carmo-Fonseca, M. (1999) *J. Cell Biol.* **147,** 715–728
34. Young, P. J., Le, T. T., Dunckley, M., Nguyen, T. M., Burghes, A. H., and Morris, G. E. (2001) *Exp. Cell Res.* **265,** 252–261
35. Matera, A. G. (1999) *Trends Cell Biol.* **9,** 302–309
36. Will, C. L., and Luhrmann, R. (2001) *Curr. Opin. Cell Biol.* **13,** 290–301