Gemin3: A Novel DEAD Box Protein that Interacts with SMN, the Spinal Muscular Atrophy Gene Product, and Is a Component of Gems

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Abstract. The survival of motor neurons (SMN) gene is the disease gene of spinal muscular atrophy (SMA), a common motor neuron degenerative disease. The SMN protein is part of a complex containing several proteins, of which one, SIP1 (SMN interacting protein 1), has been characterized so far. The SMN complex is found in both the cytoplasm and in the nucleus, where it is concentrated in bodies called gems. In the cytoplasm, SMN and SIP1 interact with the Sm core proteins of spliceosomal small nuclear ribonucleoproteins (snRNPs), and they play a critical role in snRNP assembly. In the nucleus, SMN is required for pre-mRNA splicing, likely by serving in the regeneration of snRNPs. Here, we report the identification of another component of the SMN complex, a novel DEAD box putative RNA helicase, named Gemin3. Gemin3 interacts directly with SMN, as well as with SmB, SmD2, and SmD3. Immunolocalization studies using mAbs to Gemin3 show that it colocalizes with SMN in gems. Gemin3 binds SMN via its unique COOH-terminal domain, and SMN mutations found in some SMA patients strongly reduce this interaction. The presence of a DEAD box motif in Gemin3 suggests that it may provide the catalytic activity that plays a critical role in the function of the SMN complex on RNP.

Key words: helicase • nuclear bodies • DnRNP biogenesis • splicing • spinal muscular atrophy

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PINAL muscular atrophy (SMA) is a common autosomal recessive disease that is the leading hereditary cause of infant mortality. SMA is characterized by degeneration of motor neurons of the anterior horn of the spinal cord resulting in muscular weakness and atrophy (Roberts et al., 1970; Pearn, 1980; Czeizel and Hamula, 1989). SMA results from deletions or mutations in the survival of motor neurons gene (SMN), which is duplicated as an inverted repeat on human chromosome 5 at 5q13 (Bruzacek et al., 1990; Melki et al., 1990, 1994; Lefebvre et al., 1995; reviewed in Burghes, 1997). The telomeric copy of the SMN gene (SMN1) is deleted or mutated in >98% of SMA patients (Bussaglia et al., 1995; Chang et al., 1995; Cobben et al., 1995; Hahnen et al., 1995, 1996; Lefebvre et al., 1995; Rodrigues et al., 1995; Vlasco et al., 1996). The SMN protein is expressed in all tissues of mammalian organisms, but particularly high levels are expressed in motor neurons (Coovert et al., 1997; Lefebvre et al., 1997). 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 (Coovert et al., 1997; Lefebvre et al., 1997).

The SMN gene encodes a 294 amino acid protein that does not have extensive sequence similarity to other known proteins, however, it recently has been reported that SMN contains a sequence similar to the ribonucleoprotein consensus sequence RNP1 (Bertrand et al., 1999). SMN is part of a multiprotein complex and one protein of the complex, SIP1 (SMN interacting protein 1), has been characterized in detail so far (Li et al., 1997). SMN and SIP1 are found both in the nucleus and the cytoplasm of somatic cells. In the nucleus, SMN and SIP1 are concentrated in bodies called gems, which are similar in size and number to coiled bodies and are often associated with...
them (Liu and Dreyfuss, 1996; Liu et al., 1997). In addition to SMN and SIP1, the large cytoplasmic complex of which they are part of also contains additional proteins, including some of the Sm proteins that are common components of spliceosomal small nuclear ribonucleoproteins (snRNPs; Liu et al., 1997). The presence of the Sm proteins in the SMN–SIP1 complex is a result of a direct interaction between SMN and several of the Sm proteins (Liu et al., 1997; Pellizzoni et al., 1999). The role of the cytoplasmic pool of the SMN–SIP1 complex has been studied by antibody microinjection experiments in Xenopus laevis oocytes. These experiments revealed that SIP1 has a critical role in the assembly of snRNPs, a process which takes place in the cytoplasm where the SM proteins combine with snRNA that were exported from the nucleus (M. Attia and D. Robert, 1985; M. Attia, 1988; Lührmann et al., 1990; Fischer et al., 1997). Once properly assembled and modified, the snRNPs recruit the necessary nuclear import receptors and translocate into the nucleus where they function in pre-mRNA splicing (M. Attia, 1986, 1988; Lührmann et al., 1990; Nemanic V. Evagar and D. Ahlberg, 1990; Zieve and Sauterer, 1990). In contrast to the inhibitory effects of anti-SIP1 antibodies, the anti-SMN antibodies show some stimulation of snRNP assembly (Liu et al., 1997). By transfection of a dominant negative form of SMN (SMNΔN27) in HeLa cells, we found that SMN also plays a critical role in the cytoplasmic assembly of snRNPs (Pellizzoni et al., 1998). In the nucleus, the SMNΔN27 protein causes a striking rearrangement of the snRNPs, colocalizing with the mutant SMNΔN27 in enlarged gems (Pellizzoni et al., 1998). Using in vitro experiments, we have shown that SMN is required for pre-mRNA splicing, likely for the regeneration or recycling of snRNPs (Pellizzoni et al., 1998). SMN mutants found in SMA patients lack this activity because they are defective in their interaction with the SM proteins (Pellizzoni et al., 1999). Unlike the several recycling factors described so far that are essential for splicing, SMN and SIP1 do not contain DEAD/D/DEAH motifs (reviewed in Staley and Guthrie, 1998).

Here, we report the molecular cloning and characterization of a protein designated Gemin3 (for protein component of gem number 3) that associates with SMN in vitro and in vivo. Gemin3 is a novel DEAD box protein and is, therefore, a putative RNA helicase. We have produced mAbs to Gemin3 and show by immunofluorescence microscopy that it colocalizes with SMN in gems. Like SMN and SIP1, Gemin3 can be isolated in a complex with several spliceosomal snRNPs. We further found that Gemin3 interacts directly with SMN and with several of the spliceosomal snRNPs core Sm proteins, including the B and D2-3 proteins. The unique COOH-terminal domain of Gemin3 mediates its interaction with SMN and its localization to gems. The discovery of a DEAD box protein, a likely RNA helicase, in the SMN complex is of particular interest as the functions revealed so far suggest that this complex has crucial activities in the biogenesis of RNPs. To perform such functions, including assembly of the snRNPs and the regeneration of active components of the spliceosome, it would be expected that the SMN complex can affect structural changes in its RNP targets. Of the known components of the SMN complex, the DEAD box protein Gemin3 is the most likely protein to have the capacity to perform such a function. Importantly, SMN proteins with mutations found in SMA patients show a significantly reduced interaction with Gemin3, suggesting that the SMN complexes in these patients will be deficient in this protein.

**Materials and Methods**

**Identification of p105 Protein by Mass Spectrometry**

The p105 protein was communoprecipitated with anti-SMN mAb 2B1 and the band was excised from a single-dimensional Coomassie stained polyacrylamide gel and in-gel digested with trypsin (unmodified, sequencing grade; Boehringer Mannheim Corp.) as described in Shevchenko et al. (1996). Tryptic peptides were recovered from gel pieces by extraction with 5% formic acid and acetonitrile. The combined extracts were pooled together, dried in a speed vac, redissolved in 5% formic acid, and analyzed by nanoelectrospray tandem mass spectrometry (nano-ES MS/MS) as described in Wilms et al. (1996). Nano-ES MS/MS was performed on a API III triple quadrupole instrument (PE Sciex) equipped with a nano-ES ion source developed in EMBL (Wilms and Mann, 1996).

Comprehensive protein and expressed sequence tag (EST) databases were searched using PeptideSearch v. 3.0 software developed by M. Mann and P. Mortensen (University of Southern Denmark, Odense, Denmark). No limitations on protein molecular weight and species of origin were imposed.

**Cloning of the Gemin3 cDNA**

Several peptides of the p105 band analyzed by MS identified a human EST sequence (clone #A 309340) using the peptide sequence tag algorithm. The EST clone was assumed to be incomplete since it lacked a start codon and encoded for a putative protein of only 456 amino acids. The cloning of the full-length Gemin3 cDNA was achieved by hybridization screening of a human leukemia 5′-STRETCH PLUS cDNA library (from CLONETECH) using a subfragment (EcoRI–EcoRI) of the EST clone #A 309340 as a probe. The EST EcoRI fragment contained a region of the Gemin3 open reading frame (ORF) encoding amino acids 368–548. Twelve independent partial cDNA clones with insert sizes ranging from 1.2–2.5 kb were isolated, all of which contained overlapping regions of the same ORF that encoded for a putative protein of 824 amino acids. Since the clone lacked an in-frame stop codon upstream of the start codon, we performed two successive rounds of rapid amplification of 5′-cDNA ends by PCR (5′-RACE PCR) to extend further upstream the cloning of the Gemin3 cDNA. In brief, total mRNA from HeLa-S3 cells was prepared using the TRIZOL reagent (Life Technologies, Inc.). For the generation of cDNA, 2 μg of mRNA was reverse-transcribed using Superscript™ II reverse transcriptase (Life Technologies, Inc.). The reaction was primed with Gemin3 cDNA-specific primers, GSP1 (5′-TTTACACCTCTCAGGGC) or GSP2 (5′-CTTGGGGCTTCTTCAGGG), in the first or second round of RACE, respectively. The cDNA were then purified using a GlassMAX spin cartridge, tagged with dCTP and TdT, and amplified by PCR using the Gemin3 specific primer GSP2 (5′-TCTTCTTCTTCTTCTTTCC) and the Universal Amplification primer (UAP) from Life Technologies, Inc. (5′-CUACUAUCUAUCAGGCACCCGTTTGCTACGTAGTAC). PCR products were cloned into the pCR 2.1 vector by TA cloning (Invitrogen) and sequenced. The two independent 5′-RACE experiments yielded products terminating at the same 5′ position. The extended 5′-Gemin3 cDNA contained an in-frame stop codon upstream of the start codon. By in vitro transcription–translation, we confirmed that the cloned cDNA encoded the full-length Gemin3 protein (see below).

**Production of Proteins In Vitro**

The [35S]methionine-labeled proteins were produced by an in vitro coupled transcription–translation reaction (Promega Biotech) in the presence of [35S]methionine (Nycodemed A mersham, Inc.). 6His-Gemin3 and 6His-SMN fusion protein was expressed from a pET bacterial expression system in the Escherichia coli strain BL21(DE3) and purified using nickel chelation chromatography using Novagen His-bind buffer. GST–Gemin3 fusion protein was expressed from a GST expression vector pGEX-5X-3 (Pharmacia Biotech, Inc.) in the E. coli strain BL21 and purified using glutathione-Sepharose (Pharmacia Biotech, Inc.) according to the manufacturer’s protocol.
Production of mAbs to Gemin3

A n-t Gemin3 antibodies 11G9 and 12H12 were prepared by immunizing Balb/C mice with 6His-tag COOH-terminal domain of Gemin3 (from amino acids 368-384) purified from nickel chelation chromatography using Novagen His-1Bind buffer kit. Hybridoma production, screening, and ascites fluid production were performed as previously described (Choi and Dreyfuss, 1994).

Immunoprecipitation and Immunoblotting

Immunoprecipitations of in vitro translated proteins were carried out in the presence of 1% Emgipeg BB buffer as previously described (Choi and Dreyfuss, 1984). Immunoprecipitations of SMN, the Sm proteins, and Gemin3 from cell lysates were carried out using total HeLa lysate in the presence of 1% Emgipeg BB buffer as previously described (Choi and Dreyfuss, 1984). Immunoprecipitations and purifications of the SMN, Gemin2, Sm, and Gemin3 complexes were carried out using total HeLa lysate in the presence of 0.5% Triton X-100 as previously described (Pinol-Roma et al., 1988). For immunoblotting, proteins were resolved on 12.5% SDSPolyacrylamide gels and transferred to a nitrocellulose membrane (Schleicher and Schuell, Inc.) using a BioTrans Model B Transblot apparatus (Gelman Science) according to the manufacturer’s instructions. The membranes were then incubated in blocking solution (PBS 5% nonfat milk) for at least 1 h at room temperature, rinsed with cold PBS, and then incubated in blocking solution with primary antibody for at least 1 h at room temperature. The membranes were subsequently washed three times in PBS containing 0.05% NP-40, and bound antibodies were detected using the peroxidase-conjugated goat anti-mouse IgG plus IgM (Jackson Immunoresearch Laboratories). The antibody-decorated protein bands were visualized by an ECL Western blotting kit (Nycomed Amersham, Inc.) after washing three additional times with PBS containing 0.05% NP-40.

Cell Culture and Treatments

HeLa cells were cultured in DMEM supplemented with 10% FBS (both from GIBCO BRL). HeLa cells, plated on glass coverslips, were transfected by the standard calcium phosphate method. Following overnight incubation with DNA, cells were washed and fresh medium was added. Transfected cells were fixed and processed by immunofluorescence staining after an additional 24-36 h of incubation.

Immunofluorescence Microscopy

Immunofluorescence staining was carried out essentially as previously described (Choi and Dreyfuss, 1984). Double-label immunofluorescence experiments were performed by separate sequential incubations of each primary antibody followed by P5% BSA, followed by the specific secondary antibody coupled to either FITC or Texas red. All incubations were carried out at room temperature for 1 h. Laser confocal fluorescence microscopy was performed with a Leica TCS 4D confocal microscope. Images from each channel were recorded separately and, where indicated, the files were merged. A nitrobenzide in these experiments were as follows: mouse IgG1 monoclonal anti-Gemin3 (11G9 and 12H12; this work); mouse IgG1 monoclonal anti-SM N (2B1; Liu and Dreyfuss, 1996); rabbit polyclonal anti-p80 coilin (R288; A ndrade et al., 1991); mouse IgG2 monoclonal anti-Sm (Y12; Lerner et al., 1981); and SP2/O, a nonimmunoglobulin chains secreting mouse hybridoma (ATTCC). The rabbit affinity-purified anti-Exon7 antibody was made against the polypeptide encoded by the SMN exon 7 (Liu et al., 1997).

In Vitro Protein-binding Assay

Purified GST or GST fusion proteins (2 μg) bound to 25 μl of glutathione-Sepharose beads were incubated with 106 cpm of the in vitro translated protein mix in 1 ml of binding buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM EDTA, 0.1% NP-40, 2 μg/ml leupeptin and pepstatin A, and 0.5% aprotinin). A fter 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 run on SDS-PAGE. The gels were fixed for 30 min and the signal enhanced by treatment with A mplify solution (Nyloncom A mersham, Inc.). For direct in vitro binding (see Fig. 7C), purified GST or GST-Gemin3 proteins (2 μg) bound to 25 μl of glutathione-Sepharose beads were incubated with 5 μg of purified 6His-SMN or 6His-mb in 1 ml of binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.05% NP-40, 2 μg/ml leupeptin and pepstatin A, and 0.5% aprotinin). A fter 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 analyzed by SDS-PAGE and Western blot using a rabbit polyclonal anti-His-tag antibody (Santa Cruz Biotech).

Cell Fractionation and Chromatography

HeLa cells were fractionated according to Dignam et al. (1983). S100 fractions (400 μl of ~20 mg/ml protein) in buffer F (20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 500 mM KCl) were loaded on a Superose 6 HR 10/30 column (Pharmacia Biotech, Inc.). The column was then washed with buffer A (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2.5% glycerol). Fractions (0.5 ml) were collected, and 30 μl of each fraction was resolved on SDS-PAGE, followed by Western blotting.

Accession Number

The Gemin3 GenBank/EMBL/DDBJ accession number is A F171063.

Results

Gemin3, a Novel SMN-Interacting Protein with a DEAD Box RNA Helicase Domain

Immunoprecipitations from [35S]methionine-labeled HeLa cell lysates with anti-SMN and anti-SIP1 mAbs revealed the presence of several protein components in the SMN-SIP1 complex (Li et al., 1997). Among the proteins that
can be coimmunopurified with anti-SMN and anti-SIP1 antibodies, only some of the major low molecular mass proteins, identified as the Sm proteins, have been characterized so far (Liu et al., 1997; Fig. 1, A and B). In addition to SMN, SIP1, and the Sm proteins, there is a doublet at ~97 kDa, and additional bands at 175, 95, 60, and 50 kDa that coimmunopurified with the anti-SMN antibody. The two proteins of the 97-kDa doublet were eluted from the gel, digested with trypsin, and the resulting peptides sequenced by nano-ES MS as described previously (Shevchenko et al., 1996; Wilm et al., 1996; Fig. 2). In this paper, we describe the molecular cloning and characterization of the high molecular weight protein of this doublet (p105). Several peptides from this band identified a human EST sequence (clone #A 303940) using the peptide sequence tag algorithm (Fig. 2). Several additional cDNA clones were obtained by hybridization screening of a human leukemia 5'-STRETCH PLUS cDNA library, using this EST clone as a probe. We isolated 12 independent partial cDNA clones with insert sizes ranging from 1–2.5 kb, all of which contained overlapping regions of the same ORF. 5' RACE PCR was used to extend this cDNA further upstream. A cDNA clone containing the longest ORF was constructed and conceptual translation of its nucleotide sequence revealed a potential initiator methionine, preceded by an in frame stop codon. This cDNA encodes a putative protein of 824 amino acids with a calculated molecular mass of 92.2 kDa and a pl of 6.5. We then determined that this cDNA encodes the p105 protein that coimmunoprecipitates with SMN (see Gemin3 and SMN Colocalize in Gems). Thus, this is a full-length cDNA clone for a novel component of the SMN complex that we termed Gemin3, for component of gems number 3 (see below). Because of the existence of several unrelated proteins called SIP1 (Mylin et al., 1994; Zhang and Wu, 1998; Verschueren et al., 1999), we tentatively rename the SMN-interacting SIP1 (Liu et al., 1997) Gemin2, for component of gems number 2 (SMN is the first component of gems identified; Liu and Dreyfuss, 1996). Gemin3 has high amino acid sequence similarities with the RNA helicase factor in the SMN complex turned out to be negative by both coimmunoprecipitation and direct binding to several components of the SMN complex (data not shown). Peptide ions designated with asterisks were identified as peptides from immunoglobulins used for immunofinity purification. Tandem mass spectra acquired from the peptide ions having m/z 552.9 and 622.5 did not identify any protein in a protein sequence database. However when the search was performed against a comprehensive database of expressed sequence tags (dBEST), the peptide sequence V L I S T D L T S R from EST clone W 6 5 9 0 8 was identified as matching the tandem mass spectrum. A full-length sequence had been obtained (see Materials and Methods) the tandem mass spectrum acquired from the peptide ion at m/z 622.5 was matched to the peptide L N S S D P S L I G L K present in the sequence of Gemin3. B, Tandem mass spectrum, acquired from doubly charged peptide precursor ion having m/z 552.9. Continuous series of the fragment ions containing the COOH terminus of the peptide (Y ions, Pepstorff and Fohlman, 1984) is produced upon collisional fragmentation of tryptic peptides. A short stretch of the peptide sequence was deduced unambiguously by considering precise mass differences between adjacent Y ions (in bold) observed in a part of the spectrum above m/z of the parent ion. Note that L, E, and I residues have the same nominal mass and, therefore, are usually not distinguished by MS. The determined piece of a peptide sequence was combined with the masses of correspondent Y ions and with the mass of intact peptide into a peptide sequence tag (Mann and Wilm, 1994) that was subsequently used for searching protein and EST databases by the program PeptideSearch. Once the database search produced a hit, the corresponding peptide sequence was retrieved from a database and masses of the ions from the NH2-terminal fragment series (A- and B-ions) were used to verify the match. This enabled highly confident protein identification albeit a single peptide containing only ten amino acid residues was matched to the sequence of EST clone.

Figure 2. Sequencing of Gemin3 by nano-ES MS/MS. A, Part of the spectrum of the unseparated in-gel tryptic digest of p105 band. Peptide ions, designated by T, are autolysis products of trypsin and were identified by comparison with the spectrum acquired from the control sample. Other peptide ions observed in the spectrum were in turn isolated by the first mass analyzer of a triple quadrupole instrument, fragmented in the collision cell, and their tandem mass spectra acquired. Upon searching a comprehensive protein sequence database using tandem mass spectrometric data (see below) peptide ions designated with filled triangles were identified as tryptic peptides originating from PTB-associated splicing factor (P23246). The presence of PTB-associated splicing
core region of the human eukaryotic initiation factor 4A-II (eIF4A-II). eIF4A-II is a DEAD box RNA helicase that belongs to the SFII superfamily of helicase (reviewed in De la Cruz et al., 1999). A scheme depicting the modular structure of Gemin3 and the predicted amino acid sequence of Gemin3 aligned with the sequence of eIF4A-II are presented in Fig. 3. This alignment showed the presence of seven motifs in the Gemin3 protein, which are characteristic of the RNA helicase core region. Database searches with the COOH-terminal nonconserved region did not reveal significant homology to any other protein or any recognizable motifs.

Production of mAbs to Gemin3

To investigate the interaction of Gemin3 with SMN and to characterize Gemin3 further, we generated mAbs to it by immunizing mice with a purified bacterially produced recombinant 6His-Gemin3 fragment (amino acids 368–548). Two hybridomas, 11G9 and 12H12, were selected for additional studies. Several lines of evidence demonstrate that these hybridomas indeed produce mAbs that recognize Gemin3 specifically. First, both 11G9 and 12H12 recognized purified 6His-Gemin3 on Western blots, but did not recognize similarly produced and purified 6His-Gemin2 (Fig. 4 B). Finally, on an immunoblot of total HeLa lysate, both 11G9 and 12H12 recognized a single protein of ≈105 kD (Fig. 4 C). mAbs 11G9 or 12H12 did not recognize a specific protein on a Western blot with total mouse 3T3 cell lysate or Xenopus laevis XL-177 cell lysate. However, 11G9 specifically immunoprecipitated a single protein of ≈105 kD from these cell lysates (data not shown), suggesting that Gemin3, like SMN, is conserved in vertebrates.

Gemin3 and SMN Colocalize in Gems

Indirect laser confocal immunofluorescence microscopy using antibodies 11G9 and 12H12 was performed on HeLa cells to determine the subcellular localization of Gemin3. Fig. 5 A shows that Gemin3 is found throughout the cytoplasm and also displays intense staining of prominent discrete nuclear bodies that are readily discernible by differential interference contrast (DIC; Fig. 5 B). The pattern is similar to that seen for SMN and Gemin2 (Liu and Dreyfuss, 1996; Liu et al., 1997), except that the nucleoplasmic staining of Gemin3 is stronger. To determine if the nuclear structures stained by 11G9 are gems or coiled bodies, we
eral prominent bands at 175, 95, and 50 kD. The two
in addition to Gemin3, SMN, and Gemin2, there are several prominent bands at 175, 95, and 50 kD. The two
proteins were then analyzed by SDS-PAGE. As referred to in previous studies, the immunoprecipitated proteins
were analyzed by SDS-PAGE and Western blot experiments. The anti-Gemin3 mAb 11G9 and 12H12 are specific for
cogins described so far. Compared with visual observation, the confocal photographs in Fig. 5, slightly overestimates
the nuclear staining and underestimates the cytoplasmic staining for Fig. 5, C and D.

Gemin3 Is in a Complex with SMN, Gemin2, and the Spliceosomal Sm Proteins
To characterize further the Gemin3 complex, immunoprecipitations using anti-Gemin3 mAb bs and [35S]methionine
labeled HeLa cells were carried out in the presence of either Triton X-100 or the more stringent detergent, Empigen BB (Matunis et al., 1994). The immunoprecipitated proteins were then analyzed by SDS-PAGE. As references for these immunoprecipitations, we also included an immunoprecipitation with the anti-Sm mAb Y12 (Lerner and Steitz, 1979; Lerner et al., 1981) and an immunoprecipitation with the anti-SMN mAb 2B1. As shown in Fig. 6 A, several proteins can be coimmunoprecipitated with Gemin3 and the pattern of immunoprecipitated proteins is very similar to that obtained with the anti-SMN antibody. In addition to Gemin3, SMN, and Gemin2, there are several prominent bands at 175, 95, and 50 kD. The two

Figure 4. mAbs 11G9 and 12H12 are specific for Gemin3. A, Myc-tagged Gemin3, hnrRNP A1, and SMN proteins were produced in rabbit reticulocyte lysate in the presence of [35S]methionine. The labeled proteins were immunoprecipitated using mAb 11G9 and 12H12, and the immunoiprecipitated material analyzed by SDS-
PAGE and autoradiography. 10% of the in vitro translated protein is shown on the left. B, Immunoblotting using mAb 11G9 on purified 6His-Gemin2 and 6His-Gemin3. C, Immunoblotting using mAbs 12H12 and 11G9 on total HeLa extract. Note that, in addition to the strong p105 signal, 12H12, but not 11G9, weakly detects a protein of ~55 kD. The position of the molecular weight markers is indicated on the left (in kD).

performed double-label immunofluorescence experiments using antibodies against Gemin3 and either p80 coilin as a marker of coiled bodies (Andrade et al., 1991) or SMN as a marker of gems (Liu and Dreyfuss, 1996; Fig. 5). In many cell lines, gems and coiled bodies entirely overlap by antibody staining, however, in the HeLa PV strain used here, these two bodies are frequently found separate from each other (Liu and Dreyfuss, 1996; Materia and Frey, 1998). Therefore, we used HeLa PV cells to examine whether Gemin3 is located in gems or in coiled bodies. As can be seen in Fig. 5, C and D, the nuclear structures that contain Gemin3 are clearly distinct from coiled bodies, but Gemin3 completely colocalized with SMN in gems (Fig. 5 E). The colocalization of Gemin3 with SMN strongly supports the conclusion that these two proteins exist as a complex in the cell. Gemin3 is the third constituent of gems described so far. Compared with visual observation, the confocal photographs in Fig. 5, slightly overestimates the nuclear staining and underestimates the cytoplasmic staining for Fig. 5, C and D.

To confirm the coimmunopurification results, we tested for interaction of Gemin3 with SMN, Gemin2, and the Sm proteins in HeLa cells in vivo by immunoprecipitations and Western blot experiments. The anti-Gemin3 mAb 11G9 was used for immunoprecipitation from total HeLa cell extracts, and these were then resolved by SDS-PAGE and an immunoblot was probed with the anti-SMN antibody (Liu and Dreyfuss, 1996). As shown in Fig. 6 C (lane 11G9 IP), 2B1 readily detects SMN in the 11G9 immunoprecipitates, indicating that SMN is coimmunoprecipitated with Gemin3. Because SMN is associated with Gemin2 to form a stable complex in vivo and in vitro (Liu et al., 1997), we also investigated whether Gemin3 could be coimmunoprecipitated with Gemin2. As shown in Fig. 6 C, the anti-Gemin2 mAb 257 clearly detects Gemin2 in the anti-Gemin3 11G9 immunoprecipitates (Fig. 6 C, lane 11G9 IP). In a reciprocal experiment, the Gemin3 protein could also be coimmunoprecipitated by the anti-SMN mAb 2B1 (Fig. 6 D, lane 2B1 IP) and the anti-Gemin2 mAb 257 (Fig. 6 D, lane 257 IP). Because SMN and Gemin2 are found in a complex with the Sm proteins, we asked whether Gemin3 could be coimmunoprecipitated with the spliceosomal snRNP Sm core proteins as well. Fig. 6 D shows that Gemin3 is present in the anti-Sm mAb b Y 12 im-

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munoprecipitates (Fig. 6 D, lane IP Y12) like SMN and Gemin2 (Liu et al., 1997). No Gemin3, SMN, Gemin2, or Sm proteins were detected in a SP2/O immunoprecipitate (data not shown). These results demonstrate that Gemin3, SMN, Gemin2, and the Sm proteins are associated in vivo in a complex that can be immunoprecipitated by either anti-SMN, anti-Gemin2, anti-Sm, or anti-Gemin3 antibodies.

Further support for the existence in vivo of a complex that contains SMN, Gemin2, and Gemin3 was obtained from gel filtration experiments. HeLa cytoplasmic S100 extract was fractionated on a Superose 6 HR 10/30 high performance gel filtration column and each fraction was subjected to SDS-PAGE. A rabbit affinity-purified antibody against exon7 of the human SMN protein (green) was used to detect SMN. Colocalization of green and red results in yellow color. Dashed lines demarcate the nucleus.

Figure 5. The Gemin3 protein colocalizes with SMN in gems. A, Laser confocal image of indirect immunofluorescence on HeLa cells using mAb 12H12 against the Gemin3 protein. Note the general cytoplasmic staining, as well as nucleoplasmic and discrete nuclear structures. B, DIC image of the same cell shown in A, arrows indicate gems. C and D, Superimposed laser confocal images of double-label immunofluorescence microscopy experiments using antibodies against coiled bodies marker, p80 coilin (C and D, green), anti-Gemin3 11G9 (C, red), and anti-SMN antibody 2B1 (D, red). E, Superimposed laser confocal images of double-label immunofluorescence microscopy experiments using mAb against Gemin3 (red) and a rabbit affinity-purified antibody against exon7 of the human SMN protein (green). Colocalization of green and red results in yellow color. Dashed lines demarcate the nucleus.

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Figure 6. Gemin3 is in a complex with SMN, Gemin2, and the spliceosomal Sm proteins. A and B, Immunoprecipitations of \(^{35}\)S-methionine-labeled HeLa cell using mAbs specific to SMN (2B1), Gemin3 (11G9), and the snRNP core Sm proteins (Y12). The immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography (24-h exposure). The immunoprecipitations were performed in the presence of Empigen BB or Triton X-100 as indicated. The several proteins immunoprecipitated are indicated on the right. B, A longer exposure (36 h) of the bottom part of the immunoprecipitation in the presence of Triton X-100 to visualize the Sm proteins more clearly. C and D, Gemin3, SMN, Gemin2, and the Sm proteins can be coimmunoprecipitated in vivo. C, mAbs against Gemin3 coimmunoprecipitate SMN and Gemin2. Immunoprecipitation from total HeLa extract was done with mAb 11G9 and the immunoprecipitated proteins were analyzed by Western blot using 2B1 (anti-SMN) or 2S7 (anti-Gemin2) antibodies. D, mAbs against SMN, Gemin2 and the Sm proteins coimmunoprecipitate Gemin3. Immunoprecipitation from total HeLa extract was done with mAbs against SMN (lane 2B1 IP), Gemin2 (lane 2S7 IP), or the Sm proteins (lane Y12 IP). The immunoprecipitated proteins were analyzed by Western blot using the anti-Gemin3 mAb 12H12. The positions of the molecular weight markers are indicated on the left (in kD). The positions of the light chain (l.c) and heavy chain (h.c) of the antibodies used for immunoprecipitation are indicated. E, Gemin3, SMN, and Gemin2 are found in a complex of 800 kD or more in the cytoplasm. HeLa cytoplasmic S100 extract was fractionated on a Superose 6 H R 10/30 column. The fractions were analyzed by SDS-PAGE, and the Gemin3, SMN, and Gemin2 proteins were detected by Western blot. The fraction number and the molecular weight standards are indicated.
demonstrating that they are components of a large macromolecular complex (Fig. 6 E). Interestingly, a second pool of SMN-Gemin2, lacking Gemin3, is observed in a lower molecular weight complex that peaks at \( \sim 150 \) kD, suggesting that at least two different SMN-Gemin2 subcomplexes exist in vivo. However, we cannot exclude the possibility that the 150-kD subcomplex corresponds to a fraction of SMN-Gemin2 that dissociates from Gemin3 during cell fractionation and/or chromatography. We reported previously that a SMN-Gemin2 complex migrates at \( \sim 300 \) kD after filtration of a cytoplasmic S100 extract on a TSK-GEL G3000-SW column (Liu et al., 1997). The Superose 6 HR 10/30 gel filtration column used here allowed us to obtain a better resolution of the cytoplasmic SMN complex and to better estimate its size as \( \sim 800 \) kD.
Gemin3 Interacts Directly with SMN and the Spliceosomal Sm Proteins In Vitro

To further analyze the Gemin3 complex, we performed in vitro protein-binding assay between Gemin3 and several components of the SMN complex. For in vitro binding assays, Gemin3 was produced as a fusion protein with glutathione S-transferase (GST), and SMN and Gemin2 were produced and labeled with \([^{35}S]\)methionine by in vitro transcription and translation in rabbit reticulocyte lysate. Purified GST or GST–Gemin3 fusion immobilized on glutathione-Sepharose were incubated with labeled SMN or Gemin2 proteins. After extensive washing, bound proteins were eluted by boiling in SDS-containing sample buffer and the eluted material was analyzed by SDS-PAGE and detected by fluorography. Full-length SMN, but not Gemin2, bound specifically to immobilized GST–Gemin3 (Fig. 7 A), but not to GST alone (data not shown). To investigate whether Gemin3 interacts with Sm proteins, purified GST or GST–Gemin3 recombinant proteins were used for binding assays with in vitro \([^{35}S]\)methionine-labeled Sm proteins B, D1, D2, D3, E, F, and G (Lehmeier et al., 1994; Herrmann et al., 1995; Raker et al., 1996). The results, shown in Fig. 7 B, demonstrate that the Sm proteins B and D3 bind to GST–Gemin3, whereas there is no detectable binding to GST alone (data not shown). D2 binds Gemin3 only weakly and we note that the profiles of Sm protein binding to SMN and Gemin3 are not identical (see Liu et al., 1997). For example, SMN binds to D1 whereas Gemin3 does not (Fig. 7; Liu et al., 1997; Pellizzoni et al., 1999).

To address the possibility that some component of the rabbit reticulocyte lysate mediates these interactions, wild-type full-length SMN and SmB were produced as recombinant \(6\)H is-tagged proteins and incubated with GST or GST–Gemin3. After several rounds of washing, bound proteins were solubilized by boiling in SDS sample buffer, resolved by SDS-PAGE, immunoblotted, and probed with a rabbit polyclonal antibody specific to the \(6\)H is-tag. As shown in Fig. 7 C, SMN and SmB bind specifically to Gemin3, but not to GST alone. We conclude that both SMN and SmB interact directly with Gemin3.

To further characterize the interaction between Gemin3 and SMN, we first tested whether SMN carrying two well-characterized mutations found in SMA patients, the SMN and SmB interact directly with Gemin3. We conclude that both GST–Gemin3 or the myc-Gemin3 protein interact specifically with GST–SMN, but not with GST alone. The myc-D328Gemin3 and myc-DN548 Gemin3 mutant proteins clearly do not interact with GST–SMN. Thus, the COOH-terminal domain of Gemin3 (amino acids 456–547) mediates the interaction of SMN with Gemin3.

Gemin3 Interacts with SMN Via its Unique COOH-Terminal Domain

The unwinding activity of DEAD box RNA helicases may not be sequence specific. The target specificity of these proteins is, at least in some cases, provided by their interaction with specific proteins of the RNP substrate. These interactions appear to be mediated via the unique auxiliary domain that each RNA helicase contains (Hamm and Lamon, 1998; Staley and Guthrie, 1998). Therefore, we investigated the role of the unique COOH-terminal domain of Gemin3 (amino acids 430–825) in the interaction and cellular localization of this novel DEAD box RNP helicase. To do so, we constructed three deletion mutants of Gemin3 and first tested their binding to GST–SMN. Wild-type and mutant myc-Gemin3 constructs were transcribed and translated in rabbit reticulocyte lysate in the presence of \([^{35}S]\)methionine, and the resultant translated products were assayed for binding to GST–SMN as described above. As shown in Fig. 8 B, the wild-type myc-Gemin3 protein and myc-DN368C277Gemin3 mutant proteins interact specifically with GST–SMN, but not with GST alone. The myc-D328Gemin3 and myc-DN548 Gemin3 mutant proteins clearly do not interact with GST–SMN. Thus, the COOH-terminal domain of Gemin3 (amino acids 456–547) mediates the interaction of SMN with Gemin3.

We then monitored the expression and cellular localization of the myc-tagged mutants in transfected HeLa cells. Because gems represent a marker of a concentrated pool of the SMN complex in the nucleus, it is likely that the incorporation of a myc-tagged Gemin3 protein into gems results from its interaction with SMN in vivo. Double-label immunofluorescence microscopy, using anti-myc tag antibodies to detect either the transfected wild-type myc-Gemin3 or the myc-DGemin3 mutants, and the anti-SMN mAb b2p1 antibody, showed accumulation of the wild-type (data not shown) and myc-DN368C277Gemin3 mutants into gems (Fig. 8 C). However, the myc-D328Gemin3 and myc-DN548 (data not shown) Gemin3 mutant proteins accumulate essentially in the cytoplasm without any gems or nucleoplasm localization. This strongly suggests that the COOH-terminal region of Gemin3 (amin acids 456–547) is responsible for the localization of Gemin3 into gems.

Discussion

The molecular characterization of the spinal muscular atrophy gene product, SMN, demonstrated that it is concentrated in novel nuclear structures called gems (Liu and Dreyfuss, 1996; Liu et al., 1997). Coiled bodies and gems represent nuclear structures that appear to be involved in
RNA metabolism, and in many of the cell lines studied, these two bodies are often found in association (Lamond and Carmo-Fonseca, 1993; Gall et al., 1995; Liu and Dryfuss, 1996; Liu et al., 1997; Matera and Frey, 1998; Pellizzoni et al., 1998). SMN must either have an intrinsic activity or recruit other proteins that can actively affect structural transitions to the complex in certain RNP targets. Several factors that have the capacity to serve in such functions, including assembly and disassembly of components of the splicing machinery, have been described. Many of these factors are DEAD/DEAH-box RNA helicases that are essential for splicing (reviewed in Staley and Guthrie, 1998). Prp43, for instance, is required for the disassembly of the snRNP—intron lariat complex (Arenas and Abelson, 1997). Prp32 is needed to release the mature mRNA from the spliceosome (Company et al., 1991), and Prp24 acts as a recycling factor for U4 and U6 snRNP (Raghunathan and Guthrie, 1998).

Using a biochemical approach to characterize new components of the SMN complex, we have identified a novel DEAD-box RNA helicase termed Gemin3. Gemin3 forms a stable complex with SMN in vivo and in vitro, and it colocalizes with SMN in nuclear gems. Several lines of evidence suggest that Gemin3 and SMN function as a complex in vivo. SMN and Gemin3 can be communoprecipitated and both are present in a large (∼800 kD) complex that also contains Gemin2. A nontn-SM N, anti-Gemin2, or anti-Gemin3 mAb was immunoprecipitated with the spliceosomal snRNP core Sm proteins, as well as several other unidentified proteins. Gemin3 interacts directly with SMN and with several snRNP Sm core proteins, including B/B', D2, and D3. In addition, Gemin3 is uniformly distributed in the cytoplasm, where snRNP assembly takes place, and it can be specifically communoprecipitated with the cytoplasmic pool of Sm proteins (data not shown). Together, these findings suggest that Gemin3 may play an important role in spliceosomal snRNP biogenesis.

DEAD-box protein products have been found to be involved in many aspects of RNA metabolism, including pre-mRNA splicing, translation, snRNP—snRNP interactions, mRNA degradation, and mRNA transport in eukaryotes and prokaryotes (Company et al., 1991; Ohno and Shimura, 1996; Arenas and Abelson, 1997; Hamman and Lamond, 1998; Staley and Guthrie, 1998; De la Cruz et al., 1999). One of the major questions about the function of each DEAD/DEAH-box RNA helicase is the identification of the specific RNA target for it. Some of the enzymes of this family can unwind generic RNA substrates in vitro (Laggerbauer et al., 1998; Schwer and Gross, 1998; Wang et al., 1998). For these enzymes, the specificity towards particular RNA's, therefore, appears to be determined by factors that interact with their unique auxiliary domains. For example, the DEAD-box RNA helicase Prp16 is recruited to the spliceosome via its unique NH2-terminal domain (Wang and Guthrie, 1998). The specific substrate for Gemin3 has not yet been identified and this remains a central question of interest. In a series of preliminary experiments, we have not been able to detect RNA helicase or RNA-dependent ATPase activity for recombinant Gemin3, so far. It is possible that such activity will only manifest itself when Gemin3 is associated with other proteins as part of a complex or that it will be detectable once a specific RNA or RNP target is found. The interaction of Gemin3 with SMN is direct, and we found that amino ac-
ids 456–547 of Gemin3 mediate this interaction and, likely as a consequence of this, the localization of Gemin3 to the gems. Thus, we propose that Gemin3 provides the enzymatic activity of the SMN complex to affect structural transitions in its RNA targets.

The SMN protein is capable of forming an oligomer of >400 kD in vitro (Pellizzoni et al., 1999) and we show here that SMN comigrates with an ~800-kD complex that also contains Gemin2 and Gemin3. It is likely that SMN oligomerization is critical for the nucleation of this large complex. In addition to Gemin3 and Gemin2, several Sm proteins interact with SMN, and we therefore propose that SMN forms a docking platform to bring together, in the appropriate spatial arrangement, the multiple proteins that are involved in the de novo assembly and regeneration of its RNA (e.g., snRNP) substrates. Interestingly, the interaction of SMN with Gemin3 is severely reduced by mutations found in SMA patients, such as the point mutant SMN YY272C or the exon 7 deletion. Thus, the formation of the SMN platform seems critical for SMN function because SMA affects both the capacity of SMN to oligomerize, as well as to interact with several Sm proteins (Pellizzoni et al., 1999) and Gemin3. A likely consequence of these defective interactions, the function of SMN in the regeneration of the splicing machinery is abolished (Pellizzoni et al., 1998, 1999).

Coiled bodies contain the highest local concentration of p80 coilin and are enriched in components of three major RNA-processing pathways: pre-mRNA splicing; histone mRNA 3′ maturation; and pre-mRNA processing (reviewed in Lamond and Earnshaw, 1998). Gemin3 contains the highest local concentration of SMN, Gemin2, and Gemin3 and are often found associated with coiled bodies (Liu and Dreyfuss, 1996; Liu et al., 1997, and this work). Although the definitive function of these two nuclear bodies has not been completely elucidated, the characterization of their protein and RNA contents represents an important step toward the understanding of their functions. Further studies of Gemin3, a novel DEAD box containing protein and component of gems, should shed light on the functions of the SMN complex and gems.

We thank Dr. Eng M. Tan for the anti-p80 coilin antibody and Dr. Joan Steitz for the anti-Sm Y 12 mAb. We are grateful to members of our laboratory for stimulating discussions and, in particular, Drs. W. E. Friess, Z. Simmons Mourelatos, and Sara Nakielny for helpful discussions and critical comments on this manuscript.

This work was supported by a grant from the National Institutes of Health. G. Dreyfuss is an investigator of the Howard Hughes Medical Institute.

Submitted: 2 September 1999
R evised: 21 October 1999
A ccepted: 27 October 1999

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