

SMN-independent Subunits of the SMN Complex

IDENTIFICATION OF A SMALL NUCLEAR RIBONUCLEOPROTEIN ASSEMBLY INTERMEDIATE*

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The survival of motor neurons (SMN) complex is essential for the biogenesis of small nuclear ribonucleoprotein (snRNP) complexes in eukaryotic cells. Reduced levels of SMN cause the motor neuron degenerative disease, spinal muscular atrophy. We identify here stable subunits of the SMN complex that do not contain SMN. Sedimentation and immunoprecipitation experiments using cell extracts reveal at least three complexes composed of Gemin3, -4, and -5; Gemin6, -7, and unrip; and SMN with Gemin2, as well as free Gemin5. Complexes containing Gemin3-Gemin4-Gemin5 and Gemin6-Gemin7-unrip persist at similar levels when SMN is reduced. In cells, immunofluorescence microscopy shows differential localization of Gemin5 after cell stress. We further show that the Gemin5-containing subunits bind small nuclear RNA independently of the SMN complex and without a requirement for exogenous ATP. ATP hydrolysis is, however, required for displacement of small nuclear RNAs from the Gemin5-containing subunits and their assembly into snRNPs. These findings demonstrate a modular nature of the SMN complex and identify a new intermediate in the snRNP assembly process.

The survival of motor neurons protein (SMN) is found in metazoan cells in a complex with seven other proteins called Gemin2 to -8 (1–7). The SMN complex is found dispersed in the cytoplasm and concentrated in nuclear bodies called gems (8). Reduced levels of SMN in humans result in spinal muscular atrophy (SMA),² characterized by severe motor neuron degeneration and death in early development (9–11). The SMN complex plays an essential cellular role in the assembly of spliceosomal snRNPs, a process that is disrupted in SMA (12–15).

Small nuclear ribonucleoproteins (snRNPs) are the major constituents of the cellular mRNA splicing apparatus (16, 17). Each snRNP consists of one or two small nuclear RNAs (U1, U2, U4/U6, and U5 snRNAs) bound to both a set of proteins com-

mon to all snRNPs and a set of proteins particular to each snRNP (17). The common proteins, the Sm proteins (SmB/B', SmD1, SmD2, SmD3, SmE, SmF, and SmG) form a heptameric ring around a conserved uridine-rich sequence on the snRNAs called the Sm site (18–20). The SMN complex functions in the assembly of the Sm core onto the Sm site of snRNAs (12–14). To accomplish this, the SMN complex binds directly to both snRNAs and Sm proteins in the cytoplasm. Each component of the SMN complex, except Gemin2, binds directly to Sm proteins, and SMN protein itself binds to symmetrical dimethyl-arginine-containing tails on SmB/B', SmD1, and SmD3 (1–6). The SMN complex also directly and specifically binds snRNAs, independent of its interactions with Sm proteins, via Gemin5, which binds to specific features, including the Sm site, at the 3'-end of snRNAs (21). In an ATP-dependent manner, the SMN complex assembles the seven Sm proteins into a ring onto the Sm site of snRNAs.

The SMN complex is large, ~60 S, quite stable for many hours, and resistant to high concentrations of salt and detergent (6, 22). To form the complex, SMN associates with at least six other proteins called Gemin. Gemin2 is a 32-kDa protein with no homology to any other proteins (5). Gemin3 is a DEAD-box RNA helicase, and Gemin4 shows no homology with other proteins (2, 3). Gemin5, the snRNA-binding protein of the complex, is a large multidomain protein containing 13 WD repeats. Gemin6 and Gemin7 adopt Sm-folds (4, 21). SMN protein binds directly to Gemin2, -3, -5, and -7 (1, 2, 4, 5). Gemin4 interacts with the complex via Gemin3, whereas Gemin6 interacts with the complex via Gemin7 (3, 6). Newly discovered Gemin8 is reported to interact directly with SMN as well as a heterodimer of Gemin6 and 7 (7, 23). In addition to these core proteins, the SMN complex is found associated with other transient or substrate proteins. Unrip, a protein found to bind the cap-independent translation factor UNR, associates with the SMN complex only in the cytoplasm via binding to Gemin6 and Gemin7 and may play a role in sequestering the SMN complex in the cytoplasm for snRNP assembly (7, 24–26). Additionally, each of the Sm proteins and snRNAs are associated with the SMN complex (5).

Here we identify several stable SMN-independent subunits of the SMN complex. Gemin5, the RNA-binding protein of the complex, is found abundantly outside of the SMN complex, and some of it is associated with the DEAD-box helicase Gemin3 and Gemin4. These SMN-free complexes bind to snRNA both *in vitro* and in cell extract. Additionally, Gemin6 and Gemin7 form a complex with unrip. These findings demonstrate the modular nature

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² The abbreviations used are: SMA, spinal muscular atrophy; SMN, survival of motor neurons; snRNP, small nuclear ribonucleoprotein; shRNA, short hairpin RNA; RNAi, RNA interference; AMP-PNP, 5'-adenylyl- β , γ -imidodiphosphate; eIF2 α , -3, -4E, and -4G, eukaryotic initiation factor 2 α , 3, 4E, and 4G, respectively.

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of both the SMN complex and the snRNP assembly process and suggest that an increase in the relative amount of the SMN-independent subunits may have modifying activities in SMA.

EXPERIMENTAL PROCEDURES

Antibodies—The following antibodies were used in the experiments: mouse monoclonal anti-SMN (2B1, 62E7), mouse monoclonal anti-Gemin2 (2S7, 2E17), mouse monoclonal anti-Gemin3 (12H12), mouse monoclonal anti-Gemin4 (17D10), mouse monoclonal anti-Gemin5 (10G11), mouse monoclonal anti-Gemin6 (6H5), rabbit polyclonal anti-Gemin6, mouse monoclonal anti-Gemin7 (6E2), mouse monoclonal anti-Sm (Y12), mouse monoclonal anti-PABP (10E10), rabbit polyclonal anti-eIF4G (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal anti-FXR1 (6BG10), and mouse monoclonal anti-JBP1 (6G8) (1–4, 6, 27).

RNA Interference—A cell line was constructed in which HeLa PV cells were transfected with a pSILENCER 2.1-U6 hygro vector (Ambion) expressing an shRNA hairpin containing an oligonucleotide targeting SMN (5'-GAAGAAUACUGCAGCU-UCC-3'). Construction of a control cell line expressing a nontargeting shRNA was described previously (21). The cell lines were cultured in the presence of 200 $\mu\text{g}/\text{ml}$ hygromycin B (Invitrogen).

Preparation of RNA Constructs—All RNAs were prepared via standard T7 transcription techniques from a pcDNA plasmid in the presence of [α - ^{32}P]UTP.

Protein Purification—Immunoprecipitations were carried out as described previously (28) with the following modifications. The 10G11 immunoprecipitations were washed extensively in RSB-150 (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 2.5 mM MgCl_2) with 0.01% Nonidet P-40.

Sucrose Gradient Sedimentation—500 μl of HeLa whole cell or cytoplasmic extract at 4 mg/ml were loaded onto a 10-ml 5–20% sucrose gradient and centrifuged at 41,000 rpm for 3 h in an SW 41 Ti rotor in a Beckman Coulter OptimaTM XL-100K ultracentrifuge such that 100 S is at the bottom of the gradient. Twenty 0.5-ml fractions were collected, and 20 μl of each fraction was analyzed by SDS-PAGE and Western blot. For immunoprecipitation experiments, the indicated fractions were pooled and immunoprecipitated as described above.

For the longer run experiments, the sucrose gradient was centrifuged at 29,000 rpm for 15 h such that 40 S is at the bottom of the gradient. The fractions were then pooled by twos and immunoprecipitated with the stated antibodies as described above.

Arsenite and Heat Shock Treatment and Indirect Immunofluorescence—HeLa PV cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). For immunofluorescence, cells were seeded onto glass coverslips the day before treatment. A solution of sodium arsenite was added directly into the culture medium to a final concentration of 1 mM, and the cells were then maintained at 37 °C in a 5% CO_2 humidified atmosphere for an additional 30 min prior to fixing. For heat shock, cells were maintained at 42 °C for 30 min prior to fixing. Indirect immunofluorescence on HeLa PV cells was performed as previously described (6).

RNA-binding Assays—5–20% sucrose gradients were run as described above. The 20–35 S fractions 3–6 or the 60–80 S fractions 10–13 were pooled together and immunoprecipitated as described above with anti-Gemin5 antibody 10G11. RNA binding assays were performed by the addition of ^{32}P -labeled U4 and U6 snRNAs to the immunoprecipitated complexes immobilized on beads as described previously (21, 29).

snRNP Assembly Assays—Biotin-labeled U4 snRNA was added to 2.5 mg of HeLa cytoplasmic extract and either 10 mM ATP or 10 mM AMP-PNP under conditions described previously (15). Following the assembly reaction, the mixture was loaded onto a 5–20% sucrose gradient and fractionated as described above. Each fraction was immunoprecipitated in a 96-well format using either anti-Sm (Y12) or anti-Gemin5 (10G11) mouse monoclonal antibodies immobilized on Dynabeads M-280 sheep anti-mouse IgG magnetic beads (Dyna-Bio-ASA, Oslo, Norway). Anti-Sm immunoprecipitations were performed as described previously, whereas anti-Gemin5 immunoprecipitations were performed similarly but in RSB-150 (10 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM MgCl_2) with 0.01% Nonidet P-40. Following extensive washing, co-precipitated biotin-labeled snRNA was detected by chemiluminescence resulting from incubation with horseradish peroxidase-coupled avidin as described previously (15).

RESULTS

Reduction of SMN by RNAi Results in SMN-independent Gemin Subunits—The SMN protein interacts directly with Gemin2, -3, -5, and -7 (1, 2, 4, 5). Only Gemin4 and Gemin6 do not directly bind SMN, interacting instead indirectly through Gemin3 and Gemin7, respectively (3, 6). This network of interactions places SMN at a lynchpin position at the heart of the complex. Since SMA results from a long term reduction of the SMN protein, we assessed the effect a stable, long term reduction of SMN would have on the state of the other proteins of the SMN complex.

To address this, a cell line was constructed in which SMN is reduced by stable expression of shRNAs as well as a control cell line expressing nontargeting shRNA. In the SMN RNAi cell line, SMN was effectively reduced by ~80% relative to that of the control cells, similar to what was previously observed when SMN was reduced by transient RNAi (data not shown) (30). Immunoprecipitation of the SMN complex via either SMN or Gemin2 from this cell line resulted in the purification of fewer SMN complexes relative to that of the control cells, but the relative composition of the proteins associated with SMN was unchanged (Fig. 1A). On the other hand, immunoprecipitation of Gemin5 from cells with reduced SMN showed that Gemin5 co-precipitated less SMN and Gemin2 but still co-precipitated nearly unaltered amounts of Gemin3 and Gemin4 when compared with precipitations from the control cells (Fig. 1A). These data demonstrate that Gemin5 can form a stable complex with Gemin3 and Gemin4 in the absence of SMN. This is particularly interesting, since Gemin5 was recently shown to interact *in vitro* not only with SMN but also weakly with Gemin3 and Gemin4 (31).

Similarly, Gemin6 was immunoprecipitated from the SMN RNAi cell line. In this case, Gemin6 still effectively co-precipi-

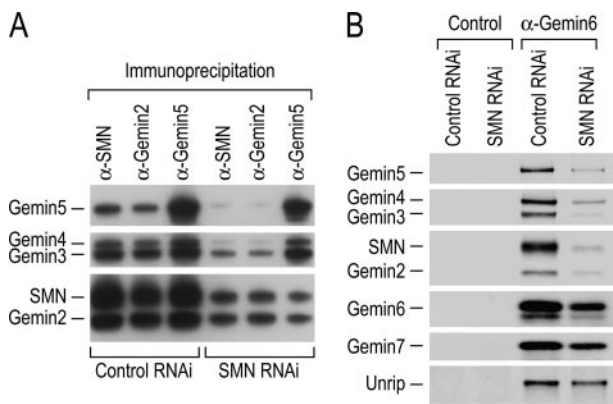


FIGURE 1. Reduction of SMN by RNA interference reveals SMN-independent subunits. *A*, SMN, Gemin2, and Gemin5 were immunoprecipitated from extracts from both a cell line stably expressing shRNA targeting SMN (lanes 4–6) and a control cell line expressing nontargeting shRNA (lanes 1–3). Immunoprecipitated proteins were observed by Western analysis. *B*, Gemin6 was immunoprecipitated as from cells expressing shRNA as described in *A*. Control immunoprecipitations were performed using mouse nonimmune antibody.

tated Gemin7 and unrip but co-precipitated much less SMN, Gemin2, Gemin3, Gemin4, and Gemin5 (Fig. 1*B*). Previous data have shown that Gemin6 and Gemin7 can directly interact *in vitro* and also that unrip can directly interact with a Gemin6-Gemin7 heterodimer *in vitro* (1, 24, 25). These data demonstrate that Gemin6, Gemin7, and unrip form a stable complex outside of the SMN complex in cells with reduced SMN.

Gemin Subunits Form in Wild-type Cells—The data above demonstrate that at least two distinct subunits of the SMN complex exist when SMN is reduced. It was unclear whether these complexes exist in wild-type cells or if they only exist as a consequence of SMN reduction. To address this, we attempted to purify the subunits from wild-type HeLa cells.

HeLa cell lysates were first fractionated by 5–20% sucrose gradient sedimentation. As has been previously published (7, 22), SMN sediments in a broad 40–80 S peak centered in fractions 12–14 (Fig. 2*A*). Gemin6, however, sediments predominantly in an ~10 S peak in fractions 2 and 3 of the gradient, with only a small amount found in the SMN complex fractions (Fig. 2*A*). To test if these lighter fractions represent the Gemin6-Gemin7-unrip complex, Gemin6 was immunoprecipitated from fraction 2. Indeed, Gemin6 effectively co-precipitated both Gemin7 and unrip but not SMN or Gemin2 to -5 from the 10 S fraction (Fig. 2*B*). These data showed that much of the Gemin6, Gemin7, and unrip in HeLa cells forms a 10 S, SMN-free complex.

Gemin5 sediments with the SMN complex in the 40–80 S peak (fractions 9–14) of a 5–20% sucrose gradient (7). However, significant amounts of Gemin5 are also found in a 20–35 S peak (fractions 4–6) (Fig. 2*A*). Gemin3 and Gemin4 predominantly sediment in the 40–80 S fractions but are also found throughout the gradient in all fractions larger than 20 S (Fig. 2*A*). Gemin5 and SMN were immunoprecipitated from both the 20–35 S fractions and the 60–80 S fractions of a 5–20% sucrose gradient. Both Gemin5 and SMN effectively co-precipitate all components of the SMN complex from the 60–80 S fractions, demonstrating that both Gemin5 and SMN are part of the same complex, the canonical SMN complex (Fig. 2*B*).

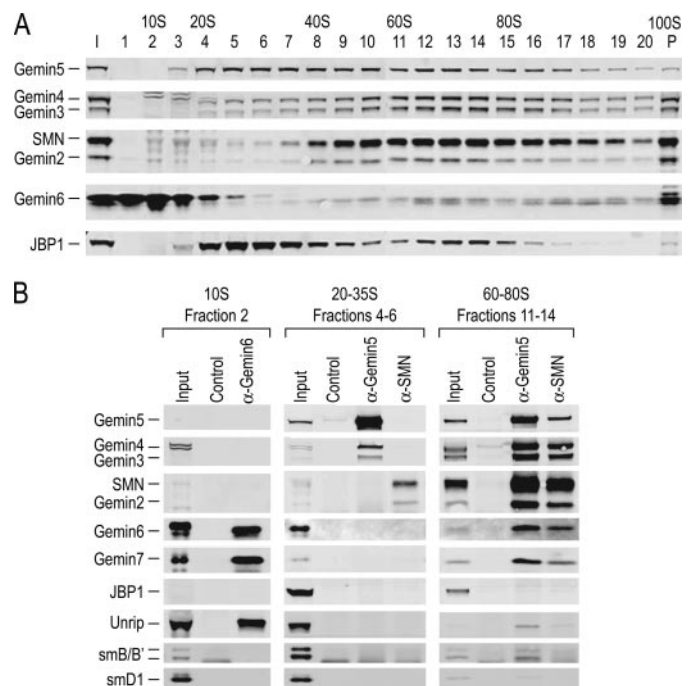


FIGURE 2. Immunoprecipitation of SMN complex subunits from sucrose gradients. *A*, HeLa cell extracts were fractionated by sucrose density gradient centrifugation. The SMN complex components in each fraction were detected by Western analysis. *B*, Western analysis of immunoprecipitations of Gemin6 (lane 3), Gemin5 (lanes 6 and 10), and SMN (lanes 7 and 11) from the 10 S (fraction 2, lanes 1–3), 20–35 S (fractions 4–6, lanes 4–7), and 60–80 S (fractions 11–14, lanes 8–11) fractions of the gradient shown in *A*. Control, immunoprecipitations using mouse nonimmune antibody.

However, Gemin5 in the 20–35 S fractions co-precipitated Gemin3 and Gemin4 but not SMN or Gemin2, -6, or -7, despite the fact that some SMN and Gemin2 were detectable in those fractions (Fig. 2*B*). Conversely, SMN only co-precipitated Gemin2 from the 20–35 S fractions, despite the fact that much Gemin5, -3, and -4 was found in these fractions (Fig. 2*B*). These data clearly demonstrate that the Gemin3-Gemin4-Gemin5 and SMN-Gemin2 complexes are separate complexes, although both sediment in roughly the same fractions. Also, although the 20 S methylosome also sedimented in these fractions, the JBP1/PRMT5 methyl-transferase did not co-precipitate with either Gemin5 or SMN (Fig. 2*B*), showing that Gemin3-Gemin4-Gemin5 and SMN-Gemin2 complexes are not components of the 20 S methylosome.

The ratio of Gemin5 to Gemin3 and -4 in the 20–35 S complex was significantly different from the ratio of these proteins within the SMN complex (Fig. 2*B*). This suggested that there was more Gemin5 in these fractions than Gemin3 and Gemin4. If the relative stoichiometry of Gemin5, -3, and -4 remains the same within the SMN complex and in the Gemin3-Gemin4-Gemin5 complex, then the 20–35 S fractions probably represented both a Gemin3-Gemin4-Gemin5 complex and excess free Gemin5. To resolve these better, a 5–20% sucrose gradient was centrifuged for a longer time, such that 40 S was at the bottom of the gradient (Fig. 3*A*). The fractions were then pooled in pairs, and Gemin5 was immunoprecipitated from each fraction. Indeed, two peaks for Gemin5 were immunoprecipitated, one at 20 S and another at 30 S (Fig. 3, *B* and *C*). Gemin3 and Gemin4 both

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co-precipitated with Gemin5 in the 30 S peak (Fig. 3, B and C). SMN only began to co-precipitate with Gemin5 in the bottom fractions (approximately 40 S). These data demon-

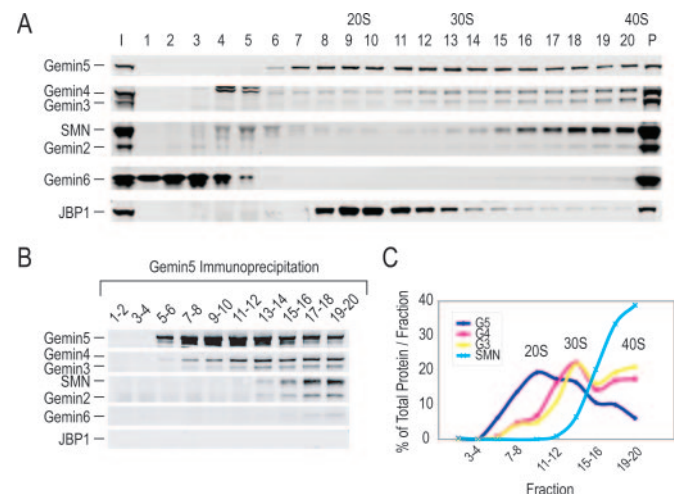


FIGURE 3. Immunoprecipitations of Gemin5 complexes from sucrose gradients. A, HeLa cell extracts were fractionated by sucrose gradient centrifugation, such that 40 S complexes were at the bottom of the gradient. The SMN complex components in each fraction were detected by Western analysis. B, fractions shown in A were pooled in pairs, and Gemin5 was immunoprecipitated. Co-precipitated proteins were analyzed by Western blot with SMN complex-specific antibodies. C, protein signal from the immunoprecipitations in B were quantified using a LI-COR Odyssey infrared imaging system, and the protein amounts are shown versus sucrose gradient fraction number.

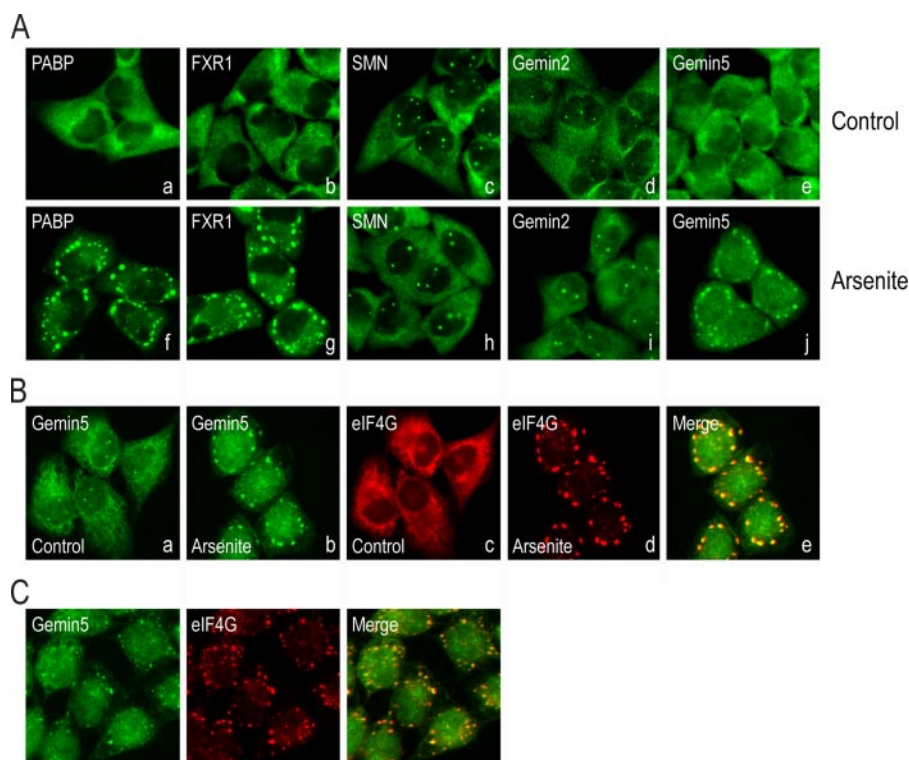


FIGURE 4. Gemin5 is recruited to stress granules in stress-activated cells. A, HeLa PV cells were either left untreated (control; a–e) or stressed for 30 min with 1 mM arsenite (f–j). After fixation, the cells were immunostained for some known stress granule markers, PABP and FXR1, and some of the SMN complex components, SMN, Gemin2, and Gemin5, as indicated in the panels. B, HeLa cells that were left untreated or exposed to treatment with 1 mM arsenite for 30 min were co-immunostained for Gemin5 (a and b, respectively) and eIF4G (c and d, respectively), a known stress granule marker. The merged view of the arsenite-treated cells is shown in e. C, HeLa cells exposed to 42 °C heat shock for 30 min were co-immunostained for Gemin5 (green) and eIF4G (red), as shown in the upper panels. The merged view is shown to the right.

strate that, in addition to the SMN complex, Gemin5 forms both a 30 S complex with Gemin3 and -4 and a 20 S complex without any other SMN complex components.

Cell Stress Reveals a Separation of Gemin5 from SMN and Gemin2—Further evidence for Gemin5 existing separate from the SMN complex comes from analyzing changes in the subcellular distribution of Gemins *in vivo* upon exposure to stressful conditions. By immunofluorescence microscopy, under normal metabolic conditions, SMN and all of the Gemins are found dispersed in the cytoplasm and in prominent nuclear bodies called Gems, precluding the use of co-localized immunofluorescence to detect separate complexes (1–6, 8). However, when exposed to supra-ambient temperatures or other environmental stresses (such as treatment with arsenite), eukaryotic cells exhibit characteristic morphological changes, including the formation of discrete cytoplasmic foci known as stress granules. The majority of the poly(A) mRNAs are recruited to these stress granules in response to phosphorylation of eIF2 α and consequent translation inhibition (32). Furthermore, in addition to the key markers of stress granules, TIA-1 and TIAR, several other proteins were identified, including components of the small ribosomal subunit, namely eIF3, eIF4E, and eIF4G, along with other proteins, such as PABP and FXR1 (33). The appearance of these cytoplasmic granules can therefore be easily detected by immunostaining with any of these marker proteins (Fig. 4A, f and g). Since previous studies have indicated that SMN protein is redistributed to granules in response to stress,

we attempted to use this strategy as a means of detecting separate complexes (34). Interestingly, induction of stress in HeLa cells by arsenite treatment caused Gemin5 to relocate to cytoplasmic granules but not SMN and Gemin2 (Fig. 4A). These structures were confirmed to be stress granules by co-immunofluorescence with eIF4G (Fig. 4B). Similar results were obtained when the cells were stressed with heat shock (Fig. 4C). Although we did not observe SMN and Gemin2 in stress granules, previous work had shown that SMN and Gemin2 are able under some circumstances to enter stress granules, a difference probably due to the specific cell type used. Although these data clearly show that Gemin5 is separate from the SMN complex under stress conditions, it is not clear if the SMN complex dissociates under stress, forming Gemin5 complexes that relocate to stress granules, or if stress causes the pool of Gemin5 that is already not associated with the SMN complex to selectively relocate to stress granules, leaving SMN complexes intact.

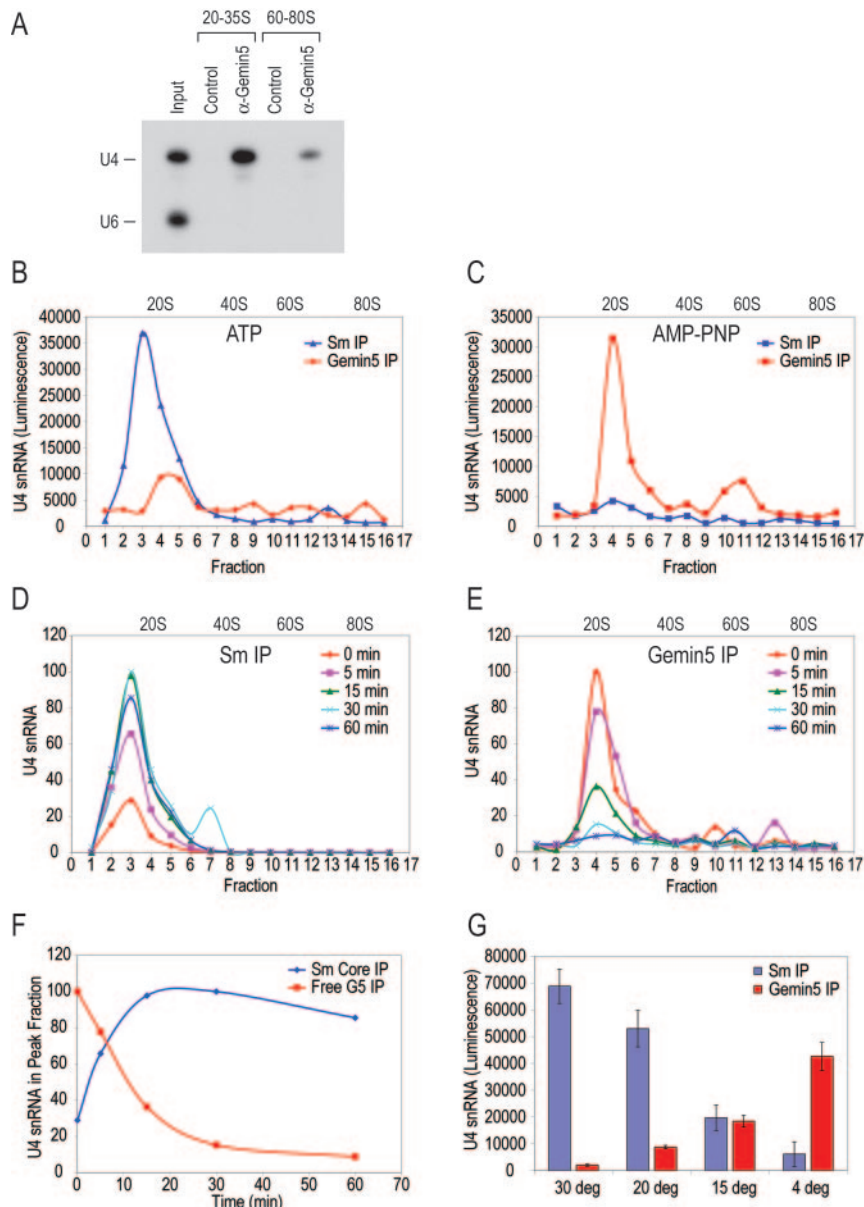


FIGURE 5. The Gemin5 subunits bind snRNA. *A*, *in vitro* RNA binding experiment in which ^{32}P -labeled U4 and U6 snRNAs were added to Gemin5 complexes immunoprecipitated using mouse monoclonal anti-Gemin5 antibodies (lanes 3 and 5) or mouse nonimmune antibodies (control lanes 2 and 4) from the 20–35 S fractions and the 60–80 S fractions of a sucrose gradient. Associated RNAs were isolated and analyzed by denaturing polyacrylamide gel electrophoresis and imaged by autoradiography. *Input*, 5% of the total RNA in the reaction. *B* and *C*, biotin-labeled U4 snRNA was added to HeLa cytoplasmic extract and incubated in the presence of either ATP (*B*) or AMP-PNP (*C*). Following the reaction, the extracts were fractionated on a 5–20% sucrose gradient as shown in Fig. 2*A*, and Gemin5 and Y12 were immunoprecipitated (*IP*) from each fraction. Coprecipitated biotin-labeled RNA was detected by chemiluminescence and is shown plotted against sucrose gradient fraction number. *D* and *E*, biotin-labeled U4 snRNA was added to HeLa cytoplasmic extract in the presence of ATP and incubated for various times. Following the reactions, the products were fractionated on a sucrose gradient, and either Sm cores (*D*) or Gemin5 (*E*) were immunoprecipitated from each fraction. Shown is the U4 snRNA associated with each protein. In each case, the maximal point was normalized to 100. *F*, plot of the U4 snRNA in the peak fractions in the sucrose gradients in fraction 3 of *E* and fraction 4 of *F*. *G*, U4 snRNA was added to HeLa cytoplasmic extract and incubated at various temperatures. Shown is the snRNA co-precipitated with either Sm cores or with Gemin5 at each temperature.

A Role for Gemin5 Subunits in snRNP Assembly—Gemin5 has been recently identified as the snRNA-binding protein of the SMN complex (21). Both Gemin5 purified from HeLa cells and recombinant Gemin5 expressed in *Escherichia coli* bind directly to snRNAs (21). Additionally, Gemin5 cross-links to snRNAs in HeLa cytoplasmic extract (21). Since much of

Gemin5 is found in the cytoplasm outside of the SMN complex, we wondered if the SMN-free pool of Gemin5 functions as a previously uncharacterized SMN-free intermediate in the snRNP assembly process.

We first wanted to verify that the Gemin5 complexes outside of the SMN complex were in a form that was capable of binding to snRNAs. To test this, HeLa cytoplasmic extract was fractionated on a 5–20% sucrose gradient as described above, and Gemin5 was immunoprecipitated from the 20–35 S fractions and the 60 S fractions as described above. ^{32}P -Labeled U4 snRNA was then added to each of the purified complexes, and direct RNA-binding assays were performed. As shown in Fig. 5*A*, both intact 60 S SMN complexes isolated via Gemin5 and SMN-free 20–35 S Gemin5 complexes bound directly to U4 snRNA. Although less binding appears to be observed to the SMN complex than to the Gemin5 complex, this is simply a result of there being less Gemin5 in the SMN complex than in the Gemin5 complex used in the experiment (see Fig. 2*B*, α -Gemin5 immunoprecipitation lanes). These data demonstrate that the SMN-free pool of Gemin5 in cells has snRNA binding activity.

Since the SMN-free Gemin5 complex has snRNA binding activity, we postulated that the SMN-free Gemin5 complexes may function as intermediates in the snRNP assembly process. To test this, snRNP assembly was blocked with AMP-PNP, a nonhydrolyzable analog of ATP that completely inhibits the assembly reaction in cell extract. Following snRNP assembly with biotinylated U4 snRNA in HeLa cytoplasmic extract, the reaction products were fractionated by centrifugation on a sucrose gradient. Sm cores and Gemin5 were then

immunoprecipitated from each gradient fraction, and the associated snRNA was detected using horseradish peroxidase-conjugated avidin. As shown in Fig. 5*B*, after a 1-h incubation in the presence of ATP, the majority of the snRNA was found associated with Sm cores in an approximately 12 S U4 snRNP peak, and relatively little RNA was associated with Gemin5. AMP-

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PNP, however, has been shown to inhibit the Sm core assembly reaction. Indeed, in the presence of AMP-PNP, relatively little snRNA was found associated with Sm cores; instead, more U4 snRNA was found in the 20 S subunit of Gemin5, which does not contain SMN (Fig. 5C, fraction 4). Little snRNA was observed associated with the SMN complex in the 40–80 S fractions, suggesting that either assembly is rapid relative to the time scale of the experiment once the snRNA associates with the SMN complex or that the snRNA-SMN complex form is not stable under the conditions, precluding analysis of SMN complex-associated snRNAs in the experiment. These data demonstrate that inhibition of SMN-dependent Sm core assembly by AMP-PNP results in an accumulation of snRNA with the 20 S complex of Gemin5 outside of the SMN complex. Similar results were obtained when snRNP assembly reactions were performed at lower temperatures, which slow down the snRNP assembly reaction. As shown in Fig. 5G, lowering the reaction temperature did indeed result in decreased snRNP assembly but also resulted in an accumulation of snRNA with Gemin5.

We next asked if the Gemin5 complexes function in the snRNP assembly process in snRNP assembly reactions that were not inhibited. Time courses of snRNP assembly reactions were performed by the addition of biotinylated U4 snRNA to cytoplasmic extract in the presence of ATP under standard conditions. At various time points, the reactions were loaded onto a 5–20% sucrose gradient and fractionated. Following fractionation, Sm cores and Gemin5 were immunoprecipitated from each fraction, and the associated snRNA was detected with horseradish peroxidase-avidin. As shown in Fig. 5, D and F, assembled U4 snRNPs accumulated over time, consistent with previous experiments (15). Interestingly, the snRNA very rapidly associated with the SMN-free Gemin5 subunit prior to accumulation of Sm cores, and the amount of snRNA in the Gemin5 complex decreased over time in an inverse relationship with the accumulation of assembled snRNPs (Fig. 5, E and F). These data demonstrate that the SMN-free Gemin5-snRNA subunit is a previously uncharacterized intermediate in the snRNP assembly process.

DISCUSSION

We have identified several cellular subcomplexes, referred to here as subunits, of the SMN complex. In addition to the SMN complex that contains SMN and all of the Geminins, we have found that SMN also forms a complex in cells with Gemin2 without the other Geminins. This is the smallest cellular complex of SMN populated to a reasonable level, and SMN was not found without Gemin2. Along with the fact that the SMN protein binds directly to Gemin3, Gemin5, and Gemin7, these data suggest that SMN-Gemin2 together are the minimal subunit of the SMN complex and form the stable core of the complex. It also suggests that SMN and Gemin2 probably serve to regulate each other in some way, perhaps by directly affecting each other's cellular stability.

Gemin5 has recently been shown to bind snRNAs as part of the SMN complex as well as on its own when purified as a recombinant protein (21). Previous work has also shown that Gemin5 directly binds to SMN protein as well as to some of the

Sm proteins (4). Further, previous RNAi experiments showed that Gemin5 is required for snRNP assembly (21). We have found that much of the Gemin5 in cells resides in the cytoplasm outside of the SMN complex.

We further show here that Gemin5 binds to snRNAs outside of the SMN complex at a very early stage in the snRNP assembly process. Inhibition of snRNP assembly causes an accumulation of the Gemin5-snRNA complex outside of the SMN complex. The assembly of Sm cores requires SMN protein and takes place in the SMN complex. We therefore identify the Gemin5-snRNA subunit as a new intermediate in the snRNP assembly process. This intermediate exists only transiently when assembly proceeds in a normal fashion. However, the Gemin5-snRNA intermediate becomes populated under conditions when assembly is inhibited or slowed. Although the Gemin5-snRNA complex is an intermediate in the pathway, we cannot conclude that it is a precursor. We propose that Gemin5 binds to snRNAs outside of the SMN complex and delivers them, in an ATP hydrolysis-dependent manner, to the other subunits of the SMN complex for final assembly. Our data cannot, however, rule out a second equally interesting possibility that the SMN-free Gemin5 binds to unassembled snRNAs and functions to sequester the snRNAs until assembly is resumed.

In addition to free Gemin5 and Gemin5 in the SMN complex, we have identified a new complex of Gemin5 with Gemin3 and Gemin4. Gemin5 has been shown to bind to snRNAs at the Sm site. In order for Sm core assembly to occur, Gemin5 must be replaced at the Sm site by the seven Sm proteins. Sm core assembly by the SMN complex is ATP-dependent, and Gemin3 is a DEAD-box RNA-dependent RNA helicase (2, 13, 35). The interaction of Gemin5 with Gemin3 may serve to remodel the Gemin5-snRNA complex to facilitate Sm core assembly. It is also possible that the interaction of Gemin5 with Gemin3 and Gemin4 is an intermediate of the assembly of the SMN complex itself.

Another stable subunit contains Gemin6, Gemin7, and unrip. This was not surprising, since these three proteins, along with the newly discovered Gemin8 protein, form an interaction network *in vitro*. Additionally, while this manuscript was in preparation, Carissimi *et al.* (23) found that Gemin6, Gemin7, and unrip form a complex. It is likely that the Gemin6-Gemin7-unrip complex described here is the same. Gemin6 and Gemin7 both directly bind to Sm proteins, and unrip has been shown to sequester the SMN complex in the cytoplasm. Since RNAi experiments have demonstrated that Gemin6 is required for snRNP assembly (15), it will be interesting to determine the precise role of this subunit in snRNP assembly.

SMA results from a reduction of the SMN protein. When SMN protein is reduced, the amounts of the Gemin3-Gemin4-Gemin5 and the Gemin6-Gemin7-unrip subunits, not as part of the SMN complex, increase relative to the amounts of Geminins within the SMN complex. In addition to the functions of the subunits in snRNP assembly, it is possible that they have other cellular functions separate from snRNP assembly. For example, Gemin3 has been implicated in transcriptional regulation of several genes (36–43), and Gemin3, Gemin4, and Gemin5 have been found to interact with components of the translation machinery (44–46). It is possible that the imbalance that is

created when SMN is reduced results in an increase in the function of the independent subunits, possibly contributing to the SMA phenotype.

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