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Gemin2 Plays an Important Role in Stabilizing the Survival of Motor Neuron Complex*[S]

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The survival of motor neuron (SMN) protein, responsible for the neurodegenerative disease spinal muscular atrophy (SMA), oligomerizes and forms a stable complex with seven other major components, the Gemin proteins. Besides the SMN protein, Gemin2 is a core protein that is essential for the formation of the SMN complex, although the mechanism by which it drives formation is unclear. We have found a novel interaction, a Gemin2 self-association, using the mammalian two-hybrid system and the *in vitro* pull-down assays. Using *in vitro* dissociation assays, we also found that the self-interaction of the amino-terminal SMN protein, which was confirmed in this study, became stable in the presence of Gemin2. In addition, Gemin2 knockdown using small interference RNA treatment revealed a drastic decrease in SMN oligomer formation and in the assembly activity of spliceosomal small nuclear ribonucleoprotein (snRNP). Taken together, these results indicate that Gemin2 plays an important role in snRNP assembly through the stabilization of the SMN oligomer/complex via novel self-interaction. Applying the results/techniques to amino-terminal SMN missense mutants that were recently identified from SMA patients, we successfully showed that amino-terminal self-association, Gemin2 binding, the stabilization effect of Gemin2, and snRNP assembly activity were all lowered in the mutant SMN(D44V), suggesting that instability of the amino-terminal SMN self-association may cause SMA in patients carrying this allele.

Spinal muscular atrophy (SMA)[2] is a common autosomal recessive disease that is clinically classified into three types, I–III, based on the severity of motor neuron degeneration and the age of onset (1–3). Two nearly identical copies of the survival of motor neuron genes (*SMN1* and *SMN2*) are located on the human chromosome, 5q13, whereas other eukaryotic species have only one copy of the *SMN* gene. Homozygous deletions of, or mutations in the *SMN1* gene are responsible for SMA (4). SMN is expressed ubiquitously and is a core component of a self-assembling multiprotein complex, the SMN complex, consisting of SMN and Gemin proteins, which plays an essential role in the assembly of the spliceosomal small nuclear ribonucleoproteins (snRNPs) and in pre-mRNA splicing (5, 6).

Most of the missense mutations in SMA patients are located in exon 6 of *SMN1*; whereas nonsense and frameshift mutations are widely spread throughout the entire gene (7). Exon 6 of *SMN1* encodes a self-association domain, and deletions or mutations of this domain result in a decrease in oligomer formation of SMN, which correlates with the severity of SMA (8). SMN oligomerization is also a prerequisite for high affinity binding of the SMN complex to spliceosomal snRNPs (9). The self-association domains of SMN were identified by surface plasmon resonance analysis; both the carboxyl-terminal exon 6-encoded region and the amino-terminal exon 2b-encoded region contributed to self-association (10). This suggests a mechanism in which the self-association formation involves either linear oligomers or closed rings by SMN proteins (10), although amino-terminal self-association is not yet evaluated in other studies. Recently, two novel amino-terminal missense mutations, located in the exon 2a-encoded region of *SMN1*, were identified from SMA patients, although the biochemical features of these mutations are currently unclear (11).

The SMN complex consists of the SMN protein and seven other major components, designated as Gemin2, -3, -4, -5, -6, -7, and -8 (12–20). Gemin2 is a 30-kDa protein that was identified as a core component of the SMN complex because of its association with the amino-terminal side of the SMN protein. Despite its lack of apparent known domains, Gemin2 plays important roles in the SMN complex; the SMN-Gemin2 complex is associated with spliceosomal snRNPs U1 and U5 (12),

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3, Figs. S1–S3, and text.

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[2] The abbreviations used are: SMA, spinal muscular atrophy; SMN, survival of motor neuron; U snRNPs, uridine-rich small nuclear ribonucleoprotein; GST, glutathione *S*-transferase; ORF, open reading frame; CHO, Chinese hamster ovary; siRNA, short interfering RNA; qRT-PCR, quantitative real-time reverse transcription-PCR; WT, wild type; HA, hemagglutinin; CMV, cytomegalovirus.

and Gemin2 is essential for the formation of the SMN complex (21), even if the precise mechanism is still unknown. Additionally, gene targeting of Gemin2 in mice reveals a correlation between defects in the biogenesis of U snRNPs and motor neuron cell death (22, 23).

As well as Gemin2, the roles and the mechanisms of the other Gemin proteins in the SMN complex are not yet fully characterized, although recent analysis reveals that, in addition to the SMN protein, Gemin3, -5, -6, and -7 all associate directly with the Sm proteins (24), and Gemin2, -3, -4, -6, and -8 and SMN are important for the U1 snRNP assembly activity of the SMN complex (20, 25, 26). Battle *et al.* (27) demonstrated that Gemin5 functions as an snRNA binding protein of the SMN complex and is required for the U4 snRNP assembly activity of the SMN complex. Recent crystal structural analysis showed that a Gemin6-Gemin7 heterodimer has an Sm protein-like structure, suggesting that it plays a role in the assembly of the snRNP proteins (28). Further, a Gemin3-Gemin4 complex was identified in the RNA-induced silencing complex complex, suggesting that Gemin3 and -4 have additional roles to play in the RNA interference gene regulation system (29).

Here, we systematically explore the functions of Gemin2 to gain insight into the molecular basis of the SMN complex and show that Gemin2 significantly stabilized amino-terminal SMN self-association. This correlates with the stability of the SMN oligomer formation and snRNP assembly activity and is likely to occur through the recently identified Gemin2 self-association. We also found that the amino-terminal self-association, Gemin2 binding, stabilizing effect of Gemin2, and snRNP assembly activity were lowered in the SMA-derived amino-terminal missense mutant SMN(D44V), supporting the importance of Gemin2 in stabilizing the SMN complex.

EXPERIMENTAL PROCEDURES

cDNA Clones—The full-length cDNAs encoding the SMN complex component proteins were obtained from the RIKEN mouse cDNA bank (FANTOM) (30). GenBank™ accession numbers are AK167832 for SMN, AK007515 for Gemin2, and AK141078 for Gemin4. The full-length cDNA for human SMN (BC015308) was purchased from the Mammalian Gene Collection. The full-length cDNA for human Gemin2 was kindly provided by Dr. Hitoshi Kurumizaka (Graduate School of Science and Engineering, Waseda University, Tokyo, Japan).

Preparation of SMN Mutants—To generate missense mutations (D30N, D44V, and Y272C), human SMN cDNA was used as a template for site-directed mutagenesis by overlap extension using PCR (31). PCR products were digested with BamHI and XhoI and ligated into the pET21a vector (Novagen, Madison, WI). SMN constructs, including synonymous point mutations in the target region of the siRNA against wild type SMN mRNA, were also generated by site-directed mutagenesis by overlap extension using PCR. PCR products were digested with EcoRI and XhoI and ligated into the pCMV-HA vector (Clontech). The resulting clones were sequenced to confirm each mutation. The primers used for the preparation of SMN mutants are listed in supplemental Table S1.

Expression and Purification of the Recombinant Gemin2—The Gemin2 encoding sequence was subcloned into the pGEX-6P-1 vector (Amersham Biosciences) to express the GST-fused Gemin2 protein (GST-Gemin2). The sequence was also subcloned into the homemade pGEX-6P-1-$(His)_6$ vector to express the carboxyl-terminal $(His)_6$-tagged GST-Gemin2 (GST-Gemin2-His). The fusion proteins were expressed in *Escherichia coli* BL21-CodonPlus (DE3)-RIL (Stratagene, La Jolla, CA). The cells, harvested from 200 ml of culture, were sonicated and centrifugation at 15,000 × *g* for 20 min at 4 °C. The lysate was applied to the GSTrap FF column (Amersham Biosciences) that contains glutathione-Sepharose resin and was then washed with 1× phosphate-buffered saline (−) buffer. The bound GST-Gemin2 or GST-Gemin2-His proteins were then cleaved with PreScission™ protease (Amersham Biosciences) at 4 °C for 4 h. Then the GST tag-free Gemin2 or Gemin2-His was eluted with HBS buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl). The tag-free Gemin2 protein was subjected to gel-filtration chromatography on a Hi-Load 16/60 Superdex 200-pg column (Amersham Biosciences) with HBS buffer. The bound GST-Gemin2 was also eluted using 10 mM glutathione, 50 mM Tris-HCl, pH 8.0, to obtain the GST-tagged Gemin2.

Mammalian Two-hybrid Assay—Mammalian two-hybrid assays, including sample construction and transfection, were carried out as previously described (32), with minor modifications. The forward primers specific to each ORF were designed to have a consensus tag sequence, 5′-GAAGGAGCCGCCACCATG-3′, followed by an ORF sense-strand sequence with an annealing temperature of 60 °C. Similarly, the reverse primers were designed to have another tag sequence, 5′-CAATTTCACACAGGAAACTCA-3′, followed by an ORF antisense-strand sequence. All the gene-specific primers and other common primers used in this work (RSALSE, FSV40LPAS02, FPCMV6, FPCMV5, RSV40LPAS01, T7-RBS-KOZAK, and LGT10L) are listed in supplemental Table S2. Briefly, fragments for the human cytomegalovirus (CMV) promoter and the Gal4 DNA-binding domain (BIND) or the VP16 transcriptional activation domain (ACT), were PCR-amplified from pBIND or pACT vectors (Promega, Madison, WI) using the primer pair FPCMV6 and RSALSE. Fragments for the SV40 late polyadenylation signal (SV40LPAS) was PCR-amplified from the pBIND vector using the primer pair FSV40LPAS02 and RSV40LPAS01. Each cDNA ORF was amplified by using the corresponding ORF-specific forward and reverse primers. Overlapping PCR was carried out to obtain the assay constructs, in which each ORF fragment was connected with the BIND or ACT fragments at the 5′-end of the first PCR product and the SV40LPAS fragment at the 3′-end (bait and prey, respectively). One microliter each of the ORF fragments was mixed with 0.75 μl of BIND or ACT fragments and SV40LPAS fragments, and then amplified in 100-μl reactions using the primer pair FPCMV5 and LGT10L. All the PCR conditions were based on those in our previous report (32).

All the combinations of Gemin2 and other components were transfected to CHO-K1 cells using Lipofectamine™ 2000 (Invitrogen) together with the luciferase reporter plasmid pG5*luc*, and the reporter activity was measured after 22 h incubation. Each combination was done in triplicates, and the assay was carried out three times.

Rapid in Vitro Pull-down Assay—The PCR products encoding protein-coding sequences were used to construct samples for *in vitro* transcription/translation. The products were connected by overlapping PCR using the primer pair T7-RBS-KOZAK and LGT10L giving the final constructs with a T7 RNA polymerase promoter at the 5′-terminal. The *in vitro* pull-down assay was carried out as previously described (33). Briefly, independent *in vitro* synthesis of biotinylated and ^{35}S-labeled proteins was carried out from the corresponding PCR constructs by using the Transcend^{TM} biotinylated lysine-tRNA (Promega), redivue L-[^{35}S]methionine (Amersham Biosciences), and TnT T7 Quick-Coupled Transcription/Translation System (Promega). After ^{35}S-labeled protein synthesis was confirmed by SDS-PAGE and autoradiography, 10 μl each of biotinylated protein and ^{35}S-labeled protein were mixed, and the mixture was incubated on ice for 1 h. Dynabeads Streptavidin (Dynal Biotech, LLC, Milwaukee, WI) suspension (0.2-mg beads in 80 μl of blocking buffer, 2% (w/v) skim milk in TBST (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.68 mM KCl, 0.1% (w/v) Tween 20)) was mixed with the reaction, and the mixture was incubated in a rotary shaker for 30 min at 4 °C. The beads were isolated with a magnet and washed five times with 150 μl of ice-cold TBST. The radiolabeled proteins co-precipitated with biotinylated proteins were separated by SDS-PAGE and visualized by autoradiography.

GST Pull-down Assay—Purified GST (5 μg) or GST-Gemin2 (5 μg) was immobilized on glutathione-Sepharose 4B (Amersham Biosciences). Purified Gemin2-His (10 μg) was incubated with the immobilized GST or GST-Gemin2 in a lysis buffer (10 mM Tris-HCl, pH 7.8, 1% (w/v) Nonidet P-40, 150 mM NaCl, 1 mM EDTA) at 4 °C for 1 h. After washing the resin five times with the lysis buffer, the bound proteins were subjected to SDS-PAGE, followed by Coomassie staining or Western blot using anti-Gemin2 or anti-His antibodies.

Western Blotting—The samples were separated by 10–20% gradient SDS-PAGE gel electrophoresis and transferred to polyvinylidene fluoride membranes. Primary antibodies used were: anti-Gemin2, SMN, Gemin3, PRMT5 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Gemin7 (Abnova Corp., Taipei City, Taiwan), polyclonal IgG, anti-Sm monoclonal antibody Y12 (Lab Vision Corp., Fremont, CA), anti-Gemin2 monoclonal antibody (BD Biosciences), and anti-His monoclonal antibody (Amersham Biosciences). Horseradish peroxidase-conjugated anti-goat (Promega) or anti-mouse IgG antibodies (Amersham Biosciences) was used as secondary antibody, and the signal was detected by using the ECL plus Western blotting detection system (Amersham Biosciences).

In Vitro Dissociation Assay—For *in vitro* dissociation assay, free [^{35}S]methionine in the radioisotope-labeled reaction that was not incorporated into the synthesized proteins was removed using CENTRI-SEP spin columns (Princeton Separations, Inc., Adelphia, NJ). Each 50 μl of Biotin-labeled and ^{35}S-labeled proteins was mixed with either 50 μl of *in vitro* synthesized unlabeled protein or 50 μl of the reticulocyte lysate, and the mixture was incubated on ice for 1 h. Dynabeads Streptavidin suspension (1-mg beads in 150 μl of the blocking buffer) was mixed with the reaction and incubated for 30 min at 4 °C. The beads were captured by a magnet and washed once with 500 μl of ice-cold TBST within 2 min of the capture, followed by re-suspension in 300 μl of TBST. Every 15 min, 50 μl of the suspension was removed. The beads were captured, and radioactivity remaining on the beads was measured by a liquid scintillation counter.

Sedimentation Analysis—HeLa cells (two 10-cm cultured dishes) were used for sedimentation analysis of the SMN oligomer. The siRNAs for Gemin2 and the negative control (GCAGCUCAAUGUCCAGAU and CCCGGACCACAACGCUCUG, respectively (25)) were purchased from Invitrogen. The siRNAs were transfected into the cells by using Oligofectamine^{TM} according to the manufacturer's protocol (Invitrogen). After 44 h from the siRNA transfection, the silencing of Gemin2 in siRNA-transfected cells was evaluated by qRT-PCR and Western blot analysis. The cells were harvested and re-suspended in 500 μl of the lysis buffer, and the centrifuged at 15,000 $\times$ *g* for 20 min at 4 °C. Preparation of the sucrose density gradient was performed by using Gradient master (Biocomp Instruments, Inc., Fredericton, New Brunswick, Canada). The supernatant was separated on 6 to 38% (w/v) sucrose gradients at 17,000 rpm in the SW28 rotor (Beckman Coulter, Inc., Fullerton, CA) at 4 °C for 17.5 h, followed by 2-ml fractionations by using a Piston gradient fractionator (Biocomp Instruments). The proteins in the fractions were precipitated with 3% (w/v) trichloroacetic acid and centrifuged at 15,000 $\times$ *g* for 10 min at 4 °C. The precipitants were diluted in 50 μl of 0.1 M NaOH, and 20% of each sample was separated by SDS-PAGE and analyzed by Western blotting using anti-SMN polyclonal IgG. Svedberg values of the SMN complex were estimated by using the marker globular proteins, ovalbumin (3.5S, 44 kDa), aldolase (7.3S, 158 kDa), and thyroglobulin (19.4S, 670 kDa). Other additional ribosomal 50S (1.8 MDa) and 70S (2.7 MDa) subunits were also used in size estimation.

Immunoprecipitation—HeLa cells (in a 10-cm culture dish) were transfected with the siRNA for Gemin2 or with the negative control siRNA using Oligofectamine^{TM} according to the manufacturer's protocol. Forty-four hours after the siRNA transfection, cells were harvested and lysed by lysis buffer containing 1 mM phenylmethylsulfonyl fluoride and 10 mg/ml leupeptin. After centrifugation at 15,000 $\times$ *g* for 15 min at 4 °C, the supernatants were subjected to an immunoprecipitation with 5 μg of anti-SMN antibody. The co-precipitated Gemin2, Gemin3, Gemin7, and SmB/B′ were detected by Western blotting.

qRT-PCR—Total RNA was extracted from HeLa cells using an RNeasy mini kit (Qiagen). The extracted total RNA was reverse-transcribed using an oligo(dT) primer and ThermoScript^{TM} RT-PCR system (Invitrogen). The prepared cDNA were used as templates for qRT-PCR analysis using SYBR Green I nucleic acid gel stain (Invitrogen). The two different primer sets against each target gene are shown in supplemental Table S3. PCR amplification was carried out using the ABI Prism 7900HT instrument (Applied Biosystems, Foster City, CA), and each reaction was run in triplicate. Expression was assessed by evaluating the threshold cycle value (C_T). Firstly, the difference of threshold cycle (ΔC_T) of each target gene was calculated between the negative control siRNA-transfected cells and Gemin2 siRNA-transfected cells. Secondly, the gene

expression levels of negative control cells was set to 1.0, and the relative gene expression levels of Gemin2 siRNA-transfected cells were calculated as $2^{-\Delta C_T}$. The procedure is described in detail in supplemental data 1.

In Vitro snRNP Assembly Assay—The *in vitro* snRNP assembly assay was carried out as described previously (26, 34), with slight modification. Briefly, U1 snRNA was transcribed *in vitro* using Riboprobe Systems (Promega) in the presence of Ribo m^7G Cap Analog (Promega) and [α-^{32}P]UTP (Amersham Biosciences) for 1 h at 37 °C, followed by removal of DNA template by digestion with RQ1 RNase-free DNase (Promega). The free [α-^{32}P]UTP that was not incorporated into the synthesized U1 snRNA was removed by using MicroSpin S-200 HR Columns (Amersham Biosciences). Cytoplasmic extracts were prepared from HeLa cells using Ne-Per nuclear and cytoplasmic extraction reagents (Pierce), and the extracts were incubated with U1 snRNA for 20 min at 30 °C in RSB-100 buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.5 mM $MgCl_2$). Subsequently, the U1 RNA that was assembled by Sm proteins was immunoprecipitated with the anti-Sm monoclonal antibody Y12 (Lab Vision Corp.) or anti-Gal4 antibody (Santa Cruz Biotechnology), which was used as a negative control antibody. The antibody and Dynabeads protein G (Dynal Biotech) in 80 μl of RSB-100 buffer containing 0.1% Nonidet P-40 and 0.2 unit/μl RNasin RNase inhibitor (Promega), were added to the reaction mixtures, and the mixture was incubated in a rotary shaker for 1 h at 4 °C. The beads were isolated with a magnet and washed five times with 200 μl of ice-cold RSB-100 buffer containing 0.1% Nonidet P-40. Half of the precipitated U1 RNA was measured using a liquid scintillation counter, and the other half was subjected to 7 M urea-6% polyacrylamide gel and detected by autoradiography. After subtraction of the count for immunoprecipitation with anti-Gal4 antibody (negative control), the relative assembly activity was compared with the assembly activity of the wild-type cells. The average count for the negative control was 82 cpm (<1% of the count in the wild type).

RESULTS

Identification of a Novel Protein-Protein Interaction, the Gemin2 Self-association—Gemin2 is a core component of the SMN complex, contributing to the activity of the SMN complex through an uncharacterized molecular mechanism (12, 22, 35). To gain insight into Gemin2 and its mechanisms, we tried to purify recombinant Gemin2 using a size-fractionation column, and we found that the main fraction of Gemin2 was ~65 kDa, whereas minor fractions of 30 and 120 kDa were apparent (Fig. 1*A*). Assuming that Gemin2 is a globular protein, the observed molecular mass for the main fraction was twice as heavy as the theoretical molecular mass of 30 kDa, which would suggest that Gemin2 forms a homo-dimer (Fig. 1*A*).

To confirm the result *in vivo*, we explored the self-association of Gemin2 using a mammalian two-hybrid system (32). We made constructs that expressed Gemin2, SMN, and Gemin4 as fusion proteins with the Gal4 DNA binding domain or the VP16 trans-activation domain. Together with the reporter plasmid, the Gal4-Gemin2 construct was transfected into CHO-K1 cells in combination with VP16-fusion protein constructs, and the interaction between the expressed fusion proteins was detected by measuring the luciferase reporter activity the next day. We found that Gemin2-Gemin2 showed high reporter activity, comparable to that of SMN-Gemin2, a previously known interaction (Fig. 1*B*). We also applied a rapid *in vitro* pull-down method that we recently developed, which uses *in vitro* biotinylated proteins instead of tagged proteins as the pull-down drivers (33). We synthesized Gemin2 protein *in vitro* using the rabbit reticulocyte lysate system and thereby demonstrated Gemin2 self-association (Fig. 1*C*).

The observed binding, derived from the mammalian two-hybrid system and the *in vitro* pull-down method with proteins produced in the reticulocyte lysate, could be indirect via additional proteins. Because of this, we further confirmed binding using purified recombinant Gemin2; we expressed and purified recombinant Gemin2 with a GST tag (GST-Gemin2) or His tag (Gemin2-His) and subjected the recombinant protein to a rational *in vitro* pull-down assay using glutathione-Sepharose resin (Fig. 1*D*). The purified Gemin2-His was successfully pulled down by GST-Gemin2, which indicates that Gemin2 is able to directly self-associate.

Domain Mapping of Gemin2 Self-association—To identify the binding domain for the Gemin2 self-association as compared with the Gemin2 binding domain for the SMN protein, we systematically constructed Gemin2 deletion mutants, and these were subjected to a binding assay using the mammalian two-hybrid system (Fig. 2*A*). We found that these binding domains are very similar and that they reside almost throughout in the Gemin2 protein. The binding availability seems to be sensitive to deletion at the carboxyl terminus, because both self-association and SMN binding drastically decreased in the mutant Gemin2$^{1-252}$, a deletion mutant lacking 17 amino acid residues at the carboxyl terminus of Gemin2. Interestingly, the binding availability is slightly different in the amino-terminal deletions. The reporter activity for Gemin2 self-association decreased in Gemin2$^{90-269}$ and was almost undetectable above the background signal in Gemin2$^{99-269}$, whereas the reporter activity for Gemin2-SMN association did not change drastically in these mutants but decreased in Gemin2$^{108-269}$. The difference in binding properties of the Gemin2 mutant was also confirmed using an *in vitro* pull-down method (Fig. 2*B*). Thus we obtained a Gemin2 mutant that exhibited reduced Gemin2 self-association activity without drastically affecting Gemin2-SMN association.

Self-association of SMN Protein at the Amino and Carboxyl Termini—It is well established that SMN protein self-associates via its carboxyl-terminal exon 6-encoded region wherein many point mutations are reported in SMA patients (11, 36). In addition, one report using *in vitro* surface plasmon resonance analysis focused on another self-association region of the SMN protein that resided in an amino-terminal exon 2b-encoded region, closely located to the region where the SMN protein associates with Gemin2 (10). Despite its importance for oligomerization of the SMN protein, self-association via the amino terminus has not been evaluated in other works. Thus, we carried out domain mapping for SMN self-association using both *in vivo* and *in vitro* assays.

We divided the SMN protein into three regions: amino-terminal, middle, and carboxyl-terminal, encoded by exons 1–2b

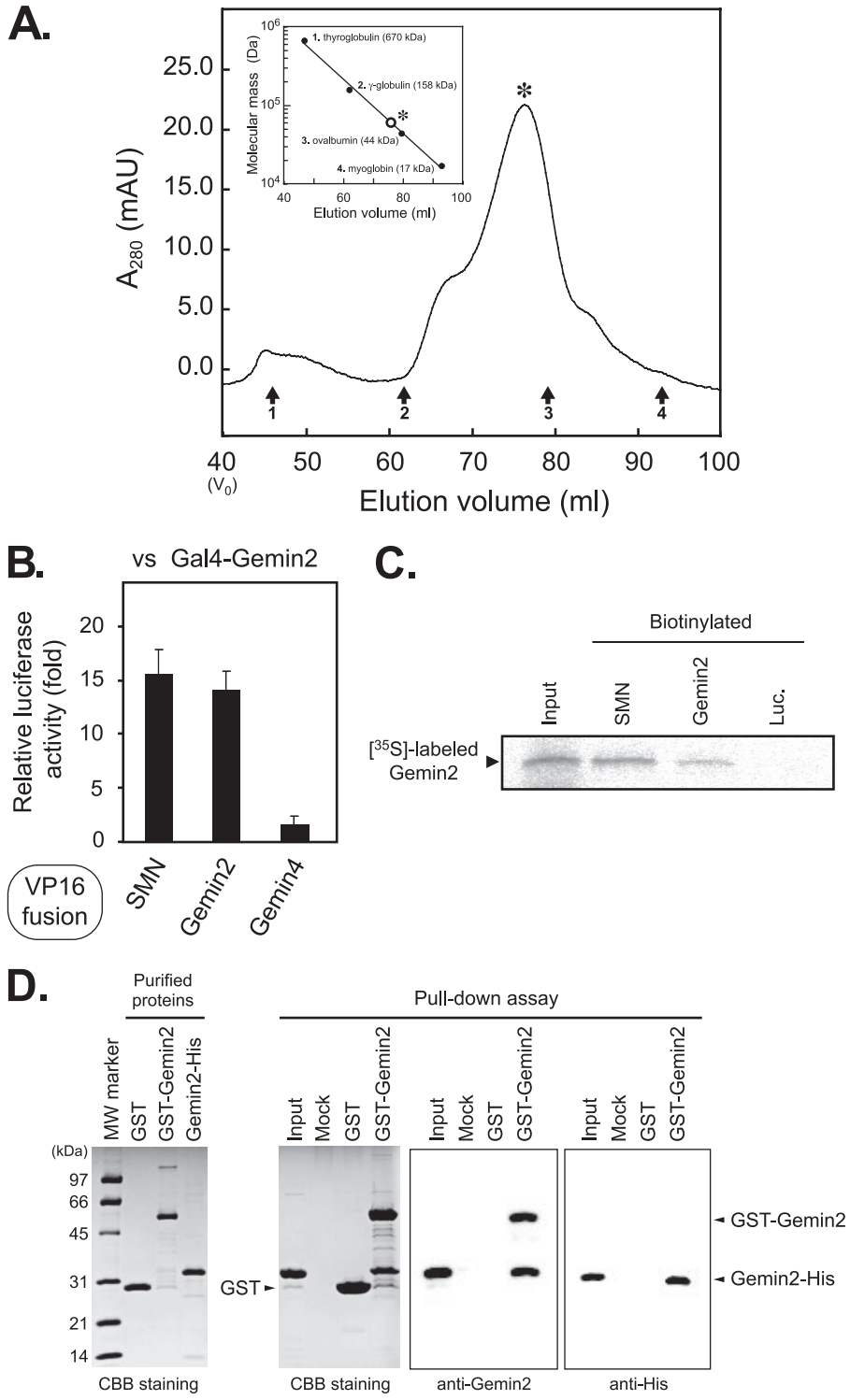
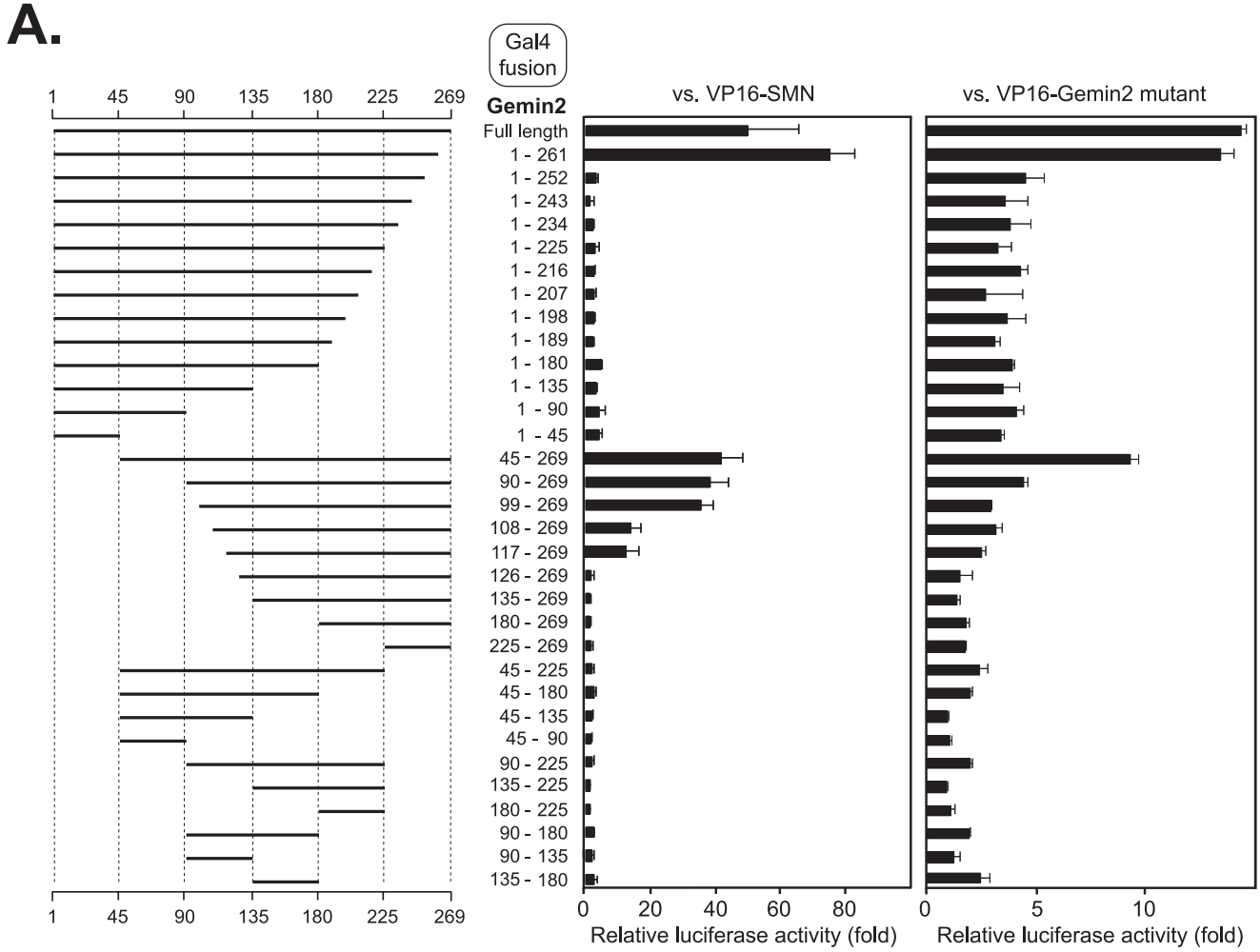


FIGURE 1. **Identification of a novel protein-protein interaction; Gemin2 self-interaction.** *A*, gel-filtration analysis of Gemin2. The chromatogram demonstrates the elution of purified recombinant Gemin2 (*, the major elution peak of the protein). The *arrows* indicate the elution point of each molecular standard protein, and *numbers* correspond to the proteins shown at the *inset graph*. The *inset graph* indicates the semi-log plots for the molecular mass of the standard proteins and Gemin2 against the elution volume of these proteins in gel-filtration chromatography. *B*, the result of the mammalian two-hybrid assay. This experiment was independently conducted three times, and the *errors bars* represent standard deviation. Gal4-Gemin2 and VP16-SMN, Gemin2, and Gemin4 were expressed in CHO-K1 cells. The combination of Gal4-Gemin2 and VP16-Gemin4 was examined as a negative control. Protein-protein interaction was determined by measurement of luciferase reporter activity. *C*, the result of *in vitro* pull-down assay. *In vitro* translated ^{35}S-labeled Gemin2 was incubated with *in vitro* translated biotinylated SMN, Gemin2, and Luc (luciferase as a negative control) and formed complexes that were captured with streptavidin beads. Proteins that remained bound to the beads were analyzed by SDS-PAGE and visualized by autoradiography. 10% of ^{35}S-labeled protein used in the assay was loaded as an input. *D*, Gemin2 self-association using the purified recombinant Gemin2. Purified GST, GST-Gemin2, and Gemin2-His were resolved by SDS-PAGE and visualized by Coomassie staining (*left panel*). *Right panels* show the results of the GST pull-down assay. Purified GST or GST-Gemin2 were immobilized on glutathione-Sepharose and incubated with purified Gemin2-His. After resolution by SDS-PAGE, the bound proteins were visualized by Coomassie staining or Western blot using an anti-Gemin2 or an anti-His antibody. 30% of the Gemin2-His used in each reaction were loaded as an input.



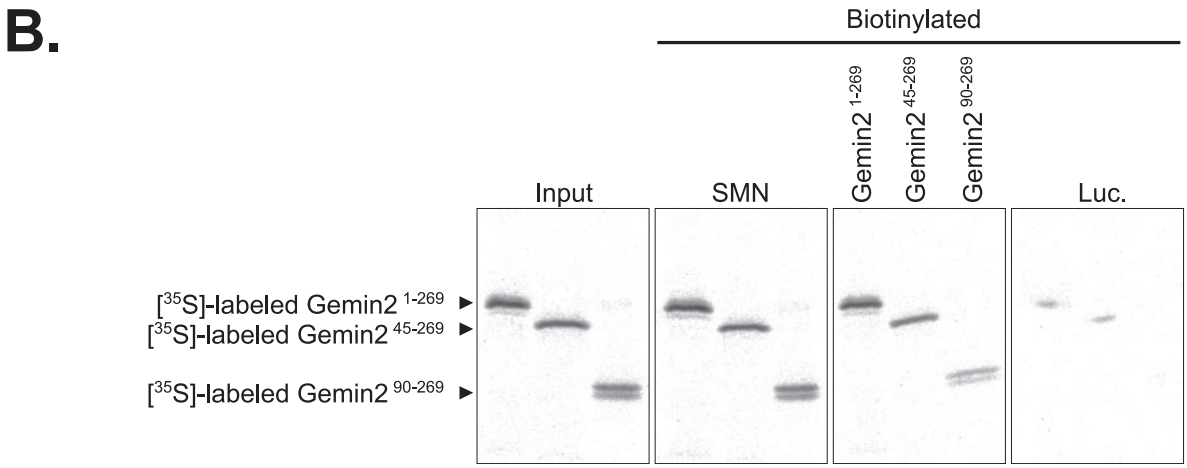


FIGURE 2. **Interaction domain mapping for Gemin2 self-association and Gemin2-SMN association.** *A*, schematic representation of the full-length and deletion mutants of Gemin2 (*left panel*). The interaction was examined using a mammalian two-hybrid assay. Gal4-fused proteins for full-length Gemin2 or Gemin2-deletion mutants were expressed in CHO-K1 cells with VP16-SMN (*middle panel*) or VP16-Gemin2 or VP16-Gemin2 mutants (*right panel*). Protein-protein interactions were determined with the same procedures as in Fig. 1*B*. The experiment was independently conducted three times, and the *errors bars* represent the standard deviation. *B*, confirmation of the properties of the Gemin2 self-association and the Gemin2-SMN association in the mutant Gemin2$^{90-269}$. The interaction assay was performed by the same method as in Fig. 1*C*.

(SMN$^{exon1-2b}$), exons 3–5 (SMN$^{exon3-5}$), and exons 6 and 7 (SMN$^{exon6-7}$), respectively, and explored their interaction with full-length SMN protein using the mammalian two-hybrid system (Fig. 3*A*). We found clear reporter signals in both the amino- and carboxyl-terminal regions but not in the middle region, consistent with the two self-association sites previously reported (10). The reporter signals became stronger when we used the regions encoded by exons 1–5 (SMN$^{exon1-5}$) and exons 3–7 (SMN$^{exon3-7}$), suggesting that these self-association sites may be more stable when present in a longer form. Next we

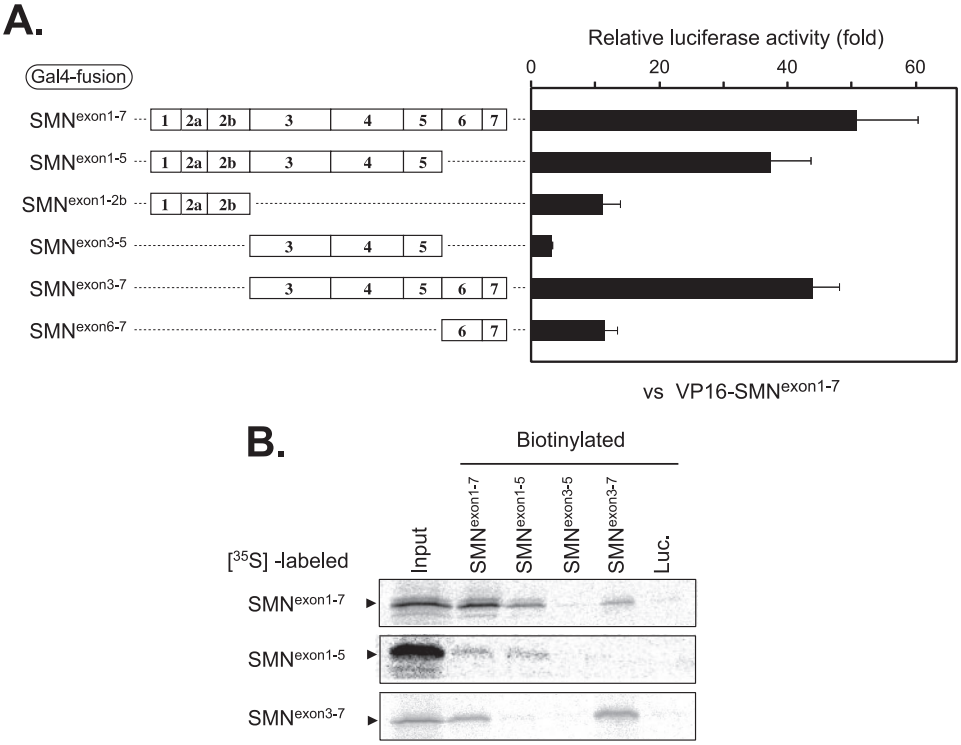


FIGURE 3. **Interaction domain mapping of SMN.** *A*, identification of SMN self-association domains by mammalian two-hybrid assay. This experiment was independently conducted three times, and the *errors bars* represent the standard deviation. Gal4-fused proteins for full-length SMN ($SMN^{exon1-7}$) or SMN-deletion mutants were expressed in CHO-K1 cells with VP16-$SMN^{exon1-7}$. Protein-protein interactions were determined using the same procedures as in Fig. 1*B*. *B*, confirmation of SMN self-association by *in vitro* pull-down assay. The interaction assay was performed by the same method as in Fig. 1*C*.

explored the *in vitro* bindings; in addition confirming the *in vivo* bindings, we could show self-associations of $SMN^{exon1-5}$ and $SMN^{exon3-7}$ (Fig. 3*B*).

Gemin2 Stabilizes the Amino-terminal Self-association of the SMN Protein—Because the amino-terminal region of SMN is responsible for both its self-association and the association with Gemin2, it is conceivable that these interactions together may assist the formation of the SMN complex. To evaluate this hypothesis, we examined the stability of the amino-terminal self-association of SMN in the presence or absence of synthesized Gemin2, using the *in vitro* dissociation assay system based on our *in vitro* pull-down assay (Fig. 4). After association with *in vitro* synthesized biotin-labeled protein and ^{35}S-labeled protein, the complex was captured by streptavidin beads and suspended with assay buffer. The remaining ^{35}S-labeled protein that was attached to the beads was measured to assay stability at different time points. We found that the amino-terminal SMN self-association was unstable; only 35% of the original [^{35}S]$SMN^{exon1-5}$ remained after 60 min of incubation (*open circles* in Fig. 4*A*). On the other hand, the carboxyl-terminal SMN self-association (*open triangles*) seemed very stable; nearly 100% of bound [^{35}S]$SMN^{exon3-7}$ remained after 60-min incubation. Further, assays for SMN-Gemin2 and Gemin2 self-association revealed relatively stable properties (*open diamonds* and *open squares*, respectively). The amino-terminal SMN self-association became significantly ($p < 0.01$) more stable in the presence of synthesized whole Gemin2 protein (Fig. 4*B*); of the original [^{35}S]$SMN^{exon1-5}$ >90% remained after 60-min incubation (*closed circles* in Fig. 4*B*).

From the domain mapping experiment we obtained $Gemin2^{90-269}$, a mutant that reduced Gemin2 self-association activity without drastically affecting Gemin2-SMN association, and we applied the mutant to the dissociation assay. First we examined the stability of Gemin2 self-association and Gemin2-SMN association in the mutant. As expected, the mutant showed a less stable Gemin2 self-association in comparison with that of full-length Gemin2 protein, whereas the stability of the Gemin2-SMN association was similar in the mutant and full-length Gemin2 protein (supplemental Fig. S1). Next we explored the effect of Gemin2 mutation on the stabilization of amino-terminal SMN self-association. The stabilizing effect was weak even in the presence of $Gemin2^{90-269}$ (*crosses* in Fig. 4*B*), indicating that Gemin2 helps to stabilize amino-terminal SMN self-association through Gemin2-SMN association and Gemin2 self-association. The result is represented schematically in Fig. 4*C*.

Gemin2 Knockdown Lowers SMN Oligomerization and in Vitro snRNP Assembly Rates—Our results indicate that Gemin2 plays an important role in the stabilization of the SMN complex through SMN interaction and a novel self-interaction. The SMN complex forms oligomers in mammalian cells, which are considered to be important for the function of snRNP assembly. We therefore explored the requirement of Gemin2 for SMN oligomerization and the snRNP assembly by applying Gemin2 siRNA in HeLa cells. First we confirmed the effect of Gemin2 siRNA to transcripts of the SMN complex components by qRT-PCR analysis (Fig. 5*A*). As expected, Gemin2 siRNA treatment decreased the Gemin2 transcript to 10% of that in untreated cells, whereas transcripts for other components were unaffected. A resulting decrease in Gemin2 protein expression was confirmed by Western blotting (Fig. 5*B*). We then subjected the cytoplasmic soluble fraction of the siRNA-treated and untreated cells to sedimentation analysis using sucrose density gradient ultracentrifugation and detected the SMN protein by Western blotting. Gemin2 siRNA (siGemin2) treatment drastically changed the SMN distribution (*middle lane* in Fig. 5*C*); most of the cytoplasmic SMN protein resided in fractions between 3.5S and 19.4S (estimated molecular mass of 44–670 kDa with a peak of 158 kDa), as opposed to untreated (*WT*) and negative control siRNA (*siControl*) treated cells (*top* and *bottom* in Fig. 5*C*), where cytoplasmic SMN protein mainly resided in molecular fractions larger than 19.4S (estimated molecular mass of larger than 670 kDa with a peak of 1.8 MDa), indicating that most of the cytoplasmic SMN protein is composed of the oligomerized complex, consistent with a previous report (21).

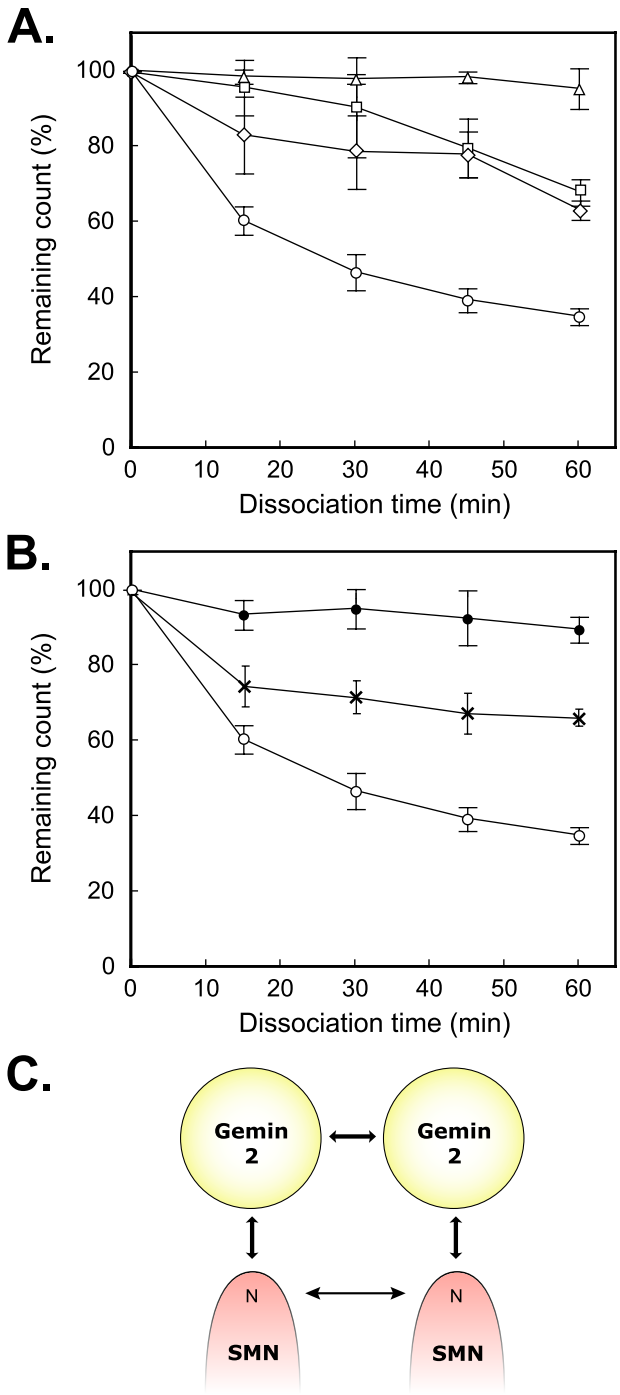


FIGURE 4. **Stabilization of amino-terminal SMN self-association in the presence of Gemin2.** *A* and *B*, *in vitro* dissociation assay was performed as described under "Experimental Procedures." Each plot indicates the relative remaining count of pull-downed ^{35}S-labeled proteins attached to streptavidin beads against the count at zero time. The sample combinations are as follows: ○, biotin-SMN$^{exon1-5}$ and [^{35}S]SMN$^{exon1-5}$; ●, biotin-SMN$^{exon1-5}$, [^{35}S]SMN$^{exon1-5}$, and non-labeled Gemin2; ×, biotin-SMN$^{exon1-5}$, [^{35}S]SMN$^{exon1-5}$ and non-labeled Gemin2$^{90-269}$; △, biotin-SMN$^{exon3-7}$ and [^{35}S]SMN$^{exon3-7}$; □, [^{35}S]Gemin2 and biotin-Gemin2; ◇, [^{35}S]Gemin2 and biotin-SMN$^{exon1-5}$. The experiment was independently conducted three times, and the *errors bars* represent the standard deviation. *C*, schematic representation of protein interactions between SMN and Gemin2. The relative binding stability is shown by the *relative thickness* of the *arrows*.

The precise stoichiometry of the components of the SMN complex is still unknown. However, because the amount of SMN and Gemin2 is far greater than that of the other components, it is highly likely that the core of the native SMN complex has a simple protein composition comprising only two proteins, SMN and Gemin2 (21). Thus, it is unlikely that the drastic change that was observed is only due to molecular weight loss of Gemin2 and/or dissociation of the other components from the oligomerized complex. This strongly suggests that the decrease in Gemin2 destabilized the oligomerized formation of the SMN complex. Nonetheless, it is interesting to explore the effects of Gemin2 knockdown on the other components in the SMN complex. We explored the components that are co-immunoprecipitated with the SMN protein both in Gemin2 siRNA-treated and untreated cells. Useful antibodies were only commercially available for a few components, but we found that the level of co-immunoprecipitated Gemin3, Gemin7, and SmB/B′ following Gemin2 siRNA treatment (Fig. 5*D*). These results indicate that Gemin2 plays important roles not only in the stabilization of SMN oligomerization but also in the stabilization of other components of the SMN complex.

Next we explored the effect of Gemin2 knockdown on SMN function of snRNP assembly, because the SMN complex plays a role in the formation of the Sm protein-U1 RNA complex. Cytoplasmic extracts from the 48-h siRNA-treated and untreated cells were incubated with *in vitro* synthesized ^{32}P-labeled U1 RNA and immunoprecipitated using an anti-Sm antibody. The *in vitro* snRNP assembly activity was determined to measure co-immunoprecipitated ^{32}P-labeled U1 RNA as Sm protein-U1 RNA complexes. The siRNA treatment did not affect the amount of Sm protein in the extracts (supplemental Fig. S2*A*). Gemin2 siRNA treatment (siGemin2) significantly decreased snRNP assembly, with 40% assembly when compared with the untreated (WT) cells (supplemental Fig. S2, *B* and *C*). In the negative control siRNA (*siControl*)-treated cells, snRNP assembly activity was comparable with the level in untreated cells, indicating that Gemin2 is required for efficient snRNP assembly, which is consistent with previous reports (25, 26).

A SMA-derived Mutant SMN(D44V) Reveals a Decrease in Amino-terminal Self-association, Gemin2 Binding, and the Stabilization Effect of Gemin2—Recently, Sun *et al.* (11) reported that two novel missense SMN mutants, SMN(D30N) and SMN(D44V), were identified from SMA patients where D30N and D44V denote an substitution of aspartic acid at the positions 30 and 44 by asparagine and valine, respectively. Although the authors failed to identify a significant biochemical feature of these mutations, the mutations reside within the Gemin2 binding site and are close to the amino-terminal SMN self-association site. We therefore investigated the effect of these mutations on SMN self-association and SMN-Gemin2 interaction by using human SMN cDNAs, into which point mutations corresponding to these missense mutations were introduced. These were then analyzed by mammalian two-hybrid assay and *in vitro* pull-down assay (Fig. 6). We used SMN$^{exon1-5}$ for the assay, because the removal of the carboxyl-terminal self-association site enables a clearer detection of any effect of mutation on amino-terminal SMN self-association. We found that the self-association of SMN(D44V)$^{exon1-5}$, but not of SMN(D30N)$^{exon1-5}$, was lowered as detected by luciferase

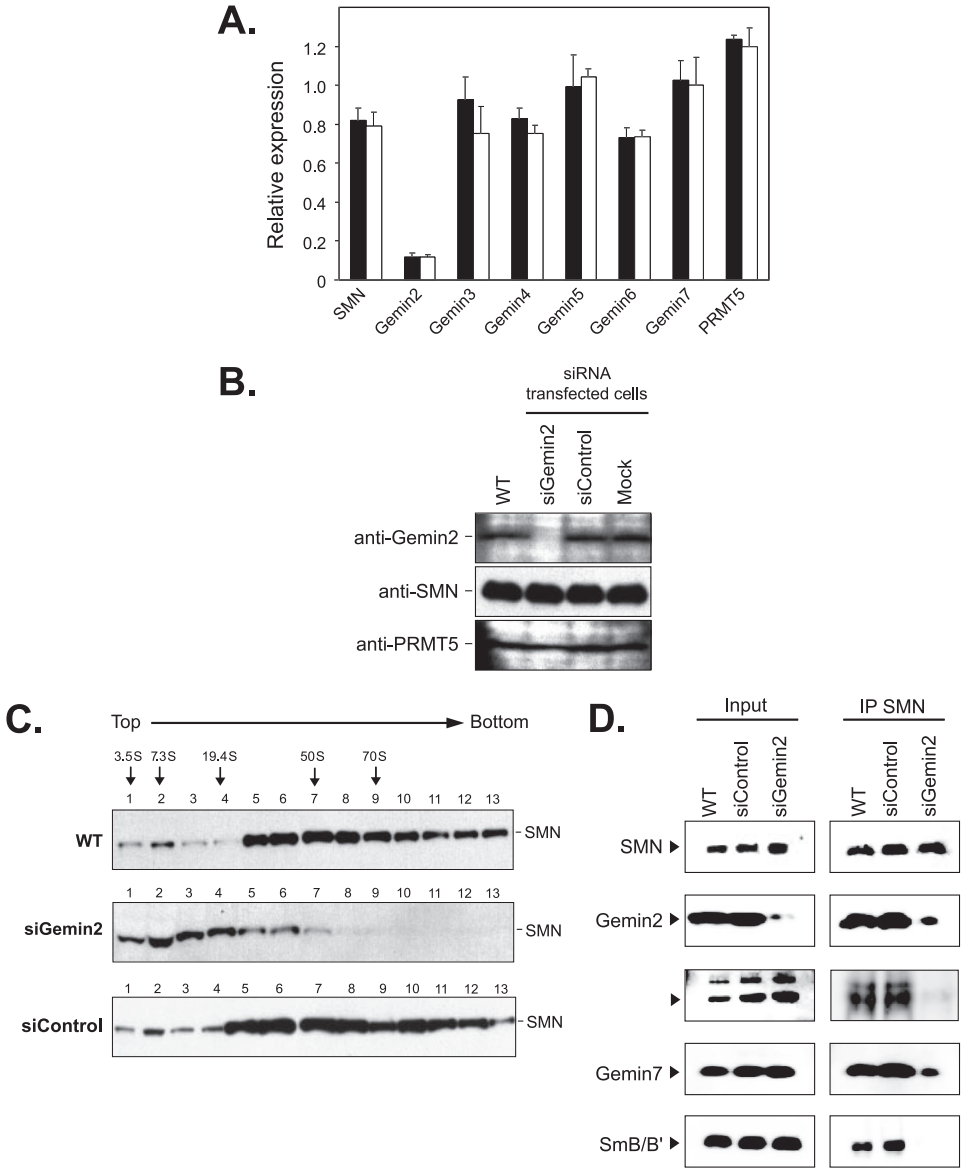


FIGURE 5. **Comparison of SMN oligomerization between untreated HeLa cells and Gemin2 knockdown cells.** *A*, evaluation of gene expression of SMN complex components in HeLa cells following Gemin2 knockdown. The relative expression levels of the target genes were determined by qRT-PCR using total RNA from cells 44 h after transfection with Gemin2 siRNA. The *black* and *white bars* show the results of qRT-PCR analysis using two different primer sets against each target gene, and the *errors bars* represent the standard deviation. *B*, Gemin2 gene silencing with siRNA. Whole cell extracts were prepared from untreated, Gemin2 siRNA-transfected (*siGemin2*), negative control siRNA transfected (*siControl*), and mock transfected (*Mock*) HeLa cells. The cell extracts were analyzed by Western blotting using anti-SMN, anti-Gemin2, and anti-PRMT5 polyclonal antibodies. PRMT5 was used as loading control. *C*, sedimentation analysis of the cell extracts of native HeLa cells (WT, *top panel*), Gemin2 knockdown cells with siRNA (siGemin2, *middle panel*), and cells transfected with negative control siRNA (*siControl*, *bottom panel*). The cell extracts were sedimented on a 6–38% (w/v) sucrose density gradient. Fractions, indicated by *numbers 1–13* from *top* to *bottom* of the centrifuge tube, were subjected to SDS-PAGE followed by Western blotting using anti-SMN polyclonal antibody. *D*, cytoplasmic soluble fraction from the siRNAs (*siGemin2* or *siControl*) treated and untreated (*WT*) HeLa cells were immunoprecipitated with anti-SMN antibody. 2% of the input (*left panel*) and immunoprecipitates (*right panel*) were analyzed by Western blotting using anti-SMN, Gemin2, Gemin3, Gemin7, and SmB/B′ antibodies.

reporter activity (Fig. 6*A*). This decrease in interaction was also observed in SMN-Gemin2 interaction; we again detected lower reporter activity for SMN(D44V)$^{\text{exon1-5}}$ but not for SMN(D30N)$^{\text{exon1-5}}$ (Fig. 6*B*). These results were confirmed by *in vitro* pull-down assay (Fig. 6, *C* and *D*). Similar results were observed even when using full-length SMN mutant proteins in these assays, although the decrease was less pronounced (supplemental Fig. S3).

The results described above suggest that the amino-terminal self-association of SMN(D44V) is unstable even in the presence of Gemin2. To examine this hypothesis, the exon 1 to exon 5 region of wild-type and mutant SMN proteins was subjected to an *in vitro* dissociation assay in the absence and presence of Gemin2 (Fig. 7). In the absence of Gemin2, amino-terminal SMN self-association decreased to 20–30% compared with the initial level in both wild-type and mutant SMNs after 60-min incubation (*opened symbols* in Fig. 7). As expected, Gemin2 could not effectively stabilize amino-terminal self-association in SMN(D44V) (*closed squares* in Fig. 7), but it significantly ($p < 0.01$) stabilized self-association in wild-type SMN and SMN(D30N) (*closed circles* and *triangles*).

SMN(D44V) Is Defective in snRNP Assembly—It is difficult to explore the functional effect of patient-derived mutants by overexpression in cultured cells because of the presence of endogenous wild-type SMN protein. The activities of the mutant SMNs were instead explored in the absence of wild-type SMN by applying the method described by Shpargel and Matera (26). We generated HA-tagged SMN constructs, including synonymous point substitutions in the target region of siRNA against wild-type SMN mRNA. Each mutation corresponding to SMN(D44V), SMN(D30N), and SMN(Y272C) was incorporated into the construct followed by co-transfection into the cells with SMN siRNA. As shown in Fig. 8*A*, the level of endogenous wild-type SMN protein decreased in the siRNA-treated cells to 20% compared with the untreated and negative control siRNA-treated cells, and overexpression of the constructs was confirmed. We also confirmed that the siRNA treatment did not affect the amount of Sm protein in the cytoplasmic extracts. The cytoplasmic lysates of the transfected cells were then used in an snRNP assembly assay. The assembly activity decreased to 20% in cells where siRNA mediated the knockdown of endogenous wild-type SMN protein (Fig. 8A, *siSMN*) compared with untreated cells, and this effect was partially rescued by incorporating wild-type siRNA-insensitive

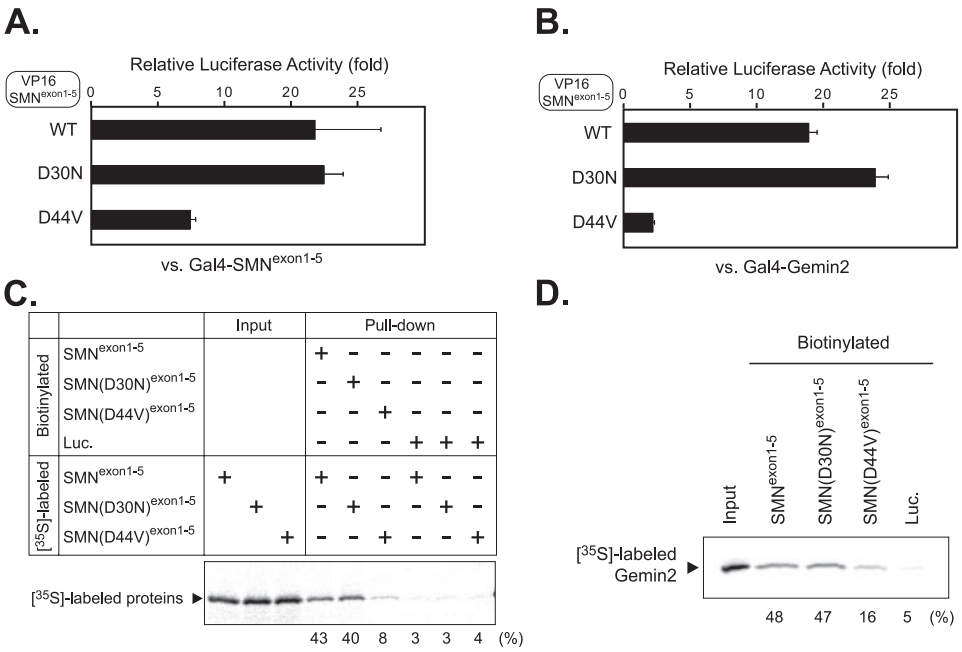


FIGURE 6. **The SMA-derived mutant SMN(D44V) caused a reduction in amino-terminal self-association and Gemin2 binding.** Assay samples for the mutant SMNs were constructed from wild-type human SMN cDNA and were subjected to *in vivo* (*A* and *B*) and *in vitro* (*C* and *D*) binding assays as described above. The *in vivo* binding assay was independently conducted four times, and the *errors bars* represent standard deviation. The lower numerical values (*C* and *D*) indicate the percentage of the pull-downed ^{35}S-labeled proteins against 10% of ^{35}S-labeled protein used in the assay.

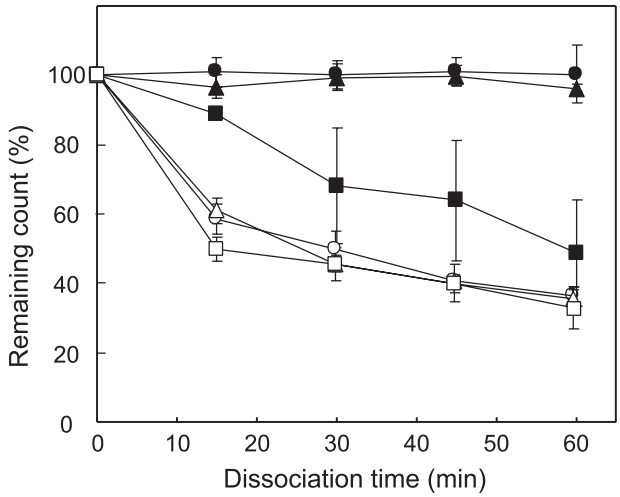


FIGURE 7. **Instability of amino-terminal self-association in the mutant SMN(D44V) in the presence of Gemin2.** The exons 1–5 region of the wild-type SMN and SMN mutants were subjected to dissociation assay in the absence and presence of Gemin2. The sample combinations were as follows: ●, biotin-SMN$^{exon1-5}$, [^{35}S]SMN$^{exon1-5}$, and non-labeled Gemin2; ▲, biotin-SMN(D30N)$^{exon1-5}$, [^{35}S]SMN(D30N)$^{exon1-5}$, and non-labeled Gemin2; ■, biotin-SMN(D44V)$^{exon1-5}$, [^{35}S]SMN(D44V)$^{exon1-5}$, and non-labeled Gemin2; ○, biotin-SMN$^{exon1-5}$ and [^{35}S]SMN$^{exon1-5}$; △, biotin-SMN(D30N)$^{exon1-5}$ and [^{35}S]SMN(D30N)$^{exon1-5}$; □, biotin-SMN(D44V)$^{exon1-5}$, and [^{35}S]SMN(D44V)$^{exon1-5}$. This experiment was carried out with a three times as high volume as in the *in vitro* dissociation assay showed in Fig. 4 because of the reduced self-association activity in SMN(D44V)$^{exon1-5}$. The assay was independently conducted three times, and the *errors bars* represent the standard deviation.

SMN constructs (Fig. 8, *B* and *C*). The rescue of snRNP assembly activity by SMN(D44V) was significantly lower ($p < 0.01$) than that of HA-SMN and was comparable to that of SMN(Y272C), the other SMN mutant at the carboxyl-terminal self-association site. Conversely, SMN(D30N) could rescue assembly activity to a similar extent as the wild-type construct.

DISCUSSION

Herein, we report that Gemin2 plays an essential role in the stabilization of amino-terminal SMN self-association of the SMN complex. Further, we found that SMN oligomerization *in vivo* and snRNP assembly activity *in vitro* was stabilized in the presence of Gemin2. It is likely that Gemin2-dependent stabilization is mediated by a novel self-interaction, where the SMN dimer-Gemin2 dimer forms a stable quaternary complex by association with each other. In fact, we revealed that a mutant Gemin2$^{90-269}$, which showed less stable Gemin2 self-association than full-length Gemin2 protein (supplemental Fig. S1), mediates a weaker stabilization effect to amino-terminal SMN self-association (Fig. 4*B*). Such multiple interaction is considered to be advantageous to the stability of the complex. It would be better if the contribution of the identified interaction could be evaluated by using Gemin2 deletion mutants lacking the property of self-association. However, it is not easy, because the interaction domains of Gemin2 for SMN binding and for Gemin2 self-association are hard to segregate, which suggests that these associations occur in a closely located region in Gemin2 (Fig. 2). We also revealed that Gemin2 plays an important role in the stabilization of other components of the SMN complex, because Gemin2 siRNA treatment partially blocked co-immunoprecipitation of Gemin3, Gemin7, and SmB/B′ using the anti-SMN antibody (Fig. 5*D*). A possible explanation is that SMN oligomerization is a prerequisite for stabilization of other components in the SMN complex. However, it is also possible that such components may be stabilized in the SMN complex through unknown associations with Gemin2. So far, the other components in the SMN complex, Gemin3, -5, and -7, are connected with SMN by very simple interaction networks. It is conceivable that many of them are associated with each other in a far more complex manner to ensure the stability of the SMN complex. In this aspect, it is intriguing that Gemin6, Gemin7, and Unrip form a stable cytoplasmic complex whose association with SMN requires Gemin8 (37).

Our results could offer a more satisfactory explanation model to explain oligomer formation of SMN proteins than previous models (35). Young *et al.* (10) reported a self-interaction at the exon2b-encoded region of SMN and proposed an oligomer model in which two self-association sites in the SMN regions encoded by exons 2b and 6 were capable of forming

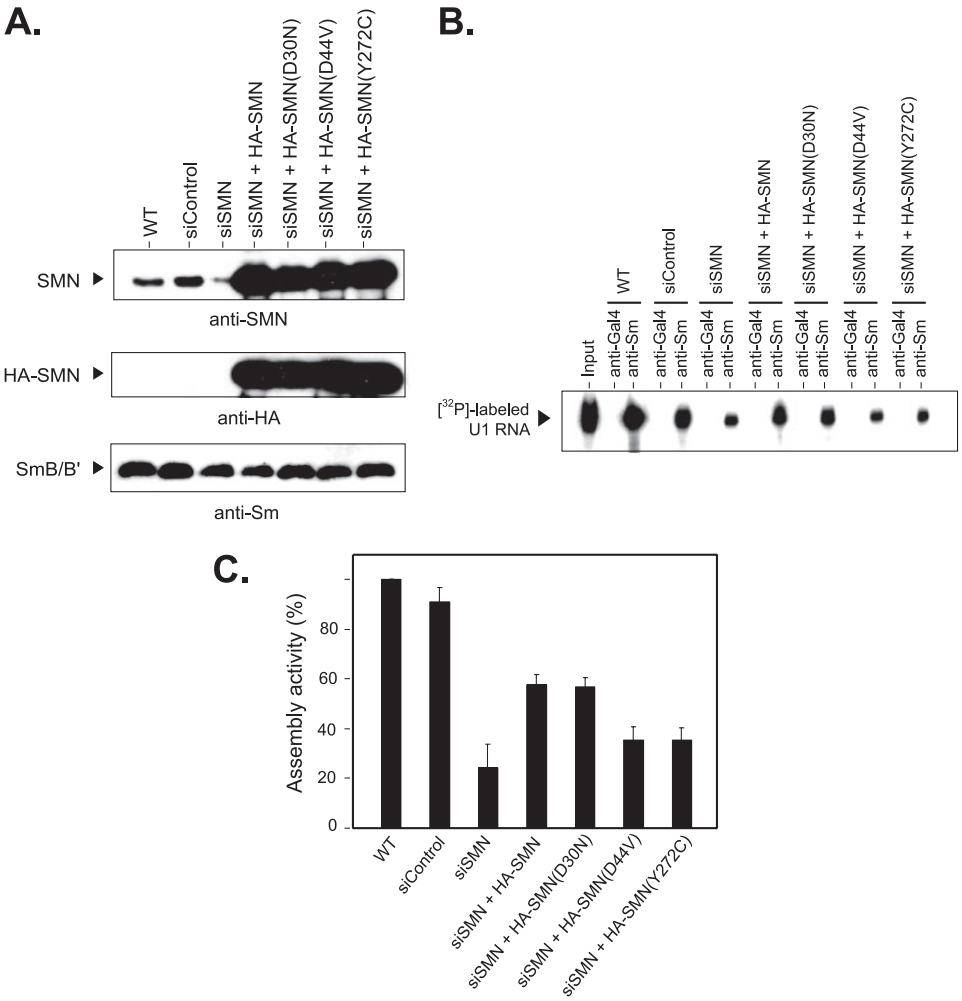


FIGURE 8. ***In vitro* snRNP assembly assay for the SMN mutants.** *A*, Western blotting of SMN, HA-SMN, and Sm proteins. The cytoplasmic extracts, prepared from untreated HeLa cells (*WT*) or HeLa cells transfected with negative control siRNA (*siControl*), SMN siRNA (*siSMN*), and SMN siRNA and various siRNA-insensitive SMN constructs, were subjected to Western blotting using anti-SMN, anti-HA, and anti-Sm antibodies. *B* and *C*, the cytoplasmic extracts were incubated with *in vitro* synthesized ^{32}P-labeled U1 RNA and immunoprecipitated using an anti-Sm antibody or negative control antibody (anti-Gal4 antibody). Half of the precipitated ^{32}P-labeled U1 RNA was separated using a 7 M urea-6% polyacrylamide gel and visualized by autoradiography (*B*), and the radioactivity of other half was measured using a liquid scintillation counter (*C*). This experiment was independently conducted four times, and the *errors bars* represent the standard deviation.

larger oligomers. However, it was uncertain whether such oligomers are stable, because surface plasmon resonance data suggested that the self-interaction at the exon2b-encoded region must be relatively weak when compared with the exon6-encoded region. In addition to the SMN self-association sites that were previously identified, and confirmed in our study (Fig. 3), we found that Gemin2 self-associates (Fig. 1) and that Gemin2 has a stabilization effect on amino-terminal SMN self-association (Fig. 4). Therefore, larger SMN protein oligomers could be formed by two independent stable self-associations of the SMN protein. Although this working hypothesis is consistent with the results obtained in this study, the effect of other components of the SMN complex, Gemin3 to -8 are currently unclear. Further studies to explore the effects of other Gemin proteins will be necessary to construct a more detailed model.

Our findings may also provide insight into the reason why missense mutations of the SMN protein in SMA patients are rarely observed in the amino-terminal half and frequently in the exon 6-encoded region. Because SMN self-association in the carboxyl-terminal, exon 6-encoded region is very stable, and there is no evidence that other components associate through this region, mutations in this region may directly affect self-association and oligomer formation, resulting in SMA. Actually, Paushkin *et al.* shows that the sedimentation of the oligomerized SMN complex shifted to smaller size objects by overexpression of the SMN proteins with missense mutation at the exon 6 (21). On the other hand, Gemin2 self-association may well work in concert with SMN self-association in the exon 2a-encoded region, which would then result in stable amino-terminal SMN self-association and stable SMN oligomer formation. Because amino-terminal SMN self-association is not stable by itself, many of the missense mutations in this region may not critically affect self-association and oligomerization. So far, no SMA patients with mutations in the *Gemin2* gene have been identified. It may be that *Gemin2* is critical for the formation of SMN complex; mutations may well be lethal in an early stage. In this aspect, it is interesting that Gemin2 is the most conserved SMN complex components (human-mouse, 94%), whereas the mean conservation rate for other SMN components is 80%.

We successfully showed that amino-terminal self-association and Gemin2 binding were decreased in SMN(D44V), but not in SMN(D30N), using both an *in vivo* mammalian two-hybrid assay and an *in vitro* pull-down assay. Conversely, Sun *et al.* failed to identify significant biochemical features in SMN(D44V) with GST pull-down assays, because in their experiments the mutation did not affect SMN self-association or SMN-Gemin2 interaction (11). A possible explanation for the different results obtained by the present study and the work reported by Sun *et al.* is that Sun *et al.* used full-length SMN protein in the binding assay; the amino-terminal mutations are unlikely to affect the stability of SMN self-association that is mediated by the carboxyl-terminal, exon 6-encoded region. Subsequently the decrease in self-association in SMN(D44V) was less clearly detected when using full-length protein than when using only the exons 1 to 5 region as used in our study (Fig. 6 and supplemental Fig. S3). It is also a possibility that the use of GST fusion proteins

as pull-down drivers in the binding assay may affect the binding ability at the amino-terminal region of the target protein. In this regard, our *in vitro* pull-down assays are less likely to affect the properties of the tag, because we use *in vitro* biotinylated proteins instead of tagged proteins as the pull-down drivers, avoiding modification of the specified region in the driver proteins.

Recently, Shpargel and Matera (26) reported that the severity of SMA is roughly correlated with snRNP assembly activity in SMA-derived missense SMN mutations, because in five of six SMA type I alleles, the severe alleles, including SMN(Y272C), showed a decreased in snRNP assembly assay, whereas in the two SMA type III alleles, mild alleles, including SMN(D30N), snRNP assembly functioned on a level comparable with the wild-type constructs. Our results for the SMN(D30N) and SMN(Y272C) were consistent with this report (26). However, we were also able to show that snRNP assembly activity was lowered in the SMA type III allele of SMN(D44V) as well as in the type I allele of SMN(Y272C). This is the first report of decreased snRNP assembly activity associated with SMA type III alleles and amino-terminal missense mutations. Together with the SMA type I allele case of SMN(A111G) whose assembly activity functioned on a level comparable with the wild-type constructs (26), our results strongly indicate that the severity of SMA is not simply determined by the snRNP assembly activity of each allele in isolation. Other SMN properties, such as nuclear import, localization, cap hypermethylation and binding activity with other proteins (38–42), should also be considered for a more complete understanding of the relationship between missense SMN mutations and disease severity. In the same way, it is necessary to consider whether the copy numbers of *SMN2* correlates with SMA severity (43). In this regard, the mutant alleles, SMN(D44V) and SMN(A111G), are expected to be good probes for the detection of other factors that are also responsible for SMA severity.

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