Gemins modulate the expression and activity of the SMN complex

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Reduction in the expression of the survival of motor neurons (SMN) protein results in spinal muscular atrophy (SMA), a common motor neuron degenerative disease. SMN is part of a large macromolecular complex (the SMN complex) that includes at least six additional proteins called Gemins (Gemin2–7). The SMN complex is expressed in all cells and is present throughout the cytoplasm and in the nucleus where it is concentrated in Gems. The SMN complex plays an essential role in the production of spliceosomal small nuclear ribonucleoproteins (snRNPs) and likely other RNPs. To study the roles of the individual proteins, we systematically reduced the expression of SMN and each of the Gemins (2–6) by RNA interference. We show that the reduction of SMN leads to a decrease in snRNP assembly, the disappearance of Gems, and to a drastic reduction in the amounts of several Gemins. Moreover, reduction of Gemin2 or Gemin6 strongly decreases the activity of the SMN complex. These findings demonstrate that other components of the SMN complex, in addition to SMN, are critical for the activity of the complex and suggest that Gemin2 and Gemin6 are potentially important modifiers of SMA as well as potential disease genes for non-SMN motor neuron diseases.

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease, which is characterized by the degeneration of motor neurons in the spinal cord (1,2). The survival of motor neurons (SMN) gene is the causative gene for this disease and is present as two inverted copies on human chromosome 5, telomeric SMN (SMN1) and centromeric SMN (SMN2) (3–5). Over 98% of SMA patients carry deletions or loss-of-function mutations in the SMN1 gene, and reduced levels of SMN protein correlate with the phenotypic severity of SMA (3). SMN2 expresses mostly as a functionally defective spliced isoform of the protein that lacks exon 7 and therefore does not protect from the disease (6). SMN is an essential gene in metazoans, including human, mouse, chicken, Caenorhabditis elegans, Drosophila and Schizosaccharomyces pombe (3,7–13).

The SMN protein oligomerizes and forms a stable multi-protein complex that consists of SMN, Gemin2 (formerly SIP1), Gemin3 (a DEAD-box RNA helicase), Gemin4, Gemin5 (a WD-repeat protein), Gemin6 and Gemin7. SMN binds directly to Gemin2, 3, 5 and 7, whereas Gemin4 and 6 require Gemin3 and 7, respectively, for interaction with SMN (14–19). In addition to these integral components of the SMN complex, numerous other proteins have been reported to bind to SMN. These proteins, referred to as SMN complex substrates, include Sm and Sm-like (LSm) proteins (14,20–22), RNA helicase A (23), fibrillarin and GAR1 (24,25), the RNP proteins hnRNP U (26), hnRNP Q (27,28) and hnRNP R (28), as well as p80-coilin (29), the protein marker for Cajal (coiled) bodies. A common feature among these substrates is the presence of a domain rich in arginine and glycine residues that is essential for the interaction with SMN. SMN, but not SMN mutants present in SMA patients, binds directly to these RG-rich substrates, indicating a strong correlation between the SMA phenotype and the defective interaction of SMN with its substrates (20–25,27–29). Strikingly, most of these substrates are associated with various types of RNP complexes, which suggests that SMN has a role in diverse aspects of RNA metabolism. Indeed, studies in Xenopus laevis oocytes and mammalian cells have shown that SMN is involved in

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several aspects of cellular RNA metabolism including snRNP assembly, pre-mRNA splicing, transcription and possibly the biogenesis of small nucleolar RNP (snoRNPs) (21,23–25,30–35). In addition, several other SMN-interacting proteins have been described that neither contain RG-rich motifs nor are they known to interact with RNPs such as ZPR1, profilin, p53 and the FUSE binding protein (36–39).

The SMN complex is present in the cytoplasm as well as the nucleus, where it is enriched in Gems, nuclear structures that resemble Cajal bodies in number and size (26). In adult tissues and most cell lines, Gems and Cajal bodies colocalize, whereas in fetal tissues and certain types of cell lines, such as HeLa PV cells, Gems and Cajal bodies are separate and distinct nuclear structures (26,40–42). Clearly, Gems and Cajal bodies are functionally related and often associated (31,40,43). Although Cajal bodies are implicated in snRNP and snoRNP biogenesis, trafficking, processing and histone mRNA 3′ processing, their precise function is still unknown (44–47).

To understand the function of the SMN complex, it is necessary to know the contribution of each of its components to the activity of the complex and to the expression of its other constituents. We have previously shown, using experiments in the chicken pre-B cell line DT40, that the level of Gemin2 protein decreases significantly upon the depletion of SMN (8). However, using this cell line, it was not possible to determine whether other Gemins are also affected by the reduction of SMN due to the lack of antibodies that recognize the chicken orthologs of these proteins. Here, we used RNA interference (RNAi) to reduce the amounts of six of the seven components of the SMN complex, namely SMN and Gemin2–6, in an effort to further characterize the function and inter-dependence of these proteins in HeLa cells. To study the role of each of these components on the activity of the SMN complex, we used a sensitive and quantitative assay for snRNP assembly (48). The findings reveal that the Gemins profoundly modulate the activity of the SMN complex.

### RESULTS

#### Efficient reduction of SMN and Gemins by RNAi

siRNAs designed to target the mRNAs of SMN and Gemin2–6 were transfected into HeLa cells. To increase the likelihood of finding efficient siRNAs for each target and to ensure that the phenotype we observe upon reduction of each of them is specific, a large number of siRNAs were tested (at least five per target). Two days after transient transfection, whole cell lysates were prepared and specific proteins were analyzed by quantitative western blotting. As shown in Figures 1 and 2, the protein levels of SMN and Gemin2–6 were significantly reduced after transfection of the respective siRNAs. However, the extent of protein reduction varied somewhat depending on the target (Figs 1 and 2). The levels of SMN, Gemin2, 3, 5 and 6 were reduced by 70–85%. RNAi of Gemin4 was less efficient and, although several different siRNAs were tested, Gemin4 could only be reduced by ~57%. Indirect immunofluorescence microscopy further confirmed the reduction of the targeted proteins and demonstrated that at least 90% of cells were transfected and affected by the siRNAs (Fig. 3) (data not shown).

#### SMN is essential for the integrity of Gems and stabilizes the Gemins

Next, we asked whether the expression levels of the SMN complex components are inter-dependent. We have previously reported that the co-expression of SMN and Gemin2, but not of either protein alone, was sufficient to form a particle of a size similar to that of the native SMN particle (49). Furthermore, we showed that Gemin2 levels were greatly diminished following depletion of SMN in chicken DT40 cells (8). Both studies suggested that SMN and Gemin2 are inter-dependent and function together as components of the SMN complex. As shown in Figures 1–3, following transient RNAi of SMN, the protein levels of most Gemins were significantly reduced compared with their levels in control cells (Fig. 2A). Moreover, the Gem staining patterns of SMN and Gemin2, 3 and 5 were almost entirely abolished (Fig. 3). Owing to the lack of suitable anti-Gemin4 or anti-Gemin6 antibodies, the sub-cellular distribution of these two Gemins following SMN RNAi could not be assessed.

In contrast to the pronounced effects of SMN reduction on the nuclear Gems and on the levels of the Gemins, RNAi of Gemin2 had more moderate effects. The levels of SMN as well as Gemin4, 5 and 6 were not significantly affected by Gemin2 RNAi, and Gemin3 levels were only moderately reduced (Fig. 2B). Furthermore, reduction of Gemin2 did not alter the distribution of SMN complex proteins in Gems (data not shown). These observations imply that SMN and Gemin2 have distinct roles within the SMN particle, with SMN being essential for the formation of Gems and critical to the expression of Gemins.

RNAi of Gemin5 and Gemin6 had no significant effect on the appearance of Gems and the stability of the other SMN complex components examined here (Figs 1–3) (data not shown). In contrast, RNAi of Gemin3 reduced the levels of Gemin4 by almost 40% (Fig. 2C) and Gemin4 RNAi, in
turn, reduced Gemin3 levels by >50% (Fig. 2D). It is thus likely that these two Gemins, which interact directly (16), stabilize one another.

Reduction of SMN, Gemin2 and Gemin6 leads to a reduction in the capacity for snRNP assembly

Using antibody-depleted cytoplasmic extracts, recent studies demonstrated that SMN, and possibly Gemin2 and 4, play critical roles in the assembly of snRNPs (30,35,50). To determine whether other components of the SMN complex also affect the activity of the complex in snRNP assembly, we carried out in vitro assembly reactions following RNAi-mediated knockdown of SMN and Gemin2–6 in HeLa cells. After transfection of siRNAs against SMN or Gemin2–6 mRNAs, cytoplasmic HeLa extracts were prepared and used for snRNP assembly on biotin-labeled U1 snRNA (48). Assembly activity was measured following Y12 immunoprecipitations of the assembled U1 snRNPs (Fig. 4). Western blotting of the RNAi-treated cytoplasmic extracts used in the assembly reactions confirmed that all siRNAs had indeed reduced the levels of their respective targets as anticipated (data not shown). As shown in Figure 4, the efficiency of snRNP assembly was decreased between 50 and 60% upon reduction of SMN, Gemin2 and Gemin6. These findings confirm the requirement of SMN and Gemin2 for efficient snRNP assembly and identify Gemin6 as an additional key component in this assembly process. Indeed, a similar reduction in the capacity for snRNP assembly is also observed in cells from SMA type I patients (48). In contrast, RNAi of Gemin3, 4 and 5 did not affect the efficiency of snRNP assembly. It is possible that the reduction of Gemin3, 4 and 5 that could be attained by RNAi in this system was too transient or, especially in the case of Gemin4, not sufficient to reflect their involvement in snRNP assembly in this in vitro assay.

To further examine the inhibitory effects of Gemin6 reduction on snRNP assembly, we tested whether the inhibitory effects of SMN and Gemin6 reduction on snRNP assembly were additive. To do so, we treated HeLa cells simultaneously with siRNAs to both SMN and Gemin6 and measured the snRNP assembly activity in extracts prepared from these cells. As expected, the activity measures showed a strong reduction, but this was not lower than that observed in cells in which either SMN or Gemin6 were each reduced alone (Fig. 4). The amount of SMN and Gemin6 proteins in the double-knockdown cells, monitored by quantitative immunoblots, showed no further reduction in either protein compared with the reduction observed for each protein when it is targeted directly alone (data not shown). It is therefore likely that the extent of reduction in activity, which is seen when each of these proteins is strongly decreased, is close to the maximal reduction achievable in these cells under the conditions of the experiments, or that the two proteins are required for (a) similar step(s) in the same pathway.

DISCUSSION

Except for SMN itself, little has been so far known about the contribution of the various components of the SMN complex to its activity and about the influence that each of them may have on the expression and on the cellular localization of the other proteins. To better understand the cellular roles
of the proteins of the SMN complex, we used RNAi to systematically reduce the amount of individual components, namely SMN and Gemin2–6, \textit{in vivo}. Gemin7, which is also part of the SMN complex, was not included in this study because we currently do not have a suitable antibody for this protein with which to assess its expression levels. In each case, we measured the activity of the SMN complex, the amount of each of the proteins and their cellular localization. These experiments were facilitated by a new quantitative assay for the activity of the SMN complex in snRNP assembly (48) and by the use of quantitative immunoblots. As expected, reduction of SMN has severe consequences on the activity of the complex. Consistent with its central role in the SMN complex as suggested by its binding to the Gemin and substrates (e.g. Sm and LSm proteins, fibrililarin), reduction of SMN causes reduction in the levels of several other Gemin. Because several of the Gemin are also found outside of the SMN complex (17,51), it is possible that some of the consequences of reduction in SMN that occur in SMA patients are the result of a deficiency in the other functions, outside of the SMN complex, that these proteins perform in cells. For example, Gemin3 and 4 are also components of microRNP complexes (miRNPs) (51), and therefore, if these proteins are reduced in tissues when SMN is reduced, it is likely that the activity of miRNPs will also be affected. In addition, SMN appears to serve as a key building block of Gems as these nuclear structures disappear when SMN is drastically reduced. In contrast to Gems, Cajal bodies, identified by p80-coilin staining, were unaltered when SMN levels were decreased (data not shown), supporting the conclusion that Gems and Cajal bodies are distinct structures (26,52).

A particularly interesting observation is that the reduction of either Gemin2 or Gemin6 decreases the activity of \textit{in vitro} snRNP assembly. Ten micrograms of cytoplasmic extracts of HeLa cells transfected with the indicated siRNAs were applied in snRNP assembly reactions using biotin-labeled U1 snRNA as RNA template. The assembly activities were measured following Y12 immunoprecipitations of the assembled U1 snRNPs and quantitation of assembled snRNPs using horseradish peroxidase-conjugated NeutrAvidin\textsuperscript{TM}. Columns represent relative snRNP assembly activities following RNAi of SMN and Gemin2–6 normalized to the assembly activity measured upon control RNAi. Error bars represent the standard deviations of three independent reactions.

![Figure 3](image3.png)

\textbf{Figure 3.} Sub-cellular distribution of SMN and Gemin2, 3 and 5 following RNAi-mediated reduction of SMN. Immuno-staining of SMN (A, E), Gemin2 (B, F), Gemin3 (C, G), and Gemin5 (D, H) in HeLa cells following control RNAi (A–D) and RNAi of SMN (E–H).

![Figure 4](image4.png)

\textbf{Figure 4.} Reduced levels of SMN, Gemin2 and Gemin6 decrease the activity of \textit{in vitro} snRNP assembly. Ten micrograms of cytoplasmic extracts of HeLa cells transfected with the indicated siRNAs were applied in snRNP assembly reactions using biotin-labeled U1 snRNA as RNA template. The assembly activities were measured following Y12 immunoprecipitations of the assembled U1 snRNPs and quantitation of assembled snRNPs using horseradish peroxidase-conjugated NeutrAvidin\textsuperscript{TM}. Columns represent relative snRNP assembly activities following RNAi of SMN and Gemin2–6 normalized to the assembly activity measured upon control RNAi. Error bars represent the standard deviations of three independent reactions.
the SMA phenotype. In the case of Gemin2, this is in agreement with the exacerbations in motor neuron degeneration observed in double heterozygous Smn+/−/Gemin2+/− mice versus Smn+/−/Gemin2+/+ mice (53). Although a previous screen for Gemin2 mutations in approximately 50 SMA patients has not revealed any mutations or reduced mRNA levels for Gemin2 (54), it will be of interest to determine Gemin2 and Gemin6 protein levels and possible mutations in a large cohort of patients with motor neuron disease, including SMA. The finding that Gemin6 plays an important role in snRNP assembly is particularly interesting in light of the recent determination of the crystal structure of Gemin6 and Gemin7 (55). The structure revealed that Gemin6 and 7 each contains a domain that highly resembles the conserved domain, the Sm domain, common to all the Sm and LSm proteins, despite the lack of a canonical Sm sequence motif in these Gemins (55). It is thus conceivable that Gemin6 and Gemin7, which interact with Sm proteins, play a role in organizing Sm proteins for assembly onto snRNAs, possibly by serving as an Sm-like dimer surrogate around which individual Sm proteins and/or sub-complexes are arranged for binding to the Sm site. Another possibility that we consider is that Gemin6 and 7, rather than interacting with Sm proteins, associate with each other to form a ring structure, which serves as a scaffold that organizes the other components of the SMN complex so that they can facilitate the formation of an Sm core. Further studies will be required to understand the specific mechanism of snRNP assembly that the SMN complex mediates, and the specific roles of Gemins in this process. Furthermore, in light of our findings here, the possibility that Gemin2 and Gemin6, as well as the other Gemins, are potential genetic modifiers of SMA deserves further consideration.

MATERIALS AND METHODS

Cell culture

HeLa PV cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen).

RNA interference

Following the guidelines by Elbashir et al. (56), 21-nt RNA duplexes (siRNAs) were designed to target SMN or Gemin2–6 mRNAs. For each target transcript, at least five siRNAs were designed and tested and, in general, found to behave similarly in all subsequent analyses. The following siRNA sequences yielded the most efficient protein knockdowns of each target: GAAGAAUACUGCAGCUUCC for SMN; GCAGCUCAGUCGUAAG for Gemin2; GCCUUGA GUGUCAUGUC for Gemin3; ACUCCCGAGAGAC CAUU for Gemin4; GCAUAGUGGUGUAAUUUGA for Gemin5 and AUCUACAGCCACGCCUUCG for Gemin6. In addition, an siRNA initially designed to target Y14, a component of the exon junction complex (57), which failed to produce any protein reduction within 44 h, was used as a control (CCCCGACCAACGCCUCUG). All siRNAs were chemically synthesized and purified by Dharmacon Research. Transfections of siRNAs into HeLa PV cells were performed using Oligofectamine™ (Invitrogen) as specified by the manufacturer. Transfected cells were analyzed 40–44 h post-transfection.

Quantitative western blotting and indirect immunofluorescence microscopy

For western blotting, fresh cell pellets were resuspended in PBS and briefly sonicated on ice. The protein concentrations of the whole cell extracts were determined using the Bio-Rad protein assay (Bio-Rad Laboratories). Proteins were resolved on 4–12% Bis–Tris NuPage pre-cast gels and transferred onto nitrocellulose membranes using a mini-gel transfer apparatus (Invitrogen). Subsequently, quantitative western analysis was performed using the Odyssey System according to the instructions of the manufacturer (Li-Cor Biosciences). Bound antibodies were detected using IRDye800CW-conjugated goat anti-mouse IgG (Rockland). The intensity of each band was measured on the Odyssey Infrared Imaging System (Li-Cor). The signals for SMN and Gemin2–6 were normalized to the protein levels of the arginine methyltransferase JBP1 that served as a loading control. Indirect immunofluorescence experiments using HeLa PV cells were performed as previously described (19).

Antibodies

The following mouse monoclonal antibodies were used: 2B1 (anti-SMN), 2E17 (anti-Gemin2), 12H12 (anti-Gemin3), 17D10 (anti-Gemin4), 10G11 (anti-Gemin5), 6G8 (anti-JBP1) and Y12 (anti-Sm). Gemin6 was detected using the affinity-purified rabbit polyclonal antibody rG6 (19). Antibodies 17D10 and rG6 are only suited for western blotting but not for immunofluorescence.

In vitro snRNP assembly

Biotinylated U1 snRNA was in vitro transcribed from 1 μg of linearized template DNA (34) using 5 mM biotin-UTP (Roche), 2.5 mM UTP, 7.5 mM ATP, 7.5 mM CTP, 1.5 mM GTP, 5.25 mM m7G(5′)ppp(5′)G cap Analog and 2 μL T7 RNA polymerase (Ambion) at 37°C for 6 h. The labeled RNAs were purified by electrophoresis on 6% acrylamide/7 M urea gels, precipitated with ethanol, resuspended in nuclease free water and quantitated by spectroscopy. Cytoplasmic extracts were prepared 40–44 h after siRNA transfection into HeLa PV cells, as previously described (35). These cytoplasmic extracts were used to assemble on the biotin-labeled U1 snRNA using standard reconstitution conditions at 30°C for 1 h in a 96-well plate format (34,35). Subsequently, Y12 immunoprecipitations were carried out following Y12 antibody immobilization on magnetizable Dynabeads® Protein A. Assembled U1 snRNPs were quantitated using horseradish peroxidase conjugated NeutrAvidin™ (48).

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