The activity of the spinal muscular atrophy protein is regulated during development and cellular differentiation

Francesca Gabanella¹, Claudia Carissimi¹, Alessandro Usiello²,³ and Livio Pellizzoni¹,*

¹Dulbecco Telethon Institute, Institute of Cell Biology (CNR), I-00016 Monterotondo Scalo, Rome, Italy, ²Department of Neuroscience, Karolinska Institutet, S-17177 Stockholm, Sweden and ³CEINGE Biotecnologie Avanzate, I-80145 Napoli, Italy

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Spinal muscular atrophy (SMA) is a lethal neuromuscular disease caused by reduced levels of expression of the survival motor neuron (SMN) protein. SMN is part of a macromolecular complex essential for the assembly of the small nuclear ribonucleoproteins (snRNPs) that carry out pre-mRNA splicing. Although the SMN complex has the potential to control the pathway of snRNP biogenesis, it is not known whether SMN function in snRNP assembly is regulated. Here, we analyze SMN interactions and function in mouse tissues and show that, when normalized per cell number, similar levels of the SMN complex are expressed throughout the ontogenesis of the central nervous system (CNS). Strikingly, however, SMN function in snRNP assembly in extracts does not correlate with its expression levels and it varies greatly both among tissues and during development. The highest levels of SMN activity are found during the embryonic and early postnatal development of the CNS and are followed by a sharp decrease to a basal level, which is then maintained throughout life. This downregulation takes place in the spinal cord earlier than in the brain and coincides with the onset of myelination. Using model cell systems and pulse-labeling experiments, we further show that SMN activity and snRNP synthesis are strongly downregulated upon neuronal as well as myogenic differentiation, and linked to the rate of global transcription of postmitotic neurons and myotubes. These results demonstrate that the SMN complex activity in snRNP assembly is regulated and point to a differential requirement for SMN function during development and cellular differentiation.

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease characterized by the degeneration of motor neurons and muscular atrophy (1,2). SMA is classified into three types according to age of onset and severity of the disease. The most severe and frequent form of SMA, SMA type 1, is the leading genetic cause of infant mortality. Homozygous deletions or mutations in the survival motor neuron (SMN1) gene, the SMA-determining gene, have been detected in >98% of SMA patients (3). The SMN gene is duplicated on human chromosome 5 and all SMA patients retain at least one copy of the nearly identical SMN2 gene. A single nucleotide difference between SMN1 and SMN2 genes impairs the inclusion of the sequences encoded by exon7 in most transcripts from SMN2 (4). As a consequence, the SMN2 gene produces reduced amounts of full-length SMN that cannot fully compensate for the absence of SMN1, leading to specific degeneration of motor neurons in the spinal cord (5,6).

The SMN protein is part of a macromolecular complex whose core components (Gemin2–7) are tightly associated through a network of protein–protein interactions (7). These proteins are called Geminins because of their co-localization with SMN in nuclear gems (8). The SMN complex plays a role in the metabolism of snRNPs, which are the essential ribonucleoproteins that carry out the removal of introns during pre-mRNA splicing. Major spliceosomal snRNPs are composed of one U snRNA molecule (U1, U2, U4/U6 or U5), a common core of seven Sm proteins and additional proteins specific for each snRNP (9). The pathway of snRNP biogenesis in higher eukaryotes has a cytoplasmic as well as...
a nuclear phase and requires the functions of two multiprotein complexes, the PRMT5 and the SMN complexes (7,10). Precursor snRNAs are transcribed in the nucleus and exported to the cytoplasm, where they assemble with Sm proteins. Before newly assembled snRNP are imported in the nucleus, snRNAs are further modified by hypermethylation of the 5’ cap structure and by processing of the 3’ end. In the cytoplasm, Sm proteins associate first with the PRMT5 complex, which symmetrically dimethylates specific arginine residues to increase their affinity for SMN, and then with the SMN complex (11–14). The SMN complex bound to the Sm proteins interacts with newly exported snRNAs and mediates their assembly into snRNPs (15–20). The physiological role of the SMN complex is to ensure the efficient and accurate assembly of Sm proteins with snRNAs, preventing their non-specific association with other RNAs (18).

Consistent with the essential function in snRNP assembly, SMN is ubiquitously expressed and its complete absence is incompatible with life from yeast to humans (21). Mice have only one copy of the SMN gene and knock-out of murine SMN causes early embryonic lethality (22). A number of different strategies were employed to circumvent lethality and create animal models of SMA (23). Characterization of these mouse models showed that reduced levels of SMN cause early postnatal loss of motor neurons in the spinal cord and muscle degeneration, leading to motor impairment and death shortly after birth (24–26). Therefore, reduced levels of SMN cause SMA and the severity of the phenotype inversely correlates with the expression levels of SMN in both humans and mouse models of the disease (1,2,23).

Despite the progress in the characterization of the SMN complex function in snRNP assembly and the generation of animal models of SMA, it is not known whether SMN activity is regulated in vivo, and the molecular mechanism responsible for the specific degeneration of motor neurons in the spinal cord of SMA patients remains elusive. A link between a defect of snRNP biogenesis and SMA is suggested by the impaired interaction with Sm proteins of SMN mutants of SMA patients and by the reduced snRNP assembly activity of extracts from SMA fibroblasts (27–29). However, it is not known whether or how deficiencies in a function required by all cells may lead to the preferential degeneration of motor neurons in the spinal cord. One possibility is that motor neurons have a greater requirement for SMN function in snRNP assembly when compared with other cell types. Alternatively, SMN may have an additional as-of-yet-unknown function that is specific to motor neurons and whose impairment causes SMA. It has been proposed that such a function may involve a role for SMN in the transport of specific messenger RNPs along axons (30,31). To begin addressing these issues, we carried out the biochemical characterization of SMN in mouse tissues focusing on the central nervous system (CNS). We show that SMN is associated with Gemin and Sm proteins in large macromolecular complexes and that approximately constant levels of the SMN complex are expressed in cells throughout CNS ontogenesis. Surprisingly, however, SMN function in snRNP assembly does not correlate with its expression levels. SMN activity is high during embryonic and early postnatal development of the CNS and decreases dramatically at the onset of myelination, reaching a low steady level that is maintained throughout life. Importantly, we show that tissues regulate SMN activity rather than its association with Sm proteins or snRNAs and that model systems of cellular differentiation recapitulate the tissue-specific temporal regulation of SMN function observed during mouse development. These findings reveal the unanticipated physiological regulation of the SMN complex activity in snRNP assembly during development and cellular differentiation.

RESULTS

SMN is associated with Gemin and Sm proteins throughout CNS ontogenesis

We sought to investigate the expression of the SMN complex during ontogenesis of the mouse CNS. To do so, we analyzed equal amounts of total proteins from spinal cords and brains of mice at different ages by western blot. Figure 1A shows that the components of the SMN complex are expressed throughout CNS ontogenesis. The levels of SMN and Gemin proteins as well as of SmB and several hnRNP proteins appear to decrease with age when compared with tubulin, which is used as a normalization control for equal content of total proteins (Fig. 1A). Previous studies interpreted the decrease of SMN and Gemin2 as a developmental downregulation of their expression levels in the spinal cord (32,33). However, a similar reduction is also observed for histone H1 and probably reflects the extensive increase in mass, due to the specific accumulation of proteins such as myelin basic proteins, but not in cell number that accompanies spinal cord and brain growth. Indeed, western blot analysis of total protein extracts from spinal cord and brain at different ages normalized per cell number, as indicated by equal amount of histone H1, shows similar levels of SMN throughout CNS ontogenesis (Fig. 1B). Therefore, we conclude that approximately constant levels of the SMN complex are expressed in cells during CNS ontogenesis.

Next, we analyzed the association of SMN with Gemin and Sm proteins in the CNS. To do so, spinal cord and brain extracts of mice at different ages were analyzed by immunoprecipitation experiments with antibodies against SMN complex components and Sm proteins. Western blot analysis shows that SMN is associated with Gemin and Sm proteins throughout CNS ontogenesis (Fig. 1C). The association is specific because SMN was not found in control immunoprecipitations with mouse immunoglobulins. Since Gemin3 and Gemin7 mediate the respective interactions of Gemin4 and Gemin6 with SMN (34,35), it is likely that these proteins are also present in these SMN complexes. Furthermore, sucrose gradient centrifugation experiments of spinal cord and brain extracts of mice at different ages show that most of SMN is part of large heterodisperse complexes ranging in size from 30S to 70S from embryogenesis to adulthood in the CNS (Fig. 1D). Noticeably, SMN complexes in spinal cord extracts from embryos at E18 display a greater sedimentation value than those in the corresponding brain extracts. Altogether these results indicate that SMN is associated with Gemin and Sm proteins in large macromolecular complexes throughout CNS ontogenesis.
The SMN complex mediates snRNP assembly in the spinal cord

To analyze SMN activity in mouse tissues, we used the in vitro snRNP assembly assay previously established with HeLa cells (18). In vitro transcribed radioactive U1 and U1ΔSm snRNAs were incubated with spinal cord extracts both in the absence and in the presence of either ATP or its non-hydrolyzable analog AMP-PNP. U1ΔSm was used as a control for specificity.
because it bears a mutated Sm site and cannot assemble the Sm core. SnRNP assembly was analyzed by both gel shift and immunoprecipitation experiments. An Sm site-specific RNP complex forms on U1, but not on U1ΔSm, only in the presence of extract and ATP hydrolysis (Supplementary Material, Fig. S1A). This complex is supershifted by addition of anti-Sm antibodies to the snRNP assembly reaction, indicating the presence of the Sm core (data not shown). Formation of the Sm core was further demonstrated by the Sm site- and ATP hydrolysis-dependent immunoprecipitation of U1 with anti-Sm antibodies from the same assembly reactions (Supplementary Material, Fig. S1A). An additional RNP complex containing the U1 snRNP-specific U1A protein forms on both U1 and U1ΔSm regardless of ATP.

To test whether SMN is necessary for the assembly of snRNPs in mouse tissues as is the case in HeLa cells (17,18), we performed depletion experiments of spinal cord extracts with anti-SMN antibodies or mouse immunoglobulins as a control. We carried out western blot analysis and snRNP assembly reactions, using three increasing concentrations of SMN- and mock-depleted extract (Supplementary Material, Fig. S1B). Depletion of SMN specifically abolishes Sm core formation but not U1A interaction with U1. Since SMN complexes purified from HeLa cells are also sufficient to mediate snRNP assembly (17,18), we analyzed by western blot and snRNP assembly three increasing concentrations of SMN complexes isolated from spinal cord extracts by immunoprecipitation with anti-SMN antibodies or mock immunoprecipitates as a control. Figure S1C shows that SMN complexes immobilized on beads are competent to carry out the Sm core formation on U1 in the absence of extract. Similar to the situation in spinal cord extracts, snRNP assembly with purified SMN complexes requires ATP hydrolysis and the Sm site (data not shown). These results demonstrate that the SMN complex mediates the ATP-dependent assembly of snRNPs in spinal cord extracts and these assays can be used to analyze SMN function in mouse tissues.

The highest levels of SMN activity in snRNP assembly are found in the CNS of embryos

We then sought to compare the content and the specific activity of SMN among different tissues of mouse embryos. First, we assessed the expression levels of SMN in spinal cord, brain, kidney, skeletal muscle and heart of embryos at E16 by western blot. We found no difference in the levels of SMN in total protein extracts from these tissues that were normalized for equal number of cells as indicated by similar amounts of histone H1 (Fig. 2A). Furthermore, the levels of PCNA expression indicate an overall lower rate of cell proliferation in the CNS when compared with the other tissues of embryos at E16 (Fig. 2A). We then analyzed SMN function in snRNP assembly using two increasing concentrations of extracts from the same tissues and found that SMN activity is up to 5-fold greater in spinal cord and brain extracts despite similar amounts of SMN found in all tissues (Fig. 2B; data not shown). These results demonstrate that SMN activity does not correlate with its expression levels or the overall rate of cell proliferation in embryonic tissues and indicate that a larger pool of SMN complexes competent for snRNP assembly is present in extracts from the CNS of embryos.

SMN function in snRNP assembly is regulated during CNS development

Next, we investigated SMN activity in snRNP assembly throughout CNS ontogenesis. To do so, we carried out snRNP assembly reactions using three increasing concentrations of spinal cord and brain extracts from mice of different ages normalized for equal content of SMN. Figure 3 shows that spinal cord extracts from embryos at E18, 3-day- and 1-week-old mice efficiently assemble snRNPs in a
concentration-dependent manner. Strikingly, when compared with embryos at E18, SMN function in snRNP assembly decreases ~10-fold at 2 weeks of age, reaching a low basal level that is then maintained throughout life (Fig. 3A; data not shown). A similar decrease in snRNP assembly was also observed with other snRNAs (data not shown). Importantly, immunoprecipitation experiments from snRNP assembly reactions show that similar levels of the U1A protein associate with U1 snRNA at all stages of spinal cord ontogenesis (Supplementary Material, Fig. S2), indicating that a different type of RNA–protein interaction remains constant and the observed decrease in Sm core formation is specific. In contrast to the spinal cord and despite a progressive decrease over time, SMN function in snRNP assembly in brain extracts remains relatively high until 2 weeks of age, attaining the low steady levels at 3 weeks (Fig. 3A). A more detailed time course analysis indicates that SMN activity reaches the basal level at postnatal day 10 in the spinal cord and at day 21 in the brain (data not shown). Altogether these results demonstrate that SMN function in snRNP assembly does not correlate with its expression levels in spinal cord and brain extracts and is strongly downregulated during the early postnatal phases of CNS development.

Importantly, there is a marked difference in the timing of the downregulation of SMN function between spinal cord and brain extracts. Western blot analysis of PCNA expression indicates that cell proliferation declines prior to the time of the sharp decrease of SMN activity in the CNS (Fig. 3B), so that cell proliferation in the time window surrounding the downregulation of SMN function in snRNP assembly is approximately constant. Therefore, there is no tight correlation between the postnatal rate of cell division and the levels of SMN activity in extracts from the CNS. The postnatal phase of CNS development is characterized by the progressive onset of myelination which displays a distinct timing between spinal cord and brain (36–38). As shown by the

![Figure 3. SMN function in snRNP assembly is regulated during CNS development. (A) Extracts were prepared from spinal cords and brains isolated from mice at the indicated age. SnRNP assembly reactions with radioactive U1 snRNA were carried out using three increasing concentrations (10, 20 and 30 μg of proteins) of spinal cord and brain extracts and analyzed by electrophoresis on native gels. The positions of U1 RNP complexes containing the Sm core or the U1A protein are indicated. (B) The same amounts of extracts in (A) were analyzed by western blot with anti-SMN, anti-PCNA and anti-myelin basic proteins antibodies as indicated.](https://academic.oup.com/hmg/article-abstract/14/23/3629/559461)
accumulation of myelin basic proteins (Fig. 3B), the marked temporal difference in the downregulation of SMN function in snRNP assembly between spinal cord and brain correlates with the lag in the onset of their myelination.

**Tissue-specific temporal regulation of SMN function in snRNP assembly**

We next investigated whether the regulation of SMN function in snRNP assembly is a general phenomenon by extending our analysis to additional mouse tissues. We found that in extracts from tissues such as kidney and skeletal muscle that complete their morphogenesis during embryogenesis the low SMN activity found at E16 further decreases to a basal level at 1 day after birth (Fig. 4). We also analyzed snRNP assembly in testis, which is a highly proliferative tissue characterized by a late postnatal development (39). We carried out snRNP assembly experiments with extracts from testis of 1-, 2-, 3-, 15- and 30-week-old mice normalized for SMN content (Fig. 4B). SMN activity in snRNP assembly increases up to 3-fold during the postnatal phase of testis maturation from 1 week to 3 weeks of age and decreases at later time points (Fig. 4A; data not shown). However, sustained levels of SMN activity in extracts from testis are maintained throughout ontogenesis and are consistent with the continuous cycle of cell division and differentiation associated with spermatogenesis. The level of SMN activity in testis extracts at 3 weeks of age is approximately similar to that in CNS extracts of embryos at E16 (data not shown). Altogether these results reveal a distinctive tissue-specific temporal regulation of the SMN complex function in snRNP assembly during mouse development.

**The SMN complex activity but not its association with Sm proteins or U1 snRNA is regulated in the CNS**

The SMN complex interacts directly and independently with both Sm proteins and snRNAs (7,10), and these interactions may represent potential targets for regulation of snRNP assembly. To gain insights into the mechanisms responsible for the downregulation of SMN function in snRNP assembly, we analyzed these SMN interactions in spinal cord and brain extracts during CNS ontogenesis. First, we analyzed the association of SMN with Sm proteins by immunoprecipitation with anti-Sm antibodies from spinal cord and brain extracts of embryos at E18 and 3-week-old mice, the two time points which display the greatest difference in snRNP assembly activity. Western blot analysis of the immunoprecipitates shows that at both ages, SMN is associated with similar levels of Sm proteins irrespective of its snRNP assembly activity in these extracts (Supplementary Material, Fig. S3A). Therefore, an alteration of the SMN complex association with Sm proteins does not likely account for the downregulation of SMN function in snRNP assembly in extracts from the CNS.

Next, we analyzed the association of SMN with U1 snRNA. The interaction of the SMN complex with specific sequences of snRNAs is required for efficient and specific assembly of snRNPs (18–20). In the case of U1 snRNA, the SMN complex binds to the stem-loop 1 (SL1) domain of U1 (20). Spinal cord and brain extracts from embryos at E18 and 3-week-old mice normalized for equal content of SMN were incubated with radioactive SL1 under snRNP assembly conditions. Similar amounts of SL1 are immunoprecipitated with anti-SMN antibodies from these reactions (Supplementary Material, Fig. S3B). There is no correlation between the ability of SMN to bind SL1 and its snRNP assembly activity in spinal cord and brain extracts, suggesting that the interaction of the SMN complex with U1 snRNA is not regulated during CNS development.

We then sought to determine whether regulation of snRNP assembly activity observed in CNS extracts was due directly to reduced capacity of the SMN complex to carry out snRNP core formation. To address this possibility, we isolated SMN complexes by immunoprecipitation with anti-SMN antibodies from spinal cord and brain extracts of embryos at E18 and 3-week-old mice and analyzed three increasing concentrations of these SMN complexes by western blot and snRNP assembly experiments (Supplementary Material, Fig. S3C). Similar to the situation observed in whole tissue extracts, SMN complexes purified from both spinal cord and brain extracts of embryos at E18 display greater activity in snRNP assembly than those from extracts of the same tissues of 3-week-old mice. Therefore, the downregulation of snRNP assembly in the CNS results directly from a reduced activity of the SMN complex in Sm core formation. Altogether these results indicate that tissues control the pool of SMN complexes competent for snRNP assembly by modulating the activity of SMN rather than its abundance or association with Sm proteins and snRNAs.

**SMN function in snRNP assembly is downregulated upon cellular differentiation**

To draw direct conclusions regarding the regulation of SMN function in specific cell types, we decided to analyze SMN activity in snRNP assembly using well-established model systems of neuronal and myogenic differentiation. NTera 2 (NT2) is a human embryonal carcinoma cell line that resembles neuronal precursors and can be induced to terminally differentiate into postmitotic CNS neurons (40–42). Figure 5 shows western blot analysis of three increasing concentrations of total proteins from undifferentiated NT2 cells and their differentiated neuronal counterpart NT2-N cells. NT2-N cells are terminally differentiated postmitotic neurons as indicated by lack of PCNA and expression of neuronal intermediate filaments (NF-M) as well as several additional neuronal markers (Fig. 5A; data not shown). Similar levels of SMN as well as of Sm proteins are found in extracts from both NT2 and NT2-N cells when normalized per equal number of cells as indicated by similar levels of the core histones. Therefore, as is the case throughout CNS ontogenesis (Fig. 1), the levels of SMN are approximately constant during neuronal differentiation. We then carried out snRNP assembly experiments using three increasing concentrations of extracts from NT2 and NT2-N cells normalized for equal content of SMN. Figure 5B shows that SMN activity in snRNP assembly is downregulated in terminally differentiated NT2-N neurons when compared with undifferentiated NT2 cells.
We next sought to extend our analysis to the process of myogenic differentiation. C2C12 myoblasts can be efficiently differentiated in myotubes after growth for several days in serum-free media and this is a well-studied model of skeletal myogenesis (43,44). As expected, western blot analysis of three increasing concentrations of total proteins from undifferentiated C2C12-GM cells and differentiated C2C12-DM myotubes shows that dividing C2C12 myoblasts exit from the cell cycle and terminally differentiate into myotubes, expressing skeletal muscle markers such as myosin heavy chain (Fig. 5A). Remarkably, despite the fact that similar levels of SMN and SmB are expressed during myogenic differentiation, extracts from C2C12-GM myoblasts display a greater snRNP assembly activity than differentiated C2C12-DM myotubes (Fig. 5B). There is approximately a 3-fold reduction of SMN activity in postmitotic neurons and myotubes when compared with their respective undifferentiated cells (data not shown). Altogether these results demonstrate that the SMN complex activity in snRNP assembly is downregulated upon neuronal as well as myogenic differentiation.

SnRNP synthesis is downregulated upon cellular differentiation

We next sought to analyze the consequence on snRNP biogenesis of the regulation of SMN complex activity. To do so, we employed the model systems of cellular differentiation described earlier, which recapitulate the regulation of SMN function observed in mouse tissues during development and are amenable to pulse-labeling experiments. First, we analyzed the steady-state levels of snRNPs during neuronal and myogenic differentiation. To do so, extracts from NT2 and NT2-N cells as well as from C2C12-GM and C2C12-DM cells normalized per equal content of SMN were immunoprecipitated using anti-Sm antibodies to isolate endogenous snRNPs. Bound snRNAs were labeled at the 3' end with [32P]pCp and analyzed by electrophoresis on denaturing polyacrylamide gels (Fig. 6A). Despite a 30% decrease in the amount of U2 snRNA in postmitotic neurons, the overall levels of the Sm site-containing spliceosomal snRNAs can be regarded as approximately constant in both undifferentiated NT2 cells and NT2-N neurons (Fig. 6A and B). Similarly, upon differentiation of C2C12 cells, there is a 20% reduction...
in the levels of U1 and U2 but not of other snRNAs in myotubes when compared with myoblasts (Fig. 6A and B). In conclusion, modest variations, if any, of the steady-state levels of splicesomal snRNPs occur during neuronal and myogenic differentiation.

Next, to analyze the rate of snRNP synthesis in vivo, both undifferentiated and differentiated NT2 or C2C12 cells were pulse-labeled for 6 h with \([^{32}P]\)phosphoric acid. Extracts from these cells were then immunoprecipitated with anti-Sm antibodies and bound snRNAs analyzed by electrophoresis on denaturing polyacrylamide gels. Strikingly, Figure 7A shows that the amount of newly synthesized snRNPs is dramatically reduced in differentiated NT2 and C2C12 cells when compared with their undifferentiated counterparts. Quantification of the levels of snRNP synthesis during cellular differentiation shows that in NT2-N neurons there is a ~20-fold drop in snRNP synthesis relative to undifferentiated NT2 cells, whereas C2C12 myotubes synthesize ~5-fold less snRNPs than undifferentiated myoblasts (Fig. 7B). Although these differences are greater than the reduction of SMN complex activity in snRNP assembly observed in cell extracts, there is also a remarkable decrease in the rate of global transcription upon terminal differentiation of both NT2 and C2C12 cells (Fig. 7A). The levels of global RNA synthesis (and of snRNA transcription) are 8-fold and 2- to 3-fold lower in postmitotic
neurons and myotubes, respectively, than in undifferentiated NT2 and C2C12 cells (Figure 7A; data not shown). Therefore, the extent of the decrease of snRNP synthesis in vivo correlates well with the reduction of SMN activity and of global transcription considered collectively. These results demonstrate that the rate of snRNP synthesis dramatically decreases upon neuronal and myogenic differentiation and that the activities of the transcriptional machinery and of the SMN complex contribute to the downregulation of snRNP biogenesis.

**DISCUSSION**

SnRNPs are the essential components of the spliceosome, the large macromolecular assembly that carries out the removal of introns from pre-mRNAs in the process of splicing (9).
The biogenesis of snRNPs in higher eukaryotes has a cytoplasmic as well as a nuclear phase and requires the function of the SMN complex (7,10). The SMN complex associates with Sm proteins in the cytoplasm and interacts with newly exported snRNAs to ensure their efficient and specific assembly into ribonucleoprotein particles. It is assumed that snRNP synthesis is a constitutively active process because pre-mRNA splicing takes place in all cells. Furthermore, despite the complexity of the snRNP biogenesis pathway and the opportunity for SMN to control snRNP biogenesis at multiple levels, there is no evidence that the housekeeping function of SMN is regulated in vivo. We investigated SMN interactions and function in mouse tissues and found that SMN is associated with Gemin and Sm proteins in large macromolecular complexes.
throughout CNS ontogenesis. The remarkable conservation in mouse tissues of SMN interactions identified previously in HeLa cells is consistent with its essential function in snRNP biogenesis. Strikingly, however, we showed that the activity of the SMN complex in snRNP assembly does not correlate with its expression levels and it varies greatly among tissues as well as during development and cellular differentiation. Importantly, we also demonstrated that downregulation of SMN activity upon neuronal and myogenic differentiation coincides with decreased snRNP synthesis in vivo. These findings are surprising as they reveal that the SMN complex activity in snRNP assembly, a housekeeping function thought to be uniformly required by all eukaryotic cells, is a developmentally regulated process.

We showed that the high levels of SMN function in snRNP assembly found in tissue extracts during embryonic and early postnatal development of the CNS are followed at the onset of myelination by a strong decrease to low basal levels, which are then maintained throughout life. These results suggest a more prominent demand for SMN activity in snRNP assembly during CNS development than for maintenance of its functionality later in life. Moreover, the regulation of SMN function in snRNP assembly is a general phenomenon observed in tissue extracts from the CNS as well as skeletal muscle, kidney and testis at distinct times during ontogenesis. In this respect, SMN activity bears similarity to tissue-specific functions that are spatially and/or temporally restricted to distinct cell types during differentiation or development. Despite the diversity of the biochemical and metabolic features of individual tissues, a correlation emerges between the regulation of SMN activity in tissues and the physiological changes that occur during organogenesis. Elevated SMN activity in snRNP assembly is associated with high levels of cell proliferation and metabolism typical of differentiating tissues, whereas the low basal levels of SMN activity are attained once tissues acquire an adult-like profile and reach their functional maturation.

Importantly, model systems of cellular differentiation appear to recapitulate this situation. We showed that SMN activity and snRNP biogenesis are strongly downregulated upon terminal differentiation in both NT2 neurons and C2C12 myotubes. In addition to demonstrating the correlation between reduced levels of SMN activity in snRNP assembly and decreased rate of snRNP synthesis in vivo, these results provided further insights into the control of SMN function and snRNP biogenesis. Proliferation, cell cycle exit and cellular differentiation are accomplished through highly dynamic regulatory networks involving transcriptional repression and activation of large sets of genes (44,45). Undifferentiated progenitor cells probably display high levels of SMN complex activity and snRNP synthesis to support the prominent RNA transcription needed to carry out these complex biological processes. Conversely, upon terminal differentiation, cells permanently modify their gene expression program. It appears that differentiated cells downregulate SMN activity and snRNP synthesis as a consequence of the relatively reduced burden on RNA transcription, which is probably associated with the maintenance of a specialized functional state. These results suggest the presence of regulatory networks that link the SMN complex function and snRNP biogenesis to the global control of transcription during cellular differentiation.

Although the association of the SMN complex with either Sm proteins or snRNAs represents a potential target for the in vivo regulation of SMN function, these interactions do not change with age in spinal cord and brain extracts. Instead, we found that regulation of snRNP assembly is probably accomplished through the modulation of the SMN complex activity during CNS development. Therefore, tissues appear to control the functionality of the SMN complexes competent for snRNP assembly rather than SMN abundance or its interactions with Sm proteins and snRNAs. A number of evidence argue in favor of a genuine regulation of the SMN complex activity and against the possibility that tissue homogenization might release components that would interfere non-specifically with SMN function in vivo. First, changes in the levels of SMN complex activity in snRNP assembly are observed at distinct times during development and in extracts from several different tissues, which are characterized by a diverse protein composition. Therefore, a putative non-specific inhibitory effect on snRNP assembly due to the accumulation of myelin basic proteins in CNS extracts, for example, could not account for changes of SMN activity in extracts from non-neuronal tissues such as testis, which does not express these proteins. Secondly and most importantly, in cell model systems, both downregulation of SMN complex activity and decreased snRNP synthesis in vivo occur cell-autonomously upon terminal differentiation of post-mitotic neurons and myotubes. Several different possibilities such as expression of trans-acting regulatory factors and changes in protein composition or post-translational modifications of the SMN complex may account for the observed regulation of SMN activity. The latter possibility is supported by recent findings that the SMN complex is phosphorylated in HeLa cells and that in vitro dephosphorylation impairs snRNP assembly (46). The identification of the molecular mechanism(s) responsible for the regulation of SMN activity will be the focus of future studies.

Since snRNPs are essential for pre-mRNA splicing, it is not surprising that complete loss of SMN activity is incompatible with life and that knock-out of SMN causes early embryonic lethality in mice (22). On the contrary, it is more difficult to explain why a reduction of SMN function in snRNP assembly should result in the neurodegenerative disease SMA. One difficulty in rationalizing snRNP assembly deficiencies as the cause of selective degeneration of motor neurons in SMA is the common assumption that ubiquitous housekeeping biological processes are required in all tissues to a similar extent. We showed that the levels of SMN activity in snRNP assembly differ among tissues and are most prominent in the developing CNS, due to the presence of a larger pool of SMN complexes competent for snRNP assembly in extracts from neuronal tissues. These observations provide evidence for the first time that SMN function in snRNP assembly may not be uniformly required in all cells, and may have important implications on the pathophysiological consequence of SMN deficiency in SMA patients.

Reduced levels of SMN cause specific degeneration of motor neurons in animal models of the disease and death occurs a few days after birth in SMA mice (2,25,26,47).
Partial loss of motor neuron cell bodies, paralysis and death are late features of pathogenesis in severe SMA mice models (23); nevertheless, they occur shortly after birth and before the onset of myelination, indicating that the defect leading to motor neuron dysfunction is probably accumulated during embryonic and early postnatal development of the spinal cord. Remarkably, we showed that the levels of the SMN complex activity in snRNP assembly are most prominent throughout this crucial phase of spinal cord development during which the defect leading to motor neuron degeneration is accumulated. Therefore, reduced levels of SMN may preferentially affect neuronal tissues because a greater proportion of SMN complexes need to be committed to the snRNP assembly pathway when compared with other tissues, and SMA may be the consequence of a dysfunction of snRNP biogenesis during spinal cord development. At present, however, it is not possible to draw definitive conclusions on SMN activity in motor neurons, or on their preferential sensitivity when compared with other neuronal cells, because our analysis was carried out using extracts from tissues that contain different cell types, and the levels of SMN function in snRNP assembly in brain and spinal cord extracts of embryos are similar. Future studies of SMN function in snRNP assembly in motor neurons as well as in animal models of SMA will be needed to address these issues directly.

Current therapeutic approaches aim at increasing SMN levels in motor neurons of SMA patients. However, our results indicate that the rescue of SMN protein levels may not necessarily correspond to a recovery of function because SMN activity is regulated in vivo. For example, if a reduced production of snRNPs during spinal cord development is the primary cause of SMA, the strategy of adding further SMN at a postnatal stage when its activity in snRNP assembly is downregulated may not be beneficial. Consistent also with the limited effect on survival of SMA mice in which SMN levels in motor neurons were restored postnatally (48), our results further emphasize the need for a more detailed knowledge of the molecular defect leading to motor neuron degeneration in SMA and of the window of time when SMN activity is most highly required.

MATERIALS AND METHODS

Mouse tissues and antibodies

C57BL/6 mice were sacrificed at the indicated age by cervical dislocation and tissues were collected by manual dissection, quickly frozen in liquid nitrogen and stored at −80°C until further processing. The antibodies used in this study were as follows: anti-SMN clone 8 (BD Transduction Laboratories), anti-SMN 2B1 (8), anti-SMN 7F3 (49), anti-Gemin2 2E17 (50), anti-Gemin4 17D10 (35), anti-Gemin6 (20), anti-lnRNP A1/A2 9H10 (51), anti-lnRNP R/Qs 18E4 (52), anti-Sm Y12 (Lab Vision), anti-pICln clone 32 (BD Transduction Laboratories), anti-H1 and core histones (Chemicon), anti-myelin basic proteins (Chemicon), anti-PCNA PC10 (Chemicon), anti-tubulin DM 1A (Sigma), anti-NF-M RMO 14.9 (Zymed) and purified mouse IgG immunoglobulins (Sigma).

Cell culture and treatments

Undifferentiated NT2 cells were maintained in Dulbecco’s modified Eagle’s medium with high glucose (DMEM-HG) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin. Neuronal differentiation was adapted from procedures described previously (40–42). NT2 cells were seeded in bacteriological grade Petri dishes and treated with 10 μM retinoic acid in DMEM-HG containing 5% FBS and penicillin/streptomycin twice a week for at least 2 weeks. Following trypsinization and replating on poly-D-lysine coated dishes, NT2-N neurons were selected in DMEM-HG containing 5% FBS, penicillin/streptomycin, 1 μM cytosine-d-arabinofuranoside, 10 μM uridine and 10 μM fluorodeoxyuridine for at least 2 weeks. C2C12 myoblasts were maintained in DMEM-HG containing 15% FBS and penicillin/streptomycin (GM medium). To induce myogenic differentiation, C2C12 cells were switched to serum-free DMEM-HG containing 10 μg/ml insulin and 10 μg/ml transferrin (DM medium) for 1 day (43,44). Fully differentiated myotubes were selected by growth for four additional days in DM medium containing 30 μM cytosine-d-arabinofuranoside. In pulse-labeling experiments to monitor snRNP synthesis in vivo, either undifferentiated or differentiated NT2 and C2C12 cells were incubated for 6 h with 100 μCi of [32P]phosphoric acid in the appropriate DMEM-HG media without phosphate and mitotic inhibitors.

Total extract preparation and sucrose gradient centrifugation

Total protein extracts for western blot analysis were prepared by homogenization of cells or tissues in SDS/PAGE sample buffer, followed by sonication, boiling and centrifugation for 15 min at 10 600 g at room temperature. Protein analysis was carried out by SDS/PAGE on 12.5% polyacrylamide gels and western blot. For the immunoprecipitation and sucrose gradient centrifugation experiments (Fig. 1), tissues were homogenized in RSB-100 buffer (100 mM NaCl, 10 mM Tris–HCl pH 7.4, 2.5 mM MgCl2) containing 0.1% NP-40, EDTA-free protease inhibitor cocktail (Roche) and phosphatase inhibitors (50 mM NaF, 0.2 mM Na3VO4). Extracts were passed five times through a 25G needle, sonicated and cleared by centrifugation for 15 min at 10 000 r.p.m. at 4°C. Sucrose gradient centrifugation experiments were carried out by centrifugation of spinal cord extracts on 10 ml 10–30% sucrose gradients for 4 h at 38 000 r.p.m. in an SW 41 rotor at 4°C. Fractions were collected and analyzed by SDS/PAGE on 12.5% polyacrylamide gels and western blot.

In vitro assembly of snRNPs

U1, U1ΔSm and SL1 RNAs were transcribed in vitro from linearized template DNAs in the presence of [γ-32P]UTP (3000 Ci/mmol) and purified from denaturing polyacrylamide gels according to standard procedures. Extracts for snRNP preparation were prepared as previously described with minor modifications (18). Cells or tissues were homogenized in ice-cold reconstitution buffer (20 mM HEPES–KOH pH
7.9, 50 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, 5% glycerol) containing 0.01% NP-40, using approximately a volume-to-
mass ratio of 5 μl of buffer per milligram of tissue. Extracts were passed five times through a 25G needle and centrifuged for 15 min at 10 000 r.p.m. at 4°C. Supernatants were collected and either used directly or stored in frozen aliquots at −80°C. To minimize potential variations in SMN activity that may result from different extract preparations, extracts were prepared simultaneously for each set of experiments and were not subjected to more than two cycles of freeze and thaw. Representative results from at least three independent experiments are shown. Depletion of spinal cord extracts with anti-SMN (2B1) antibodies or mouse IgG immunoglobulins as a control was carried out as previously described (18). SnRNP assembly reactions with either whole tissue and cell extracts or SMN complexes bound to beads were carried out for 1 h at 30°C in a volume of 20 μl of reconstitution buffer containing 0.01% NP-40, 10 000 c.p.m. of in vitro transcribed [α-32P]UTP-labeled snRNAs, 2.5 mM ATP and 10 μm Escherichia coli tRNA. Following addition of heparin and ura to a final concentration of 5 mg/ml and 2 M, respectively, assembly reactions were analyzed by electrophoresis on 6% polyacrylamide native gels at 4°C and by autoradiography as previously described (53).

Immunoprecipitation experiments

Antibodies were bound to protein G-Sepharose (Sigma) in RSB-100 buffer containing 0.1% NP-40 and protease inhibitors for 2 h at 4°C. Following five washes with the same buffer, immunoprecipitations were carried out as described in what follows. Immunoprecipitations from tissue extracts (Fig. 1) were carried out in RSB-100 buffer containing 0.1% NP-40 and protease inhibitors for 2 h at 4°C. After five washes with the same buffer, bound proteins were eluted by boiling in sample buffer and analyzed by SDS/PAGE on 12.5% polyacrylamide gels and western blot. For snRNP assembly experiments, SMN complexes were immunopurified from whole tissue extracts with anti-SMN (7F3) antibodies bound to protein G-Sepharose for 2 h at 4°C in reconstitution buffer containing 0.01% NP-40 and protease inhibitors, as previously described (49). Following five washes with the same buffer, immobilized SMN complexes were used directly for snRNP assembly reactions or eluted by boiling in sample buffer and analyzed by SDS/PAGE on 12.5% polyacrylamide gels and western blot. Immunoprecipitations of snRNP assembly reactions with anti-Sm (Y12) antibodies were carried out for 3 h at 4°C in RSB-500 (500 mM NaCl, 10 mM Tris–HCl pH 7.4, 2.5 mM MgCl₂) containing 0.01% NP-40 and protease inhibitors. Immunoprecipitations of snRNP assembly reactions with anti-SMN (2B1) and anti-U1A antibodies were carried out for 3 h at 4°C in RSB-100 containing 0.01% NP-40 and protease inhibitors. Immunoprecipitations of endogenous snRNPs from either undifferentiated or differentiated NT2 and C2C12 cell extracts were carried out with anti-Sm (Y12) antibodies for 3 h at 4°C in RSB-500 buffer containing 0.1% NP-40 and protease inhibitors. Following five washes with the same buffer, bound RNAs were recovered from immunoprecipitates by proteinase K treatment, phenol/ chloroform extraction and ethanol precipitation. 3’ end labeling experiments were carried out with 10 μCi of [32P]pCP and T4 RNA ligase (Roche) following manufacturer’s instructions and unincorporated nucleotides were removed by centrifugation through micro Bio-Spin P-30 columns (Biorad). RNAs were analyzed by electrophoresis on denaturing polyacrylamide gels and by autoradiography.

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