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The SMN–SIP1 Complex Has an Essential Role in Spliceosomal snRNP Biogenesis

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Summary

Spinal muscular atrophy (SMA) is an often fatal neuromuscular disease that has been directly linked to the protein product of the Survival of Motor Neurons (*SMN*) gene. The SMN protein is tightly associated with a novel protein, SIP1, and together they form a complex with several spliceosomal snRNP proteins. Here we show that the SMN–SIP1 complex is associated with spliceosomal snRNAs U1 and U5 in the cytoplasm of Xenopus oocytes. Antibodies directed against the SMN–SIP1 complex strongly interfere with the cytoplasmic assembly of the common (Sm) snRNP proteins with spliceosomal snRNAs and with the import of the snRNP complex into the nucleus. Thus, the SMN–SIP1 complex is directly involved in the biogenesis of spliceosomal snRNPs. Defects in spliceosomal snRNP biogenesis may, therefore, be the cause of SMA.

Introduction

Spinal muscular atrophy (SMA) is a common autosomal recessive disease characterized by degeneration of motor neurons in the spinal cord (Roberts et al., 1970; Pearn, 1973, 1978; Czeizei and Hamular, 1989). A human gene that is directly linked to SMA, termed Survival of Motor Neurons (*SMN*), has recently been identified (Lefebvre et al., 1995). Two copies of the *SMN* gene are located in a 500 kb inverted repeat at chromosome 5q13. In over 98% of all SMA patients, the telomeric copy of *SMN* is deleted or mutated while the centromeric copy is unaffected (Lefebvre et al., 1995). The *SMN* gene encodes a 294–amino acid protein that does not have any obvious sequence similarity to other known proteins. SMN is found in both the cytoplasm and the nucleus in somatic cells. Strikingly, SMN is highly concentrated in a novel nuclear structure, termed gems (gemini of coiled bodies) (Liu and Dreyfuss, 1996; Liu et al., 1996). Depending on the cell type, there are between two and eight gems per nucleus, often in close proximity to coiled bodies. Indeed, coiled bodies and gems display similar responses to transcriptional inhibitors and to low temperature, suggesting a functional relationship between these nuclear bodies (Liu and Dreyfuss, 1996). No definitive functions have been identified for either gems or coiled bodies, and the function of the SMN protein is unknown.

The Sm class of small nuclear ribonucleoproteins (snRNPs) U1, U2, U4/6, and U5 are major constituents of the spliceosome, the catalytic center of the pre-mRNA splicing reaction (Moore et al., 1993; reviewed by Madhani and Guthrie, 1994). Each spliceosomal snRNP consists of one (U1, U2, and U5) or two (U4/6) snRNAs, a common set of at least eight Sm proteins, termed B, B′, D1, D2, D3, E, F, and G, and specific polypeptides that are associated with only one individual U snRNP (reviewed by Lührmann et al., 1990). With the exception of U6, all spliceosomal snRNAs share two structural features: the 5′-terminal trimethylguanosine (m_3G) cap and a single-stranded, uridine-rich sequence flanked by two hairpin loops, referred to as the Sm site (Branlant et al., 1982; Reddy and Busch, 1988). The Sm site is the primary binding site for the Sm proteins. The remaining snRNA domains provide binding sites for the snRNA-specific snRNP proteins and for RNA–RNA interactions (Lührmann et al., 1990). U6 differs from the other spliceosomal U snRNAs in that it contains a γ-monomethyl cap instead of the m_3G cap and does not bind directly to Sm proteins due to its lack of an Sm site (Reddy and Busch, 1988; Singh and Reddy, 1989).

The snRNP-specific proteins have snRNP-specific functions in the splicing reaction. In contrast, the only known function for the Sm proteins is in the biogenesis of U snRNPs. The biogenesis of snRNPs U1, U2, U4, and U5 is a complex cycle that requires the bidirectional transport of these snRNAs across the nuclear envelope (DeRobertis, 1983; Mattaj and DeRobertis, 1985; Mattaj, 1986, 1988; Neuman de Vegvar and Dahlberg, 1990). The snRNAs are transcribed in the nucleus by RNA polymerase II and acquire a 5′-terminal monomethyl (m^7G) cap structure, while the Sm proteins are synthesized in the cytoplasm and do not migrate on their own into the nucleus. Instead, the snRNAs are exported from the nucleus to the cytoplasm, where the Sm proteins bind to the snRNAs' Sm site to form the Sm core. Thereafter, the m^7G cap is hypermethylated to form the m_3G cap and the assembled U snRNP is imported into the nucleus. The nuclear import of the m_3G-capped spliceosomal snRNPs thus appears to require a nuclear import signal that is generated only after both the m_3G cap and the Sm core domain have been formed (Fischer and Lührmann, 1990; Hamm et al., 1990; Fischer et al., 1993). The precise point in this cycle and the location in the cell where the association of the snRNP-specific proteins takes place are, in most cases, unknown. Much of the current knowledge of the spliceosomal snRNP biogenesis cycle has been derived from experiments in the Xenopus laevis oocyte. In the oocyte, large amounts of Sm proteins are stored in the cytoplasm, awaiting the burst of snRNA transcription that occurs during midblastula transition, at which time copious amounts of spliceosomal snRNPs are assembled (Zeller et al., 1983). Thus, when U snRNAs are microinjected into the Xenopus oocyte cytoplasm, they are assembled with Sm proteins, receive a cap hypermethylation, and are subsequently imported into the nucleus. The amphibian oocyte thus provides an excellent experimental system in which to dissect the snRNP biogenesis pathway (DeRobertis, 1983; Mattaj and DeRobertis, 1985).

Formation of the Sm core domain in the cytoplasm

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requires specific interactions among the Sm proteins to allow their binding to the Sm site. The Sm proteins D1, D2, E, F, and G form a stable, RNA-free complex prior to RNA binding (Fisher et al., 1985; Sauterer et al., 1988, 1990; Raker et al., 1996). The binding of the Sm protein complex to the Sm site results in the formation of a subcore that is then completed to the mature Sm core by addition of a complex of B, B′, and D3 proteins (Raker et al., 1996). It is not clear whether these ordered assembly steps are assisted by additional, non-snRNP factors or whether they proceed entirely by self assembly.

SMN is tightly associated in the cell with SIP1, and together these two proteins are in a complex with Sm and other snRNP-specific proteins (Liu et al., 1997). However, the function, if any, of SMN and SIP1 in the biogenesis, metabolism, or function of snRNPs could not be determined from these observations. Here we report that the SMN–SIP1 complex is associated with spliceosomal snRNAs U1 and U5 in the cytoplasm of Xenopus oocytes. Moreover, antibodies against SIP1 strongly inhibit Sm core assembly of spliceosomal snRNPs U1, U2, U4, and U5 and their transport from the cytoplasm to the nucleus. The anti-SMN antibodies we have tested also affected snRNP assembly, but in contrast to the anti-SIP1 antibodies, they stimulate formation of the Sm core domain. Thus, the SMN–SIP1 complex is directly involved in the biogenesis of spliceosomal snRNPs. As SMN contains two distinct domains through which it can directly bind to SIP1 and the Sm proteins, respectively (Liu et al., 1997), it can potentially serve to bring the SIP1–SMN–Sm complex together. These findings describe a function for the SMA disease gene product SMN and for SIP1 in a specific biochemical pathway and suggest that defects in spliceosomal snRNPs biogenesis is likely to be the molecular basis for the SMA phenotype.

Results

SMN and SIP1 Are Associated with Spliceosomal snRNAs in the Cytoplasm

In the accompanying paper (Liu et al., 1997 [this issue of *Cell*]), we have described a novel protein complex containing SMN, SIP1, and spliceosomal snRNP proteins, including several of the Sm proteins. The Xenopus oocyte provides a particularly advantageous system in which to study spliceosomal snRNP biogenesis by use of microinjections (Mattaj and DeRobertis, 1985; Mattaj, 1986). We therefore wished to determine whether SMN and SIP1 are present in these oocytes. If so, the unique features of this system can be used to investigate the possible functions of these proteins in snRNP biogenesis. Immunoblotting with monoclonal antibodies to the human SMN and SIP1 proteins (2B1 and 2E17, respectively) on Xenopus tissue culture cells detected proteins of similar size to the corresponding human proteins. cDNA cloning, sequencing, and transfection experiments confirmed that these proteins are the Xenopus homologs of SMN and SIP1 (Q. L. and G. D., unpublished data). Surprisingly, however, unlike in somatic cells, where there is more SMN and SIP1 in the nucleus, in the oocyte both proteins were found almost exclusively in the cytoplasm (Figure 1A). The high cytoplasmic concentration of SMN and SIP1 in the Xenopus oocyte is reminiscent of the large amounts of Sm proteins that are stored in the oocyte cytoplasm (Zeller et al., 1983).

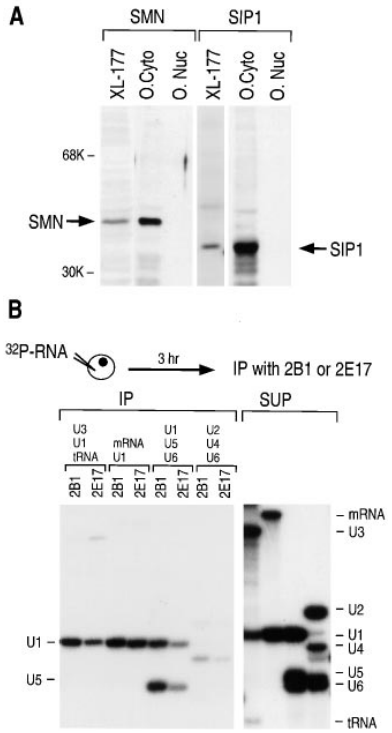

Figure 1. SMN and SIP1 Are Found in the Cytoplasm of Xenopus Oocytes and Associate with Spliceosomal snRNAs

(A) Protein from total Xenopus somatic cells (XL-177) or from oocytes dissected into nucleus and cytoplasm (O. Nuc and O. Cyto, respectively) were fractionated by SDS PAGE and analyzed by Western blotting with anti-SMN antibody (2B1) or anti-SIP1 antibody (2E17).

(B) Immunoprecipitation of spliceosomal U snRNAs with anti-SIP1 and anti-SMN antibodies. Different mixtures of the indicated in vitro synthesized ^{32}P-labeled RNAs were injected into the cytoplasm of oocytes. Three hours later, immunoprecipitations were carried out with either anti-SMN antibody 2B1 or anti-SIP1 antibody 2E17. Immunoprecipitated RNA (IP) was analyzed by gel electrophoresis. The supernatants (SUP) of the 2B1 immunoprecipitations are shown. The supernatants from the 2E17 immunoprecipitations were similar (data not shown).

In order to identify potential cellular targets for the SMN–SIP1 complex, we tested if it can associate with RNA. Various ^{32}P-labeled RNAs were generated by transcription in vitro, including mRNA, tRNA, U3 snoRNA, 5S RNA, and the spliceosomal snRNAs U1, U2, U4, U5, and U6. Different mixtures of these RNAs were then coinjected into the cytoplasm of oocytes and immunoprecipitations were carried out with anti-SMN (2B1) and anti-SIP1 (2E17) antibodies. As shown in Figure 1B, only U1 and U5 snRNAs were efficiently precipitated, indicating that they interact with SMN and SIP1. A weak but reproducible immunoprecipitation of U4 snRNA was observed. In contrast, other RNAs, including mRNA, tRNA, U3 snoRNA, U6 snRNA, U2 snRNA (Figure 1B), and 5S rRNA (data not shown) were not immunoprecipitated at significant levels with the anti-SMN or anti-SIP1 antibodies.

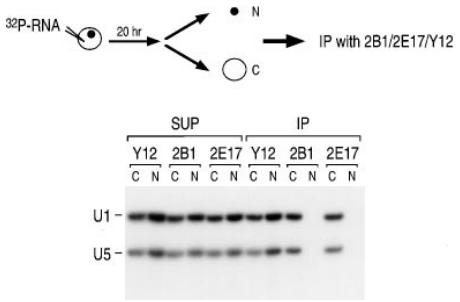

Figure 2. Anti-SMN and Anti-SIP1 Antibodies Can Immunoprecipitate U1 and U5 snRNAs Only from the Cytoplasm

A mixture of ^{32}P-labeled U1 and U5 snRNAs was injected into the cytoplasm of oocytes. After incubation for 20 hr, the oocytes were dissected into nuclear (N) and cytoplasmic (C) fractions, and RNAs from both fractions were immunoprecipitated (IP) with either the anti-Sm antibody Y12, anti-SMN antibody 2B1, or anti-SIP1 antibody 2E17. One-fifth of the total supernatant (SUP) was loaded on the gel.

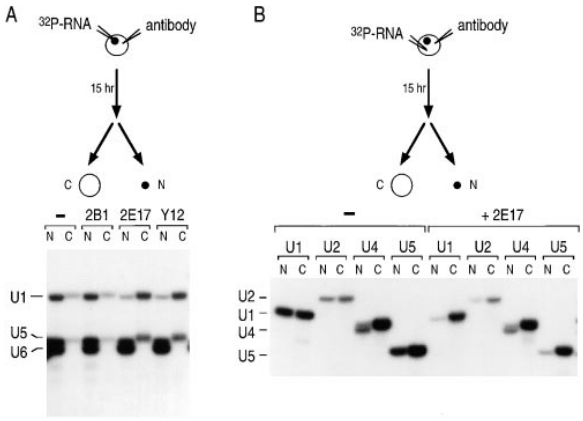

Figure 3. Anti-SIP1 Antibodies Interfere with the Biogenesis Cycle of Spliceosomal U snRNPs

(A) Oocytes received a cytoplasmic injection of either water (-), anti-SMN antibody (2B1), anti-Sm antibody (Y12), or anti-SIP1 antibody (2E17). The same oocytes were nuclear injected 1 hr later with a mixture of ^{32}P-labeled U1, U5, and U6 snRNA. Fifteen hours later, the oocytes were dissected into nuclear (N) and cytoplasmic (C) fractions, and the injected RNAs were analyzed by electrophoresis on an RNA gel.

(B) Anti-SIP1 antibodies inhibit the nuclear import of all spliceosomal snRNAs. In vitro transcribed, ^{32}P-labeled snRNAs U1, U2, U4, and U5 were injected into the cytoplasm of oocytes either alone (-) or together with anti-SIP1 antibody (2E17). Oocytes were incubated for an additional 15 hr and were then dissected into nuclear (N) and cytoplasmic (C) fractions. Injected RNAs were isolated and analyzed by electrophoresis on a denaturing RNA gel.

Since neither SMN nor SIP1 is detectable in the oocyte nucleus (Figure 1A), it seemed possible that they are not associated with mature U1 and U5 snRNPs but rather only during the cytoplasmic phase of their biogenesis pathway. To test this directly, U1 and U5 snRNAs were injected into the cytoplasm of oocytes, and the oocytes were incubated for 12 hr (Figure 2). After this incubation period, approximately 50% of the injected snRNA was transported to the nucleus while the rest was still in the cytoplasm. Immunoprecipitations from the nuclear and cytoplasmic fractions were then carried out with either anti-SMN antibody, anti-SIP1 antibody, or the anti-Sm monoclonal antibody Y12, and the coimmunoprecipitated RNAs were analyzed. Y12 recognizes a subset of the Sm proteins and was used in this experiment to monitor the assembly of the Sm core domain (Lerner et al., 1981). As previously reported (Mattaj, 1986; Fischer and Lührmann, 1990), U1 and U5 snRNAs were immunoprecipitated by Y12 in approximately equal amounts from the nucleus and cytoplasm (Figure 2). This indicated that the Sm proteins associate in the cytoplasm with the snRNA and then move as an assembled and stable snRNP complex to the nucleus. In striking contrast, however, SMN and SIP1 association with U1 and U5 snRNAs was observed only in the cytoplasm (Figure 2).

Further evidence for the physiological relevance of the interaction of SMN and SIP1 in the cytoplasm with spliceosomal snRNPs was obtained following nuclear injections of U1 snRNA. Only after export to the cytoplasm and during the cytoplasmic phase of their biogenesis could U1 snRNA be immunoprecipitated with anti-SMN or anti-SIP1 antibodies (data not shown). We conclude that the SMN–SIP1 complex interacts with U1 and U5 snRNAs in the cytoplasm but not after these snRNAs have been assembled into snRNPs and imported into the nucleus. Thus, SMN and SIP1 dissociate from the spliceosomal snRNPs either prior to nuclear entry or shortly thereafter.

Anti-SIP1 Antibodies Inhibit Spliceosomal snRNP Assembly and Nuclear Import

The association of SMN and SIP1 with U1 and U5 snRNPs in the cytoplasm suggested a role for these proteins in the biogenesis pathway of these snRNPs, i.e., in the assembly of snRNP proteins onto these snRNAs and/or in the nuclear import of these particles. We therefore examined whether anti-SMN or anti-SIP1 antibodies have an effect on the nucleo-cytoplasmic transport of spliceosomal snRNPs (Figure 3). Anti-SMN (2B1), anti-SIP1 (2E17), or the anti-Sm (Y12) were injected into the cytoplasm of oocytes. One hour later, a mixture of U1, U5, and, as a control for nuclear injection, U6 snRNA was injected into the nuclei of the same oocytes, and the incubation was continued for 15 hr. The oocytes were then fractionated, and the RNAs in the nucleus and cytoplasm were analyzed. In oocytes preinjected with water, nuclear-injected U1 and U5 snRNAs were exported to the cytoplasm and, after cytoplasmic assembly of the Sm core domain, reimported to the nucleus (Figure 3A) (Hamm et al., 1990; Neuman de Vegvar and Dahlberg, 1990; Terns et al., 1993). This was confirmed by immunoprecipitation of U1 and U5 snRNAs with anti-Sm antibodies (data not shown) and by the observation that the nuclear pool of U1 snRNA had undergone 3′ end trimming (see Figure 3A, nuclear fractions). The latter has been previously shown to occur in the cytoplasm prior to nuclear import (Neuman de Vegvar and Dahlberg, 1990; Terns et al., 1993). In oocytes preinjected with Y12, U1 and U5 were also exported to the cytoplasm; however, they were not reimported to the nucleus, resulting in the accumulation of these RNAs in the cytoplasm (Figure 3A). This is because upon binding to the Sm proteins, Y12 interferes with the subsequent steps in the biogenesis of snRNPs that are required for their nuclear import (U. F. and G. D., unpublished data; see also below). Surprisingly, a similar result

was obtained in oocytes preinjected with anti-SIP1 antibodies (Figure 3A). However, no effect on nuclear import of U1 and U5 snRNA was observed in oocytes injected with anti-SMN antibodies (Figure 3A).

We next asked whether anti-SIP1 antibodies interfere with the nuclear import of the other spliceosomal snRNPs. For this, in vitro transcribed snRNAs U1, U2, U4, and U5 were injected into the cytoplasm of oocytes, either without or with anti-SIP1 antibody, and nuclear import was then analyzed 15 hr later (Figure 3B). Without anti-SIP1 antibody injection, all snRNAs accumulated in the nucleus to approximately 50%, although the import of U4 was less efficient (Figure 3B). However, in the presence of anti-SIP1 antibody, the nuclear import of U1, U2, and U5 was almost completely inhibited, and the import of U4 was slowed down by at least 50% (Figure 3B). Thus, anti-SIP1 antibodies interfere with the nuclear import of all spliceosomal U snRNPs regardless of whether they can be efficiently immunoprecipitated with the anti-SMN or anti-SIP1 antibodies. This suggests that the interaction of SMN and SIP with some snRNAs is transient and cannot be monitored by immunoprecipitation (see Discussion).

As outlined above, assembly of the Sm core domain and the formation of the m_3G cap are required for the nuclear import of U snRNPs (Mattaj and DeRobertis, 1985; Fischer and Lührmann, 1990; Hamm et al., 1990; Fischer et al., 1993). Therefore, anti-SIP1 antibodies could inhibit U snRNP import by interfering either with the assembly of the Sm core or with the cap hypermethylation or both. Alternatively, and not mutually exclusive, the antibody could directly interfere with the snRNP transport process, e.g., by blocking transport factors. In order to determine the reason for the observed inhibition of U snRNP nuclear import, the effect of anti-SIP1 antibodies on the assembly of the Sm core domain was studied (Figure 4A). For this, anti-SIP1, anti-SMN or anti-Sm (Y12), and, as controls, nonimmune antibodies SP2/0 or water were injected into the cytoplasm of oocytes, followed by incubation for 1 hr before receiving a second cytoplasmic injection of a mixture of ^{32}P-labeled U1, U5, and U6 snRNAs. One hour later, the Sm core assembly on the injected RNAs was analyzed by immunoprecipitation with Y12 (Figure 4A). In control oocytes preinjected with either water or SP2/0, both U1 and U5 snRNAs were efficiently immunoprecipitated with Y12, indicating that these RNAs assembled with the Sm proteins. As expected, U6 snRNA was not immunoprecipitated because this RNA does not contain an Sm site and thus cannot bind Sm proteins. However, the anti-SIP1 antibody completely inhibited the Sm core assembly on U1 and U5 snRNAs (Figure 4A). Y12 itself strongly reduced the Sm core assembly on both U1 and U5 snRNAs in a similar fashion (Figure 4A). The anti-SMN antibody 2B1, in contrast, did not inhibit but rather slightly stimulated assembly of Sm proteins (Figure 4A). If the snRNAs are injected 1 hr prior to injection of the anti-SIP1 antibody, no interference with subsequent Y12 immunoprecipitation is observed. The observed inhibition of Sm core assembly by preinjection of anti-SIP1 is therefore not due to a nonspecific occlusion of the Sm epitope by the anti-SIP1 antibody (Figure 4A). Next, we tested whether anti-SIP1 antibodies could also interfere with the assembly of U2 and U4 snRNAs. As shown

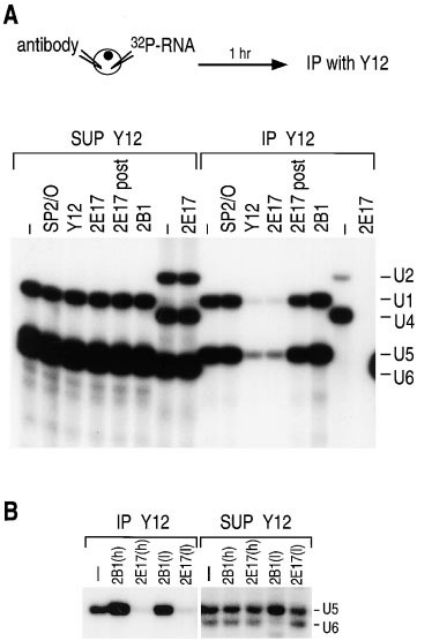

Figure 4. Anti-SIP1 and Anti-SMN Antibodies Affect the Assembly of the Sm Core Domain of Spliceosomal snRNPs

(A) Anti-SIP1 antibodies inhibit the assembly of the Sm core domain of all spliceosomal U snRNPs. Injections of either water (-), anti-Sm antibody (Y12), anti-SIP1 antibody (2E17), anti-SMN antibody (2B1), or control antibody (SP2/0) were performed into the oocyte cytoplasm. One hour later, the same oocytes were injected a second time into the cytoplasm with a mixture of ^{32}P-labeled U1, U5, and U6 snRNAs. In the experiment shown in the lane labeled "SIP1 post", U1, U5, and U6 snRNAs were injected 1 hr prior to the injection of anti-SIP1 antibody. After incubation for an additional 1 hr, the oocytes were homogenized, and RNAs were immunoprecipitated with anti-Sm antibody Y12 (IP Y12). Immunoprecipitated RNAs were analyzed by electrophoresis on an RNA gel with the corresponding supernatants (SUP Y12).

(B) The anti-SMN monoclonal antibody 2B1 stimulates the formation of the Sm core domain. Stimulation of Sm protein binding onto U5 snRNA in the presence of high concentrations of the anti-SMN antibody 2B1. A mixture of ^{32}P-labeled U5 and U6 snRNAs was injected into the cytoplasm of oocytes either with water (-) or with anti-SMN and anti-SIP1 antibodies (2B1 and 2E17, respectively) at either high (h, 3 μg/μl) or low (l, 1 μg/μl) concentrations. After a 1 hr incubation, the oocytes were homogenized, and the RNAs were immunoprecipitated with the anti-Sm antibody Y12 (IP Y12). Immunoprecipitated RNAs were analyzed by electrophoresis on an RNA gel alongside one-tenth of the supernatants (SUP Y12).

in Figure 4A, preinjection of anti-SIP1 antibodies strongly interfered with the Sm core assembly on both U2 and U4 snRNAs, while in oocytes preinjected with water, both snRNAs assembled with the Sm proteins. The inhibition of nuclear import of snRNAs U1, U2, U4, and U5 by anti-SIP1 antibodies is, therefore, at least partially due to the interference of the antibody with the Sm core domain assembly. Thus, SIP1 is a cytoplasmic assembly factor that mediates the formation of the Sm core domain on spliceosomal U snRNPs.

Anti-SMN Monoclonal Antibody Stimulates Sm Protein Binding onto the Sm Site of U snRNAs

Although SMN is in a tight complex with SIP1 and, therefore, is likely to form a functional unit with SIP1, the

anti-SMN antibodies used in the experiments described above, unlike the anti-SIP1 antibodies, did not interfere with U snRNP biogenesis. We therefore investigated further the function of SMN in snRNP assembly. As shown in Figure 4B, injection of anti-SMN antibodies (2B1) does not inhibit snRNP assembly. However, we noticed that upon injection of high concentrations (2 to 3 μg/μl) of this anti-SMN antibody, the assembly of snRNPs was often enhanced. To analyze this in more detail, a mixture of U5 and U6 snRNAs was injected along with either high (3 μg/μl) or low (1 μg/μl) concentrations of the anti-SMN antibody 2B1 or the anti-SIP1 antibody 2E17 (Figure 4B). Sm protein binding onto these RNAs was then assessed by immunoprecipitation with the anti-Sm antibody Y12 1 hr later. After 1 hr, the assembly of Sm proteins onto U snRNAs is not yet complete, thus allowing a more quantitative evaluation of the efficiency of Sm protein binding. In the absence of coinjected antibody, U5 but not U6 was precipitated, indicating Sm core formation on U5 snRNA. However, while low concentrations of 2B1 had only a slight stimulatory effect on Sm protein binding, coinjection of higher 2B1 concentrations significantly enhanced the assembly (2- to 3-fold) (Figure 4B). Coinjected anti-SIP1 antibody, in contrast, inhibited the assembly of Sm proteins onto U5 snRNA almost entirely at both low and high antibody concentrations (Figure 4B). Thus, 2B1 stimulates Sm protein binding onto U5 snRNA and hence Sm core formation, indicating that SMN is also involved in spliceosomal U snRNP assembly. We further found that 2B1 stimulates Sm protein binding onto an artificial snRNA, termed SmII RNA, that consists of the Sm site and stem/loop E of U1 snRNA and an artificial stem/loop 5′ to the Sm site. The capacity of this RNA to bind Sm protein is severely compromised, and, as a consequence of this, its nuclear import is reduced (Fischer et al., 1993). Using the same injection strategy as described above, a strong stimulation of Sm protein binding onto SmII RNA was observed, and, as a consequence, SmII was efficiently imported into the nucleus (data not shown). Taken together, these data and those presented in the preceeding sections strongly suggest that both SIP1 and SMN are directly involved in the assembly of the Sm core domain of spliceosomal U snRNPs.

Discussion

In this study, microinjection experiments in Xenopus oocytes provided important insight into the function of the SMA disease gene product SMN and its associated protein SIP1. SMN and SIP1 are tightly associated as two subunits of a heteromeric protein complex, and both are found in the ooctye cytoplasm. We have detected a complex that contains SMN, SIP1, the Sm proteins, and several additional splicesomal snRNP-specific proteins (Liu et al., 1997). Here we show that the SMN–SIP1 complex has an essential role in spliceosomal snRNP biogenesis. Several lines of evidence lead to this conclusion. First, SMN and SIP1 are specifically associated in the cytoplasm with U1 and U5 snRNAs but not with nuclear snRNPs and not with other RNAs tested so far. Thus, they are not components of mature nuclear snRNPs but rather are associated with them only during the cytoplasmic phase of their biogenesis. Second, anti-SIP1 antibodies strongly interfere with the assembly of the the Sm core domain of spliceosomal U snRNAs and with their nuclear import. Third, anti-SMN antibodies stimulate the assembly of Sm proteins onto the Sm site of spliceosomal U snRNAs. Finally, two distinct domains in SMN have been identified that mediate its interaction with several Sm proteins and with SIP1 (Liu et al., 1997), in addition to its capacity to interact with itself (Liu and Dreyfuss, 1996). The different effects of anti-SMN and anti-SIP1 antibodies on the assembly of splicesomal U snRNAs raise the possibility that SIP1 and SMN have different, although related, functions in the Sm core assembly process. This conclusion is consistent with the observation that SMN but not SIP1 directly interacts with a subset of Sm proteins (Liu et al., 1997). Moreover, SMN can likely simultaneously interact via two distinct binding domains with SIP1 and Sm proteins. Therefore, it is possible that SMN can serve to recruit the Sm proteins to the Sm site of spliceosomal snRNAs, while SIP1 may have a more direct function in mediating the assembly of the Sm proteins onto the Sm site. Alternatively, upon binding to Sm proteins, SMN may prevent their misassembly onto RNAs other than U snRNAs. It is, however, clear both from the tight association of SMN and SIP1 as well as from the effect that antibodies to both proteins have on Sm core domain assembly that both proteins play a role in snRNP assembly. We note, however, that the lack of inhibitory effect of the monoclonal anti-SMN antibodies that we used on snRNP assembly may simply be because of the location of the particular epitope, and other anti-SMN may inhibit this process. It is not clear why only U1 and U5 but not U2 and U4 snRNA can be efficiently immunoprecipitated with anti-SIP1 and anti-SMN antibodies, although the assembly of the Sm core domain of all spliceosomal U snRNPs is strongly inhibited by anti-SIP1 antibodies. One possible explanation is that the interaction of SMN and SIP1 with U2 and U4 snRNA is more transient and, therefore, can not be readily detected by immunoprecipitation. In support of the role of SIP1 in U snRNP biogenesis, SIP1 has significant sequence homology (Liu et al., 1997) to the Saccharomyces cerevisiae protein Brr1 (Noble and Guthrie, 1996a, 1996b), mutations in which result in defects in U snRNP biogenesis. Moreover, genetic criteria suggest that Brr1 interacts with the yeast homolog of the Sm D1 protein.

A great deal of information on the detailed assembly pathway of the Sm core domain has been recently obtained. Importantly, it has been shown that specific interactions among Sm proteins precede their binding to the RNA (Fisher et al., 1985; Sauterer et al., 1988, 1990; Raker et al., 1996). According to this scheme, the D1, D2, E, F, and G proteins first form an RNA-free complex that is only then capable of binding to the Sm site on the RNA. Thereafter, binding of a protein complex that includes B, B′, and D3 proteins completes the assembly of the Sm core domain. The Y12 antibody that we used in this study to monitor the assembly of the Sm core domain is not specific to one particular Sm protein but rather recognizes all of the individual Sm protein assembly intermediates (Raker et al., 1996). Thus, the fact that Y12 does not immunoprecipitate U snRNAs after

injection of anti-SIP1 antibody suggests that none of the Sm proteins was able to bind the Sm site under these conditions. It is, therefore, likely that the SMN–SIP1 complex is involved at an early stage in the Sm core assembly; i.e., in a step that proceeds the Sm protein binding to the Sm site of the snRNAs.

We have previously shown that the SMN protein also interacts with fibrillarin and with the hnRNP U protein (Liu and Dreyfuss, 1996). Fibrillarin is a common component of small nucleolar RNPs (snoRNPs) and is perhaps the snoRNPs' functional equivalent of the common Sm proteins of splicesomal snRNPs (Tyc and Steitz, 1989; Maxwell and Fournier, 1995). It is, therefore, possible that SMN and SIP1 also play a role in snoRNP assembly in a fashion similar to the one that we have found it to play in splicesomal snRNP assembly. In this case, it can be anticipated that this function will be fulfilled by the nuclear pool of SMN and SIP1 found in somatic cells, because snoRNAs remain in the nucleus and snoRNP biogenesis does not have a cytoplasmic phase (Terns and Dahlberg, 1994; Terns et al., 1995). It can, therefore, be envisioned that snoRNP assembly also involves SMN in the nucleoplasm or in gems. The close association and relationship between gems and coiled bodies, which are enriched in fibrillarin, lend further support for this thought. SMN and SIP1 may also play a role in the biogenesis of other nuclear RNPs, and further experiments will be necessary to examine this possibility.

Our findings connect the SMA disease gene *SMN* to a specific biochemical pathway and identify two proteins, SMN and SIP1, that are essential to the fundamental cellular process of splicesomal snRNP biogenesis. As motor neurons are the target tissue in SMA, it is of great interest that these cells also contain very high levels of SMN and SIP1 and have very prominently staining gems (Lefebvre et al., 1997). Significantly, severely affected (type I) SMA patients have reduced levels of the SMN protein and show no detectable immunohistochemical staining with antibodies to SMN or SIP1 in their motor neurons (Lefebvre et al., 1997). It is, therefore, possible that SMA results from a defect in snRNP assembly in motor neurons. It will be of interest to determine if indeed there is a decrease in splicesomal snRNP assembly in motor neurons of SMA type I patients. It is, however, also possible that several additional defects occur in motor neurons of SMA patients. For example, the stimulation of inappropriate assembly of Sm proteins on RNAs seen upon microinjection of anti-SMN antibodies raises the possibility that there may be missassembly of Sm proteins on various RNAs in patient cells. The biogenesis of other RNPs, in addition to splicesomal snRNPs, may also be affected in these patients. Experiments are in progress to address these questions.

Experimental Procedures

Western Blot Analysis

Oocytes were defolliculated and dissected into nuclear and cytoplasmic fractions (see below). The nuclei were precipitated in ethanol. The cytoplasmic fractions were homogenized in 5:1 buffer (Fischer et al., 1993) and centrifuged for 15 min at 4°C. The supernatant was transferred to a new test tube and precipitated with four volumes of acetone. After centrifugation, the pellets were washed with 70% ethanol, dried, and resuspended in SDS sample buffer. For Western blot analysis, proteins were separated on an SDS-polyacrylamide gel (12.5%) and subsequently transferred to a nitrocellulose membrane (Schleicher and Schuell, Inc., Keene, NH) using a BioTrans Model B Transblot apparatus (Gelman Science) according to the manufacturer's instructions. After protein transfer, the blotting membrane was incubated in phosphate-buffered saline (PBS) containing 5% nonfat milk for 1 hr at room temperature, rinsed with PBS, and then incubated in the same solution with the primary antibody for 1 hr at room temperature. The membrane was then washed three times with PBS containing 0.1% Tween 20, and bound antibodies were detected using peroxidase-conjugated goat anti-mouse IgG plus IgM (Jackson ImmunoResearch Laboratories). Proteins were visualized using an ECL Western blotting detection kit (Amersham) after washing the membrane three times in PBS containing 0.1% Tween 20.

Oocyte Injections

Injections were carried out as described in Fischer et al. (1993). In brief, oocytes were incubated for 3 hr in modified Barth's solution containing 0.2% collagenase type II (Sigma). Defolliculated stage V and VI oocytes were collected and usually used on the same day for microinjection.

In a typical injection experiment, 30 nl of ^{32}P-labeled RNA (1×10^6 cpm/μl; total concentration of 0.7 μM) was injected either into the nucleus or into the cytoplasm. For the antibody inhibition experiments, oocytes were preinjected with antibody (1 μg/μl or 3 μg/μl in Figure 4B) and incubated for 1 hr before they received a second injection of [^{32}P]RNA. Nucleo-cytoplasmic transport of injected RNAs was monitored by dissection of the oocytes into nuclear and cytoplasmic fractions. Both fractions were incubated for 20 min in homogenization buffer, and the RNAs were isolated and analyzed by electrophoresis on denaturing RNA gels as described in Hamm et al. (1990).

Immunoprecipitation of RNA–Protein Complexes

For immunoprecipitation of RNA–protein complexes (Fischer et al., 1993), the injected oocytes were homogenized in 300 μl of ice-cold PBS (pH 7.4). The insoluble fraction was pelleted by centrifugation, and the clear supernatant was transferred into a new 1.5 ml Eppendorf tube containing antibodies bound to protein G-Sepharose (Pharmacia). This mixture was incubated with constant shaking for 1 hr at 4°C and subsequently washed five times with 1 ml aliquots of ice-cold PBS. Bound RNAs were isolated by phenol extraction for 1 hr, precipitated with ethanol, and analyzed by denaturing gel electrophoresis.

Plasmid DNA In Vitro Transcription and Translation

Plasmids coding for U1, U2, U4, U5, and U6 snRNAs have been described in Hamm et al. (1990) and Fischer et al. (1993, 1995). Plasmids encoding dihydrofolate reductase mRNA (DHFR) and tRNA^{met} were described in Jarmolowski and Mattaj (1993) and Jarmolowski et al. (1994) and in Fischer et al. (1995). A plasmid coding for the human U3 snoRNA was a kind gift of C. Marshallsay. Clones encoding the Sm proteins B, D1, D2, D3, E, F, and G are described in Raker et al. (1996). The clone encoding SIP1 is described in Liu et al. (1997). In vitro transcription of ^{32}P-labeled RNAs was carried out exactly as described in Fischer et al. (1993). Labeled RNA was precipitated in ethanol and resuspended in water. For nuclear injection, RNA was dissolved in water containing 10 mg/ml dextran blue (MW 1,000,000). In vitro translation of ^{35}S-labeled proteins was carried out using a combined transcription and translation kit (TnT) (Promega) according to the instructions of the manufacturer.

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