Cell, Vol. 90, 1013–1021, September 19, 1997, Copyright ©1997 by Cell Press

The Spinal Muscular Atrophy Disease Gene Product, SMN, and Its Associated Protein SIP1 Are in a Complex with Spliceosomal snRNP Proteins

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Summary

Spinal muscular atrophy (SMA), one of the most common fatal autosomal recessive diseases, is characterized by degeneration of motor neurons and muscular atrophy. The SMA disease gene, termed Survival of Motor Neurons (*SMN*), is deleted or mutated in over 98% of SMA patients. The function of the SMN protein is unknown. We found that SMN is tightly associated with a novel protein, SIP1, and together they form a specific complex with several spliceosomal snRNP proteins. SMN interacts directly with several of the snRNP Sm core proteins, including B, D1–3, and E. Interestingly, SIP1 has significant sequence similarity with Brr1, a yeast protein critical for snRNP biogenesis. These findings suggest a role for SMN and SIP1 in spliceosomal snRNP biogenesis and function and provide a likely molecular mechanism for the cause of SMA.

Introduction

Spinal muscular atrophy (SMA) is characterized by degeneration of anterior horn cells of the spinal cord, leading to progressive symmetrical limb and trunk paralysis and muscular atrophy. It is the second most common fatal autosomal recessive disorder after cystic fibrosis and the most common genetic cause of childhood mortality (Roberts et al., 1970; Pearn, 1973, 1978; Czeizei and Hamular, 1989). Childhood spinal muscular atrophies are divided into severe (type I, Werdnig-Hoffman disease) and mild forms (type II and III) according to the age of onset and the severity of the disease (Munsat, 1991; Crawford and Pardo, 1996). The Survival of Motor Neurons (*SMN*) gene (Lefebvre et al., 1995) has been shown to be the SMA disease gene, and it is deleted or mutated in over 98% of SMA patients (Bussaglia et al., 1995; Chang et al., 1995; Cobben et al., 1995; Hahnen et al., 1995, 1996; Lefebvre et al., 1995; Rodrigues et al., 1995; Velasco et al., 1996; Lefebvre et al., 1997). The *SMN* gene encodes a protein of 296 amino acids with a calculated molecular mass of 32 kDa (Lefebvre et al., 1995). The sequence of the protein does not show any significant homology to any other protein in the databases.

Recently, in the course of studies of the functions of heterogeneous nuclear ribonucleoproteins (hnRNPs) (Dreyfuss et al., 1993), we found that the SMN protein interacts with fibrillarin, an RNA-binding protein involved in rRNA processing, and with several other RNA-binding proteins (Liu and Dreyfuss, 1996). By use of monoclonal antibodies to SMN, we have also found that it has a unique cellular localization. SMN shows general localization in the cytoplasm and is particularly concentrated in several prominent nuclear bodies called gems. Gems are novel nuclear structures. They are related in number and size to coiled bodies and are usually found in close proximity to them (Liu and Dreyfuss, 1996). Coiled bodies are prominent nuclear bodies found in widely divergent organisms, including plant and animal cells (Bohmann et al., 1995a; reviewed in Gall et al., 1995). Coiled bodies contain the spliceosomal U1, U2, U4/U6, and U5 snRNPs, U3 snoRNAs, and several proteins, including the specific marker p80-coilin, fibrillarin, and NOP140 (Bohmann et al., 1995a, and references therein; Gall et al., 1995). Expression of p80-coilin mutants and microscopic observations suggests a close association between coiled bodies and the nucleolus (Raska et al., 1990; Andrade et al., 1991; Bohmann et al., 1995b). However, the specific functions of coiled bodies are not clear. Current ideas propose that coiled bodies may be involved in processing, sorting, and assembly of snRNAs and snoRNAs in the nucleus. The close association of gems and coiled bodies raises the possibility that the SMN protein and gems are also involved in the processing and metabolism of small nuclear RNAs (Liu and Dreyfuss, 1996).

The biogenesis of snRNPs is a complex, multistep process (DeRobertis, 1983; Fisher et al., 1985; Mattaj, 1988; Feeney et al., 1989; Neuman de Vegvar and Dahlberg, 1990; Zieve and Sauterer, 1990). Spliceosomal snRNAs that contain the Sm site (a short, single-stranded, eight-to-ten-nucleotide uridine-rich sequence) are first exported to the cytoplasm, where they associate with the Sm proteins (B, B′, D1, D2, D3, E, F, and G) (Mattaj and DeRobertis, 1985). Next, in a reaction that requires the assembled Sm core domain (comprising the Sm proteins bound to the Sm site), the 7-methylguanosine (m^7G) cap of the snRNAs is hypermethylated to yield 2,2,7-trimethylguanosine (m_3G) (Mattaj, 1986). In addition, varying numbers of nucleotides are trimmed from the 3′ end of several of the snRNAs. Proper Sm core assembly, cap hypermethylation, and 3′-end processing are important for nuclear import of the assembled snRNP particles (Fischer and Lührmann, 1990; Hamm et al., 1990). Finally, just before or after the nuclear import, many internal nucleotides are modified and more than 30 snRNP-specific proteins associate with the individual snRNP precursors to complete their biogenesis (Mattaj, 1988; Lührmann et al., 1990; Neuman de Vegvar and Dahlberg, 1990; Zieve and Sauterer, 1990). However, the detailed mechanism of how the Sm core proteins and the snRNP-specific proteins form functional assembled snRNPs is not clear.

Here we report the molecular cloning and characterization of a protein designated SIP1 (for SMN-interacting protein 1) that forms a stable heteromeric complex with SMN in vivo and in vitro. SIP1 is a novel protein, and it colocalizes with SMN in gems and in the cytoplasm. We

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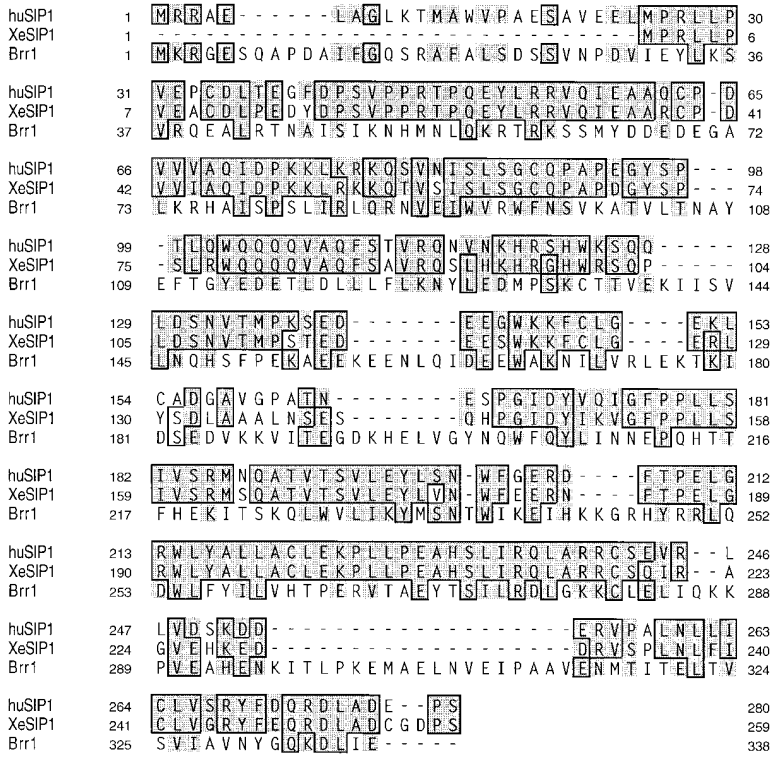

Figure 1. Amino Acid Sequence Alignment of Human SIP1 (huSIP1) and Xenopus SIP1 (XeSIP1)

Also shown is the amino acid sequence alignment of SIP1 with the S. cerevisiae Brr1 protein. The boxes indicate identical amino acids, and the borderless gray boxes indicate similar amino acids.

have isolated a large protein complex (ca. 300 kDa) that contains both SMN and SIP1 together with several spliceosomal snRNP proteins. We have found that SMN interacts directly with several spliceosomal snRNP core Sm proteins, including B/B′ and the D and E group proteins. Interestingly, we found that SIP1 has limited but significant similarity to the recently described yeast protein Brr1, which has been shown to play a role in the production of spliceosomal snRNPs (Noble and Guthrie, 1996a, 1996b). SMA may, therefore, be the result of a genetic defect in spliceosomal snRNP biogenesis in motor neurons.

Results

SIP1, a Novel SMN-Interacting Protein

Using SMN as a bait in a yeast two-hybrid screen of a HeLa cDNA library, we isolated ten independent partial cDNA clones with insert sizes from 1 kb to 1.3 kb, all of which contained the same open reading frame. The longest of these clones, designated 7-10, contained an insert of 1.3 kb that was completely sequenced. Using the BLAST search program to search the GenBank database, an EST (clone #Z64761) (Cross et al., 1994) that is identical to the 5′ end of clone 7-10 and extends further upstream was identified. Conceptional translation of this cDNA revealed another potential methionine 24 amino acids upstream of the first methionine of clone 7-10. Immediately upstream of this methionine is a stop codon. We are not certain which methionine is the actual initiation methionine for the full-length cDNA. The 3′-untranslated region is very AU-rich and contains a putative polyadenylation site AAUAAA. Thus, this is likely the full-length cDNA clone for the novel protein that interacts with SMN, which we term SIP1. The predicted amino acid sequence of SIP1, along with the sequence of the Xenopus laevis homolog that we isolated as described below, is presented in Figure 1. SIP1 encodes a protein of apparently 279 amino acids (including the potential 24 amino acids predicted by the EST sequence) with a calculated molecular mass of 32 kDa and pI of 5.3.

To examine the interaction of SIP1 with SMN and to characterize SIP1 further, we generated monoclonal antibodies to the SIP1 protein by immunizing mice with purified recombinant 6His-tag SIP1 (starting with the second methionine). Two of these monoclonals, 2E17 and 2S7, were further characterized in detail and shown to react with SIP1 specifically by both immunoprecipitation and Western blotting (data not shown). 2E17 reacted also with a protein of similar size in Xenopus, and using this as the primary antibody, we screened a Xenopus oocyte cDNA library and obtained the Xenopus homolog of SIP1. The predicted amino acid sequence of Xenopus SIP1 is presented in Figure 1. Interestingly, all of the eight clones we obtained from the library screen lack the first 24 amino acids that are potentially found in the human EST clone. Xenopus SIP1 is highly similar to human SIP1, the two proteins being ~90% identical in amino acid sequence. BLAST searches did not reveal significant homology to any other protein in the databases. However, we subsequently noticed a yeast protein, termed Brr1, that has significant similarity to SIP1 (Figure 1), and this is discussed below (see Discussion).

SIP1 Interacts with SMN In Vitro and In Vivo

In order to confirm the yeast two-hybrid results, we tested for interaction of SIP1 with SMN both in vitro and

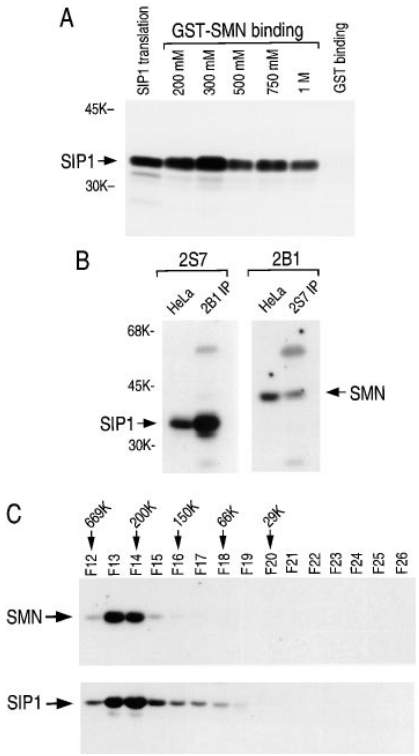

Figure 2. SIP1 Interacts with SMN In Vitro and In Vivo

(A) SIP1 interacts with GST-SMN in vitro. The interaction is resistant to a 1 M salt (NaCl) wash. Under the same conditions, SIP1 does not bind to GST alone even at low salt (200 mM NaCl) concentration.

(B) SIP1 and SMN are associated in vivo. Immunoprecipitation from total HeLa extract was done with monoclonal antibodies 2B1 against SMN and 2S7 against SIP1. The immunoprecipitated proteins were analyzed by Western blot using 2S7 and 2B1, respectively (lane 2B1 IP and lane 2S7 IP).

(C) SMN and SIP1 form a complex of ca. 300 kDa or more in the cytoplasm. HeLa cytoplasmic S100 extract was fractionated on a G3000-SW column. The fractions were analyzed by SDS-PAGE, and the SMN and SIP1 proteins were detected by Western blot.

in HeLa cells in vivo. For the in vitro binding assay, SMN was expressed as a fusion protein with glutathione S-transferase (GST), and SIP1 was produced and labeled with [^{35}S]methionine by in vitro transcription and translation in rabbit reticulocyte lysate. Purified GST or GST-SMN fusion proteins immobilized on glutathione-Sepharose were incubated with labeled SIP1 protein. Following washing at various salt concentrations (200 mM to 1 M), bound proteins were dissociated by boiling in SDS-containing sample buffer, and the eluted material was analyzed by SDS-PAGE. As shown in Figure 2A, full-length SIP1 bound specifically to immobilized GST-SMN but not to GST alone. This binding appears to be very avid because it is not disrupted at 1 M NaCl. Immunoprecipitation experiments were performed to examine if SMN and SIP1 interact in vivo. Anti-SMN monoclonal antibody 2B1 (Liu and Dreyfuss, 1996) was used to immunoprecipitate SMN from total HeLa cell extract. The immunoprecipitates were then resolved by SDS-PAGE and immunoblotted with monoclonal antibody 2S7 against SIP1. As shown in Figure 2B (lane 2B1 IP), 2S7 readily detects SIP1 in the 2B1 immunoprecipitates, indicating that SIP1 is coimmunoprecipitated with SMN. In a reciprocal experiment, the SMN protein could also be coimmunoprecipitated by the anti-SIP1 monoclonal antibody 2S7 (Figure 2B, lane 2S7 IP). This result was confirmed with other monoclonal antibodies to SMN and to SIP1. As shown in Figure 2 and from data not shown, there is no crossreactivity between the anti-SMN and anti-SIP1 antibodies. These results indicate that SMN and SIP1 are associated in vivo and can be coimmunoprecipitated by either anti-SMN or anti-SIP1 antibodies.

Further indication of the existence in vivo of a complex containing both SIP1 and SMN was obtained from gel filtration experiments. HeLa nuclear and cytoplasmic S100 extracts were fractionated on a high performance gel filtration column, TSK-GEL G3000-SW, and each fraction was subjected to SDS-PAGE. SMN and SIP1 were detected in the column fractions by immunoblotting with specific monoclonal antibodies. Figure 2C shows the results of the cytoplasmic fractionation. SMN and SIP1 comigrate as a peak of ca. 300 kDa, suggesting that they are part of a large macromolecular complex. The observed size suggests that this complex contains either multiple copies of the SMN and SIP1 proteins or additional components. This complex is very stable, as it resists dissociation by 4 M urea, and it is observed in both the nuclear and cytoplasmic fractions.

SIP1 and SMN Colocalize in Gems in the Nucleus and in the Cytoplasm

Indirect immunofluorescence microscopy using the monoclonal antibodies 2E17 and 2S7 was performed on HeLa cells to determine the cellular localization of SIP1. Figures 3A and 3B show, by standard light microscopy immunofluorescence, that SIP1 is found throughout the cytoplasm and also displays intense staining of prominent discrete bodies in the nucleus. This pattern is very similar to that seen for the SMN protein (Liu and Dreyfuss, 1996), except that the nucleoplasmic staining of SIP1 is somewhat stronger than that seen for SMN. In order to determine if the intensely staining nuclear structures are gems or coiled bodies, we performed double-label laser confocal immunofluorescence experiments using antibodies against p80-coilin (Figure 3C), fibrillarin (Figure 3D), snRNP proteins (Y12, Figure 3E) found in coiled bodies, and SMN (Figure 3F) found in gems. Figures 3C–3E show the double labeling with the coiled body markers and with 2S7. The nuclear structures that contain SIP1 are clearly different from coiled bodies, but the two bodies are, in most cases, closely associated. However, the staining with the anti-SIP1 monoclonal antibody 2S7 and a rabbit serum raised against exon 7 of SMN show that SMN and SIP1 completely colocalize in gems (Figure 3F). The weak signal in the cytoplasm makes it impossible to determine whether SMN and SIP1 also completely colocalize in the cytoplasm. However, we believe this is very likely because of the tight association of SMN with SIP1 described above (see also accompanying paper, Fischer et al., 1997 [this issue of *Cell*]). The colocalization of SMN with SIP1 further supports the conclusion that these two

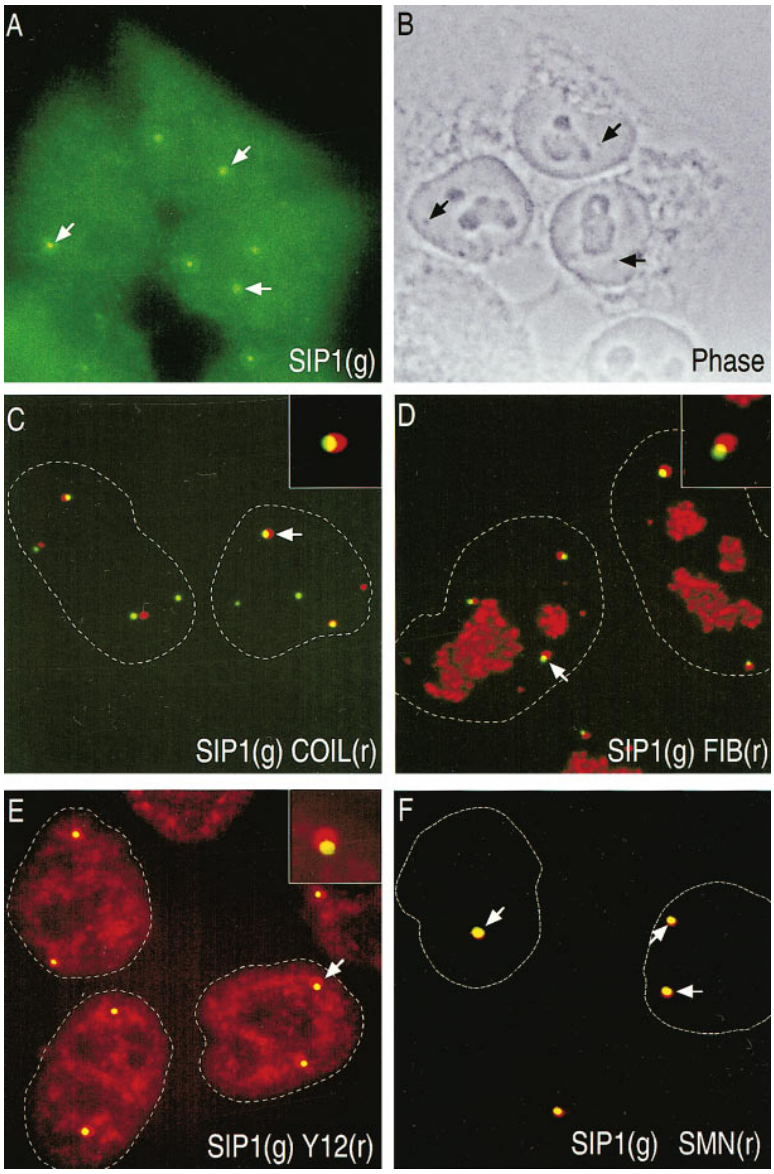

Figure 3. The SIP1 Protein Colocalizes with SMN in Gems

(A) Light microscopic image of indirect immunofluorescence experiment on HeLa cells using monoclonal antibody 2S7 against the SIP1 protein. Note the general cytoplasmic staining and discrete nuclear structures.

(B) Phase contrast image of the same field as in (A).

(C–E) Superimposed laser confocal images of double-label immunofluorescence microscopy experiments using antibodies against SIP1 (green) and antibodies against coiled body markers, p80-coilin ([C], red), fibrillarin ([D], red), and anti-Sm antibody Y12 ([E], red).

(F) The superimposed confocal images of double-label immunofluorescence microscopy experiments using monoclonal antibody against SIP1 (green) and a rabbit polyclonal serum raised against exon 7 of the human SMN protein (red). Colocalization of green and red results in yellow color.

proteins exist as a complex in the cell. SIP1 is thus the second constituent of gems described so far.

The SMN–SIP1 Complex Contains Spliceosomal snRNP Proteins

The observation that SMN and SIP1 are in a complex of ca. 300 kDa prompted us to search for other protein components in this complex. To do this, we carried out immunoprecipitations using anti-SMN and anti-SIP1 monoclonal antibodies from ^{35}S-labeled HeLa cell lysates, and the immunoprecipitated proteins were then analyzed by SDS-PAGE. As shown in Figure 4A, similar patterns were obtained with anti-SMN and anti-SIP1 antibodies. Several proteins can be specifically coimmunopurified by anti-SMN and anti-SIP1 antibodies. Besides SMN and SIP1 proteins, there is a prominent doublet at ~97 kDa, a group of proteins of ~28 kDa, and a group of proteins of ~15 kDa. This protein complex is quite stable, since it is resistant to SDS/Triton X100/deoxycholate-containing buffer and to a high salt wash (500 mM NaCl). As a reference for these immunoprecipitations (and for reasons discussed below), we also included a lane showing an immunoprecipitation with the monoclonal antibody Y12 against the Sm proteins common to spliceosomal snRNPs (Figure 4A, lane Y12) (Lerner and Steitz, 1979; Lerner et al., 1981). To characterize further this complex, we used two-dimensional nonequilibrium pH gradient gel electrophoresis (NEPHGE). Figure 4B shows the major proteins that are specifically found in the anti-SMN (2B1) isolated complex but not in control (SP2/0), and these are labeled as SIP1, SIP2, SIP3, and SIP4. A group of basic, low molecular weight proteins in the anti-SMN immunoprecipitate show the same pattern as some of the Sm proteins in immunopurified snRNPs. For direct comparison, Y12 immunoprecipitate from HeLa nuclear fractions was analyzed in parallel, and U1A, Sm B/B′, D1–3, E, F, and G proteins of snRNPs migrate in exactly the same pattern as those

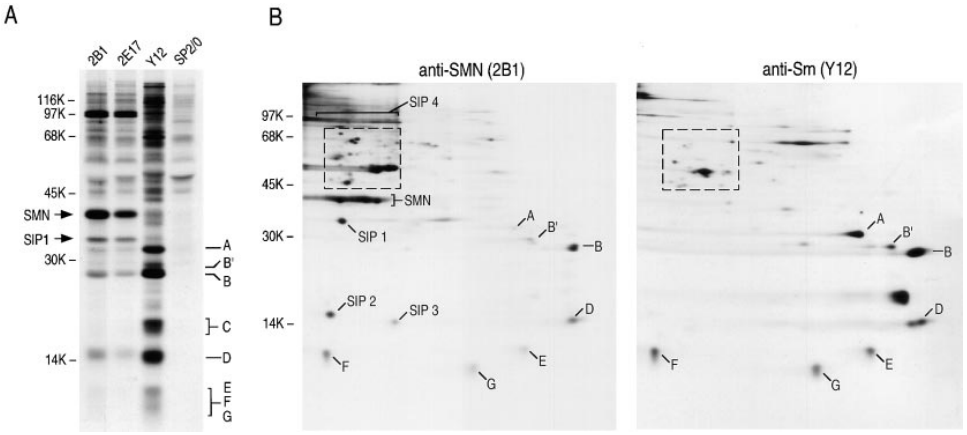

Figure 4. Immunoprecipitation of SMN- and SIP1-Containing Complexes with Monoclonal Antibodies against SMN and SIP1

(A) Immunoprecipitation using monoclonal antibodies 2B1 (lane 2B1) and 2E17 (lane 2E17) from [^{35}S]methionine-labeled HeLa total cell extract shows a similar pattern. Control antibody Y12 immunoprecipitates Sm proteins and some snRNP-specific proteins (lane Y12), while SP2/0 shows the background of immunoprecipitation (lane SP2/0).

(B) Two-dimensional nonequilibrium pH gradient gel electrophoresis (NEPHGE) analysis of SMN-immunoprecipitated complex (2B1 panel) and the Sm-immunoprecipitated complexes containing core Sm proteins and some U snRNP-specific proteins immunoprecipitated with monoclonal antibody Y12 from HeLa nuclear fractions (Y12 panel). The dashed boxes indicate background proteins that are also seen in control SP2/0 immunoprecipitations (data not shown).

proteins in the SMN complex. Immunoblotting experiments with monoclonal antibodies against the U1 snRNP-specific protein U1A and the anti-Sm monoclonal antibody Y12 confirmed that these proteins are indeed the spliceosomal snRNP proteins. Immunoprecipitations with Y12 starting with either total HeLa extract or from nucleoplasm also showed that SMN and SIP1 could be detected in Y12 immunoprecipitates by immunoblotting (data not shown). The immunoprecipitations shown in Figure 4 were carried out using nucleoplasm as the starting material. Similar results, although with considerably higher background, were obtained from cytoplasmic or whole cell extracts, and predigestion with RNases did not reduce the signal (data not shown). This suggests the SMN–SIP1–Sm protein complexes are found both in the nucleus and in the cytoplasm. Though we have not been able to determine definitively whether the immunoprecipitated complexes contain snRNAs (the resistence of the complexes to RNase digestion does not preclude this possibility, as the RNAs may not be fully accesible), experiments described in Fischer et al. (1997) demonstrate that SMN and SIP1 can immunoprecipitate labeled snRNAs.

The SMN Protein Interacts with Sm B/B′, Sm D, and Sm E Proteins Directly

Most of the snRNAs in snRNP complexes are resistant to RNase digestion and this, therefore, makes it difficult to determine if the SMN–SIP1–snRNP protein complexes result from protein–protein or protein–RNA interactions. To investigate whether SMN interacts with snRNP proteins directly, we used in vitro translated [^{35}S]methionine-labeled SMN in a far-Western blot assay to probe SDS-PAGE-resolved proteins of purified snRNPs. The protein composition of purified snRNPs is shown in Figure 5 (lane snRNPs). The result of probing these proteins with [^{35}S]SMN (Figure 5, lane [^{35}S]SMN) indicates that SMN binds specifically and directly to Sm B/B′ and also one or several of the Sm D proteins. When the same experiments were done with in vitro translated SIP1, we did not detect any specific binding of SIP1 to snRNP proteins, although, in the same assay, SIP1 binds strongly to recombinant SMN protein (data not shown). These findings suggest that the association of SMN with snRNPs occurs via direct protein–protein interaction between SMN and Sm B/B′ and one or more of the D group proteins, although we cannot entirely exclude the possibility that some component in the rabbit reticulocyte lysate mediates this interaction. To study this further and to examine the binding in solution to other Sm proteins, we used in vitro translated and ^{35}S-labeled Sm proteins B, D1, D2, D3, E, F, and G and tested their ability to bind to recombinant GST-SMN fusion protein immobilized on glutathione-Sepharose beads (Lehmeier et al., 1994; Herrmann et al., 1995; Raker et al., 1996). As shown in Figure 5B, all Sm proteins except for F and G bound efficiently to GST-SMN, whereas there was no detectable binding to GST alone. However, similar experiments failed to detect any interaction between the Sm proteins and SIP1 (data not shown).

SMN Contains Two Distinct Binding Sites for the Sm Proteins and for SIP1

The experiments described above showed that SMN interacts with both SIP1 and several of the Sm proteins, and we therefore tested whether binding of SMN to Sm proteins and SIP1 was mutually exclusive or could occur at the same time via two different binding sites on the SMN protein. To analyze this, peptides were synthesized corresponding to the two most conserved regions of SMN (determined by comparing the sequence of the human SMN with that of Xenopus SMN, which we cloned and sequenced; data not shown) located at the N terminus (aa 13–44) and the C terminus (aa 240–267), assuming that these domains may be involved in important

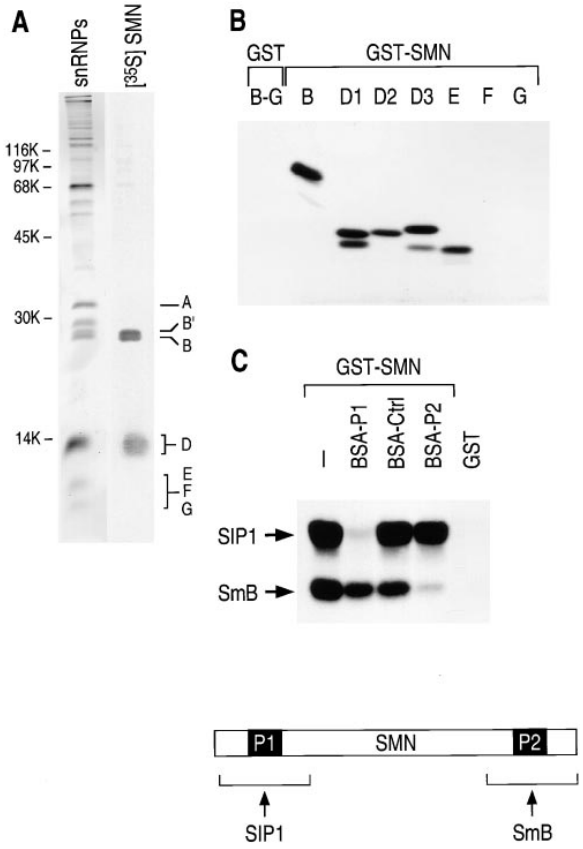

Figure 5. The SMN Protein Can Bind to Sm B/B′, D1–3, and E Proteins Directly

(A) Purified snRNP proteins were analyzed on SDS-PAGE (lane snRNPs) and transferred to a nitrocellulose membrane. The membrane was then probed with 2×10^6 cpm of in vitro translated ^{35}S-labeled SMN protein. After washing away the nonspecific binding proteins, the membrane was exposed to an X-ray film (lane [^{35}S]SMN). We can not distinguish among the three D proteins in this gel system. SMN has two distinct binding domains for the Sm proteins and SIP1, respectively.

(B) In vitro translated [^{35}S]-labeled Sm proteins B, D1, D2, D3, E, F, and G were incubated for 30 min at 4°C with GST-SMN immobilized on glutathione-Sepharose beads. The beads were then washed six times with binding buffer (see Experimental Procedures). Proteins that remained bound to the beads were eluted by boiling in SDS-PAGE sample buffer and analyzed by SDS-PAGE followed by fluorography.

(C) In vitro translated [^{35}S]-labeled Sm B and SIP1 proteins were mixed, and binding to GST-SMN was carried out as described above either in the absence (-) or presence of BSA-coupled peptides corresponding to amino acids 13–44 (P1) or 240–267 (P2) of human SMN or to an unrelated control sequence from HIV-1 Rev NES (ctrl). After washing the beads, bound proteins were eluted by boiling in SDS-PAGE sample buffer and analyzed by SDS-PAGE followed by fluorography.

protein–protein interactions. These regions are also conserved in several candidate SMN orthologs identified in divergent organisms (Talbot et al., 1997). These peptides were then coupled to BSA (termed BSA-P1 and BSA-P2, respectively) and used as competitors in the binding assays for SMN to SIP1 and the Sm proteins. Without competitors, SIP1 and Sm B bound to GST-SMN (Figure 5C). However, BSA-P1 completely abolished binding of SIP1 to SMN, while the binding of Sm B was unaffected (Figure 5C). In contrast, BSA-P2 strongly inhibited the binding of Sm B to SMN but had no effect on the binding of SMN to SIP1. As a nonspecific control, we used BSA coupled to HIV1 Rev NES (BSA-Ctrl) (Fischer et al., 1995), and this, in contrast, had no effect on the binding of SMN to either protein (Figure 5C). Additional experiments showed that the corresponding domains of SMN are alone sufficient for binding to SIP1 and Sm B (data not shown). Similar results were obtained for the other Sm proteins. Thus, these experiments define two independent binding sites for SIP1 and the Sm proteins on SMN. SMN may therefore serve as a critical bridge between the Sm proteins and SIP1 and nucleate the formation of the SMN–SIP1–Sm complex.

Discussion

Molecular characterization of the protein product of the gene that causes SMA, *SMN*, has led to the discovery of a novel nuclear structure, called gem, in which SMN is concentrated (Liu and Dreyfuss, 1996). Gems appear by size, number, and proximity to be related to coiled bodies, another subnuclear structure of unknown function. Previous studies suggested that gems, like coiled bodies, are involved in RNA metabolism, and, consistent with this, SMN was found to interact with several RNA-binding proteins and possibly also with RNA directly (Liu and Dreyfuss, 1996; Liu et al., 1996). However, the specific function of SMN remained obscure. Here, in a yeast two-hybrid screen using SMN as the bait, we have identified a novel protein, SIP1. SIP1 forms a stable complex with SMN in vivo and in vitro, and it colocalizes with SMN in gems and in the cytoplasm. Several lines of evidence suggest that SMN and SIP1 function as a complex in vivo. The interaction of SMN and SIP1 in vitro is resistant to 1 M NaCl, suggesting that they interact avidly. A similarly strong interaction is found for several other protein complexes, such as the FMR1/FXR family protein (Zhang et al., 1995) and the Sm E, F, G complex (Raker et al., 1996). The 300 kDa complex that contains SMN and SIP1 is stable even in 4 M urea. Also, SMN and SIP1 can be coimmunoprecipitated with specific monoclonal antibodies. Further, a yeast two-hybrid screen using SIP1 as the bait under high stringency conditions (15 mM 3-aminotriazole) isolated from a human library only SMN clones (Q. L. and G. D., unpublished data). Finally, SMN and SIP1 colocalize in gems, suggesting that these two proteins function together. As loss or mutation of SMN leads to motor neuron degeneration, it will be interesting to determine if mutations in or loss of SIP1 also causes neurodegenerative disease. Chromosomal mapping and experiments to generate SIP1-null mutation in the mouse and determine their effect are under way.

Immunopurification of the 300 kDa complex showed that it contains, besides SMN and SIP1, spliceosomal snRNP core proteins including B/B′, D, E, F, and G, the snRNP-specific protein U1A, and several other unidentified proteins. Furthermore, we found that SMN interacts directly with several spliceosomal snRNP core Sm proteins, including B/B′, D1–3, and E. These data suggest

that the SMN–SIP1 complex plays an important role in spliceosomal snRNP biogenesis and/or function. Although BLAST searches of sequence databases with the SIP1 protein did not find significant homology to any other proteins, we noticed by visual inspection limited but significant homology between SIP1 and the yeast protein Brr1 (Noble and Guthrie, 1996a, 1996b). The sequence alignment of the two proteins is shown in Figure 1. Brr1 has been shown to be involved in snRNP biogenesis in Saccharomyces cerevisiae. Deletion of Brr1 causes destabilization of newly synthesized spliceosomal U2 snRNA, and Brr1 interacts genetically with the yeast Sm D1 protein (Noble and Guthrie, 1996b). Searching the yeast genome sequence for possible SMN homologs has not identified any proteins with significant similarity. However, if Brr1 is the yeast homolog of vertebrate SIP1, one would expect that computer searches would also not be able to pick up the homology between the yeast SMN and the human SMN. It is also possible that there is no SMN homolog in S. cerevisiae and that although SIP1 functions with SMN in metazoan snRNP biogenesis, Brr1 fulfills the functions of both in yeast. To find out if SIP1 and Brr1 are functional homologs, it will be interesting to see if human SIP1 can rescue the phenotype of Brr1 deficiency in yeast. SMN, because it can interact with SIP1 and with the spliceosomal snRNP Sm proteins via different domains, can potentially serve as the key bridging component to bring together the various components of the complex. It is therefore particularly interesting to note that many SMA patients have deletions or point mutations encompassing exons 6 and 7 of SMN, the region we show here to be involved in binding of SMN to the Sm proteins. Several missense mutations in the the region of SMN corresponding to the P2 peptide have recently been described (Hahnen et al., 1996), and it will be of interest to determine if such mutations exhibit altered binding to the Sm proteins. Important information about the functions of the SMN–SIP1-containing complex will also likely come from the characterization of the unidentified proteins SIP2, SIP3, and SIP4.

Together, these observations provide an important advance in understanding the molecular mechanism of SMA as well as in the fundamental process of spliceosomal snRNP biogenesis. It is particularly intriguing that motor neurons in the spinal cord contain an extremely high concentration of SMN and SIP1 compared with other tissues and that these cells also exhibit the most prominent gems and coiled bodies. However, in motor neurons of SMA type I patients, there are no detectable gems, while coiled bodies appear normal (Lefebvre et al., 1997). Thus, motor neurons probably have a very high requirement for SMN and gems, and these cells may, therefore, be most sensitive to reduced amounts of the protein or to mutations in it, which result in their degeneration. It is, however, also possible that SMN has a specific function in motor neurons. In this vein, we note that there is a neuronal-specific Sm protein, the SmN protein (Latchman, 1990).

Experimental Procedures

Yeast Two-Hybrid Screening

The human HeLa cDNA library, yeast strains, and yeast plasmids pGBT9, pGADGH, pVA3, and pTD1 were from Clontech, Inc. The manipulation of yeast and the library screening were carried out according to the conditions suggested by the manufacturer. In brief, the coding region of SMN was cloned into the pGBT9 vector. The S. cerevisiae HF7c reporter strain was first transformed with pGBT9-derived SMN construct and subsequently with the HeLa cDNA library. Approximately 6×10^6 transformants were seeded on eight 150 mm plates containing synthetic medium lacking histidine, leucine, and tryptophan. His^+ colonies were grown on synthetic medium plates lacking leucine and tryptophan and then assayed for β-galactosidase activity by filter assay as described by the manufacturer. Of 6 million transformants screened, 146 were His^+ $LacZ^+$ colonies. These positives fell into ten groups. One of these groups has ten independent cDNA clones that all encode identical sequences of SIP1. The library plasmid was recovered from these clones into the Escherichia coli HB101 strain. True positive clones were confirmed by their ability to transactivate HIS3 and LacZ reporters when cotransforming HF7c with pGBT9 containing SMN.

Production of Monoclonal Antibodies to SIP1

Anti-SIP1 antibodies 2S7 and 2K9 were prepared by immunizing Balb/C mice with His6-tag SMN protein purified from nickel chelation chromatography using a Novagen His-Bind buffer kit. Hybridoma production and screening and ascites fluid production were performed as previously described (Choi and Dreyfuss, 1984).

Production of Proteins In Vitro

The [^{35}S]-labeled proteins were produced by an in vitro transcription–translation reaction (Promega Biotech) in the presence of [^{35}S]methionine (Amersham). His6-SMN fusion protein was expressed from a pET bacterial expression system in the E. coli strain BL2l(DE3)pLysS and purified using nickel chelation chromatography using the same kit as described above. GST-SMN fusion protein was expressed from a GST expression vector pGEX-5X-3 (Pharmacia) in the E. coli strain BL21 and purified using glutathione-Sepharose provided by Pharmacia Biotech according to the manufacturer's protocol.

In Vitro Protein-Binding Assays

Purified GST or GST fusion protein (2 μg) was incubated with 10^6 cpm of the in vitro translated protein product and 25 μl of glutathione-Sepharose beads in 500 μl of binding buffer (50 mM Tris-HCl [pH 7.5], 2 mM EDTA, 0.1% NP40, 2 μg/ml leupeptin and pepstatin A, and 0.5% aprotinin) containing different salt (NaCl) concentrations. Following incubation for 30 min at 4°C, the resin was pelleted, washed with binding buffer, and the bound fraction was eluted by boiling in SDS-PAGE sample buffer, analyzed by SDS-PAGE, and visualized by fluorography. For the binding experiments described in Figure 5, 200 to 300 ng of GST or GST-SMN fusion protein was bound to 30 μl of glutathione-Sepharose beads dissolved in PBS (pH 7.4) and incubated with ^{35}S-labeled in vitro translated protein (ca. 1×10^5 to 2×10^5 cpm/assay) for 30 min at 4°C. The beads were subsequently washed six times with PBS/350 mM NaCl (pH 7.4), and the bound fraction was eluted by boiling in SDS-PAGE sample buffer.

Cell Culture and Treatments

HeLa cells and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS (GIBCO BRL). Low temperature incubations were carried out as follows. HeLa cells were shifted to 32°C and incubated for 24 hr prior to fixation and permeablization for immunostaining. For actinomycin D treatment, HeLa cells were incubated with 5 μg/ml of actinomycin D for 3 hr before fixation for immunostaining. For in vivo labeling with [^{35}S]methionine, 50% confluent HeLa cells growing in 100 mm plates were incubated with 10 μCi/ml [^{35}S]methionine in DMEM without methionine and supplemented with 10% FCS overnight before homogenization for immunoprecipitation.

Immunoprecipitation and Immunoblotting

Immunoprecipitation of in vitro translated SIP1 protein was carried out in the presence of 1% Empigen BB buffer as previously described (Choi and Dreyfuss, 1984). Immunoprecipitation and purification of the SMN complex was carried out using total HeLa lysate

in the presence of 0.5% TritonX-100 as previously described (Piñol-Roma et al., 1988). For immunoblotting, proteins were resolved on a 12.5% SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Schleicher and Schuell, Inc., Keene, NH) using a Bio-Trans Model B Transblot apparatus (Gelman Science) according to the instructions of the manufacturer. Filters were incubated in blotting solution (PBS, 5% nonfat milk) for at least 1 hr at room temperature, rinsed with cold PBS, and then incubated with primary antibody for at least 1 hr at room temperature. Filters were washed three times in PBS containing 0.1% Tween 20, and bound antibodies were detected using the peroxidase-conjugated goat anti-mouse IgG plus IgM (Jackson ImmunoResearch Laboratories). The protein bands were visualized by an ECL Western blotting detection kit (Amersham) after washing three times in PBS containing 0.1% Tween 20.

Immunofluorescence Microscopy

Immunofluorescence microscopy was carried out essentially as previously described (Choi and Dreyfuss, 1984) with the following exceptions. Primary monoclonal antibodies 2B1 and 1816 were diluted 1:1000 in PBS containing 3% BSA. The incubation with the first and second antibody was at room temperature for 1 hr. In double-label immunofluorescence experiments, a mixture of primary or secondary antibodies was incubated at the same time. Laser confocal fluorescence microscopy was performed with a Leica TCS 4D (Germany) confocal microscope. Antibodies used for these experiments were as follows: antibody against p80-coilin: monoclonal antibody Pd and rabbit polyserum R288; antibody against fibrillarin: human autoimmune antibody 1881. Rabbit polycolonal antibody against SMN was raised against exon 7 for SMN protein by Quality Control Biochemicals and affinity purified.

HeLa Cell Fractionation and Chromatography

HeLa cells were fractionated according to Dignam et al. (1983). Fractionation of the nuclear or cytoplasmic S100 fraction was carried out as follows. The nuclear fraction (200 μl of ca. 20 mg/ml protein) in buffer D (20 mM Tris-HCl [pH 7.4], 0.1 mM EDTA, 1 mM DTT, 20% glycerol, 500 mM KCl) or S100 fraction (400 μl of ca. 20 mg/ml protein) in buffer F (20 mM Tris-HCl [pH 7.4], 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 500 mM KCl) was loaded onto a TSK-GEL G3000-SW glass column (Toso-Haas, 08800). The column was then washed with buffer A (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 2.5% glycerol) with or without 4 M urea at 0.25 ml/min flow rate. Fractions (0.5 ml) were collected, and 15 μl of each fraction was analyzed on an SDS-PAGE.

Far Western Analyses Using [^{35}S]SMN

In vitro purified snRNP proteins were analyzed by SDS-PAGE, and the proteins were then transferred onto a nitrocellulose membrane as described above. The membrane was incubated in blotting solution (5% nonfat milk) for at least 1 hr at room temperature, rinsed with cold PBS, and then incubated with in vitro translated [^{35}S]methionine-labeled SMN (2×10^6 cpm) for 2 hr at room temperature. The membrane was washed three times in PBS containing 0.05% NP-40, and bound SMN was detected by exposing the membrane to X-ray film.

Preparation of BSA-Peptides Conjugates

BSA-peptide conjugates were prepared as described in Fischer et al. (1995). In brief, peptides starting with a N-terminal cysteine and consisting of amino acids 13–44 (CRRGAGQSDDSDIWDDTALIKAY DKAVS) and 240–267 (CEDDEALGSMLISWYMSGYHTGYYLGLKQ) of human SMN or the HIV-1 Rev NES (CLPPLERLTL) (Fischer et al., 1995) were cross-linked to BSA using sulfo-SMCC (Pierce) as a cross-linker. The cross-linked BSA conjugates were separated from unreacted peptides and cross-linking reagent by dialysis against PBS (pH 7.4) and concentrated with Centricon30 concentrator (Amicon).

Acknowledgments

We thank Dr. Eng M. Tan for the anti-p80 coilin and anti-fibrillarin antibodies, Dr. Maria Carmo-Fonseca for the anti-coilin monoclonal antibody, Dr. Joan A. Steitz for the anti-Sm Y12 monoclonal antibody, and Dr. Joseph Craft for the anti-fibrillarin antibodies. We thank Dr. Reinhard Lührmann and Veronica Raker for purified snRNP proteins and plasmids. We thank Dr. Peter Bannerman and Tracy Oliver for help with the confocal microscopy. We are grateful to members of our laboratory for stimulating discussions and, in particular, Drs. Paul Eder, W. Matthew Michael, Sara Nakielny, Victoria Pollard, and Haruhiko Siomi for helpful discussions and critical comments on this manuscript. U. F. received an AIDS scholarship and a grant from the Deutsche Forschungsgemeinschaft (SFB 286). This work was supported by the Howard Hughes Medical Institute and by a grant from the National Institute of Health.

Received April 11, 1997; revised August 7, 1997.

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