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RESEARCH ARTICLE

Hongzhi Xu · Ramesh S. Pillai · Teldja N. Azzouz ·
Karl B. Shpargel · Christian Kambach ·
Michael D. Hebert · Daniel Schümperli ·
A. Gregory Matera

The C-terminal domain of coilin interacts with Sm proteins and U snRNPs

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Abstract Coilin is the signature protein of the Cajal body (CB), a nuclear suborganelle involved in the biogenesis of small nuclear ribonucleoproteins (snRNPs). Newly imported Sm-class snRNPs are thought to traffic through CBs before proceeding to their final nuclear destinations. Loss of coilin function in mice leads to significant viability and fertility problems. Coilin interacts directly with the spinal muscular atrophy (SMA) protein via dimethylarginine residues in its C-terminal domain. Although coilin hypomethylation results in delocalization of survival of motor neurons (SMN) from CBs, high concentrations of snRNPs remain within these structures. Thus, CBs appear to be involved in snRNP maturation, but factors that tether snRNPs to CBs have not been described. In this report, we demonstrate that the coilin C-terminal domain binds directly to various Sm and Lsm proteins via their Sm motifs. We show that the region of coilin responsible for this binding activity is separable from that which binds to SMN. Interestingly, U2, U4, U5, and U6 snRNPs interact with the coilin C-terminal domain in a glutathione *S*-transferase (GST)-pulldown assay, whereas U1 and U7 snRNPs do not. Thus, the ability to interact with free Sm (and Lsm) proteins as well as with intact snRNPs, indicates that coilin and CBs may facilitate the modification of newly formed snRNPs, the regeneration of 'mature' snRNPs, or the reclamation of unassembled snRNP components.

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H. Xu · M. D. Hebert
Department of Biochemistry, The University of Mississippi Medical Center, Jackson,
MS, 39216-4505, USA

R. S. Pillai · T. N. Azzouz · D. Schümperli
Institute of Cell Biology, University of Bern,
Baltzerstrasse 4,
3012 Bern, Switzerland

K. B. Shpargel · M. D. Hebert · A. G. Matera (✉)
Department of Genetics, Case Western Reserve University,
10900 Euclid Avenue,
Cleveland, OH, 44106-4955, USA
e-mail: a.matera@case.edu
Tel.: +1-216-3684922
Fax: +1-216-3681257

C. Kambach
Paul Scherrer Institute, Life Sciences,
5232 Villigen PSI, Switzerland

R. S. Pillai
Friedrich Miescher Institute,
Maulbeerstrasse 66,
4058 Basel, Switzerland

Introduction

The nucleus contains many distinct yet dynamic structures that play roles in various aspects of RNA metabolism (reviewed in Carmo-Fonseca 2002a; Jackson 2003; Spector 2003). The most easily observable of these, the nucleolus, is well established as the site of rRNA synthesis, processing, and ribosomal subunit assembly. Other domains known as nuclear speckles are enriched in pre-mRNA splicing factors and are thought to serve as storage depots for these factors until they are needed in perichromatin fibrils, where splicing occurs concurrently with transcription (Lamond and Spector 2003). Among these splicing factors are the U1, U2, U4/U6, and U5 spliceosomal small nuclear ribonucleoproteins (U snRNPs). The Cajal body (CB), a nuclear structure discovered by Santiago Ramón y Cajal over 100 years ago, contains the highest concentration of U snRNPs in the nucleus and most likely plays a role in their metabolism (Gall 2003; Matera 2003).

The biogenesis of spliceosomal U snRNPs is a complicated process with multiple maturation steps that take place in a variety of subcellular compartments (reviewed in Carmo-Fonseca 2002b; Gerbi et al. 2003; Will and Luhrmann 2001). With the exception of the U6 RNA, the spliceosomal small nuclear RNAs (snRNAs) are synthesized by RNA polymerase II as immature precursors containing a 5′-monomethyl cap and extra nucleotides at the 3′-end. The RNA is then transported to the cytoplasm, possibly after visits to the CB and/or nucleolus (Gerbi et al. 2003; Will and Luhrmann 2001). In the cytoplasm, a

structural motif (called the Sm-binding site) within the snRNA is bound by a septet of Sm proteins (B/B′, D1, D2, D3, E, F, G) in an ordered manner that is regulated by the survival of motor neurons (SMN) protein complex. Patients with the neurodegenerative disorder spinal muscular atrophy (SMA) have mutations in the human *SMN1* gene, resulting in reduced levels of the SMN protein (reviewed in Meister et al. 2002; Paushkin et al. 2002). Although Sm assembly onto U snRNAs can occur in vitro without SMN and its associated proteins (Raker et al. 1996), recent work has shown that the SMN complex is required for proper assembly in vivo (Meister et al. 2001; Meister and Fischer 2002; Pellizzoni et al. 2002; Yong et al. 2004). After assembly of the Sm core, the snRNA is subject to cap hypermethylation and 3′-end trimming, followed by import back into the nucleus, again under the aegis of the SMN complex (Huang and Pederson 1999; Massenet et al. 2002; Mouaikel et al. 2003; Narayanan et al. 2002; Will and Luhrmann 2001).

Upon nuclear reentry, newly assembled snRNPs target first to the CBs (Sleeman and Lamond 1999), where the snRNAs are likely modified (Jady et al. 2003) and assembled into mature RNPs (Nesic et al. 2004; Tanackovic and Kramer 2005). These 2′-*O*-methyl sugar- and pseudouridine-base modifications of the U snRNPs (Darzacq et al. 2002; Jady et al. 2003; Verheggen et al. 2002) are guided by small CB-specific RNAs (scaRNAs) and are crucial for proper pre-mRNA splicing in vivo (Yu et al. 1998).

In contrast to U1, U2, U4, and U5 snRNAs, maturation of the RNA polymerase III-transcribed U6 snRNA does not include a cytoplasmic phase and may take place in the nucleolus and the CB (Kiss 2004; Stanek and Neugebauer 2004). Additionally, U6 snRNA lacks a canonical Sm-binding site, but contains a stretch of 3′ uridine residues that, following transcription termination, are initially bound by the La protein (reviewed in Wolin and Cedervall 2002). Subsequently, La is replaced by a heptameric ring composed of the Lsm (Sm-like) proteins 2–8. A slightly different subcomplex containing the Lsm proteins 1–7 plays a role in cytoplasmic mRNA degradation (reviewed in He and Parker 2000).

The U7 snRNP, which is required for histone pre-mRNA 3′-end processing (Dominski and Marzluff 1999; Muller and Schumperli 1997; Schumperli and Pillai 2004), has a noncanonical Sm-binding site. This sequence recruits the Sm proteins B/B′, D3, E, F, and G, but not D1 and D2 (Pillai et al. 2001). In the place of SmD1 and SmD2, the U7 snRNP contains U7-specific Lsm proteins, Lsm10 and Lsm11, respectively (Pillai et al. 2001, 2003). Assembly of the U7 snRNP is mediated by a specialized SMN complex that also lacks Sm D1 and D2, but contains Lsm10 and Lsm11 instead (Pillai et al. 2003). Otherwise, assembly and nuclear import of U7 snRNPs resembles the pathway for spliceosomal snRNPs (Stefanovic et al. 1995), and U7 snRNPs are predominantly localized to CBs with low levels being present in the nucleoplasm (Frey and Matera 1995; Pillai et al. 2001). Thus, both the spliceosomal U snRNPs and the U7 snRNP, along with many, but not all, of the snRNP-specific proteins are highly enriched within CBs. Exactly how these various proteins and RNAs are targeted to CBs is unknown.

The CB marker protein, coilin, is highly enriched in the CB, but the majority (70%) of the protein is diffusely localized throughout the nucleoplasm (Lam et al. 2002). Coilin is highly conserved among vertebrates and clear homologs can be found in plants (Tucker and Matera 2004), but the gene has not been identified in worms and flies. Although coilin appears to be absent from the genomes of worms and flies, structures similar to CBs have been identified in various insects including *Drosophila* (Gall et al. 1995; Jady et al. 2003; Yannoni and White 1997). Moreover, coilin is required for the accumulation of U snRNPs within the CBs of amphibians and mammals (Bauer and Gall 1997; Hebert et al. 2001; Tucker et al. 2001). Coilin knockout mice are viable when crossed onto an outbred strain, but have significant viability and fertility defects when maintained on inbred strains (Tucker et al. 2001; also our unpublished observations). Thus, coilin is not an essential protein, but its evolutionary conservation from plants to animals suggests it plays an important role, perhaps as an efficiency factor that coordinates the activities of various types of RNA-processing machineries that come together in one subnuclear locale (Gall 2000, 2003; Ogg and Lamond 2002; Tucker and Matera 2004).

Coilin contains several arginine/glycine (RG) dipeptide repeats that are required for interaction with SMN (Hebert et al. 2001). The arginines within this so-called RG box motif are symmetrically dimethylated (Boisvert et al. 2002; Hebert et al. 2002). Symmetric dimethylarginine (sDMA) modification strongly increases the affinity of SMN for RG dipeptide repeats (Brahms et al. 2001; Friesen et al. 2001). Consistent with these observations, coilin hypomethylation results in delocalization of SMN to twin structures called Gemini of CBs, or gems (Boisvert et al. 2002; Hebert et al. 2002). In addition to SMN, coilin's direct binding partners include itself (Hebert and Matera 2000), the nucleolar protein Nopp140 (Isaac et al. 1998) and SmB/B′ (Hebert et al. 2001). Coilin's ability to compete with SmB/B′ for binding sites on SMN raises the interesting possibility that locally high concentrations of coilin within the CB may play a role in snRNP targeting to this domain (Hebert et al. 2001).

We therefore examined whether or not coilin could bind to Sm proteins other than B/B′ and mapped the region(s) within the coilin C-terminal domain that are responsible for this activity. We also demonstrated that the Sm motif is the binding determinant for Sm protein–coilin interaction. The RG-rich tails of SmB/B′, D1, D3, and LSm4 are dispensable for this interaction. We also show that coilin binds directly to Lsm10 and Lsm11, which do not contain RG tails. Thus, while methylation of the RG tail-containing Sm proteins is important for their interaction with the SMN complex (Brahms et al. 2001; Friesen et al. 2001), it is not required for Sm binding to coilin. Furthermore, we identified a coilin subfragment that interacts with Sm proteins but not with SMN, thus separating the two binding ac-

tivities. Coilin can bind to posttranslationally modified Sm proteins isolated from HeLa cells or to unmodified Sm protein heterodimers and heterotrimers. Intriguingly, U2, U4, U5, and U6 snRNPs interacted with coilin, but the U1 and U7 snRNPs did not. Thus, coilin's ability to interact with free Sm (Lsm) proteins as well as intact snRNPs may facilitate snRNP biogenesis.

Materials and methods

In vitro binding assays

Glutathione *S*-transferase (GST)- and His-tagged constructs, after transformation into *Escherichia coli* BL21 (DE3)pLysS cells, were induced and purified as described (Hebert et al. 2001). In a binding reaction, ~1 μg of His-T7-tagged protein was incubated with 1 μg of the GST-fusion protein immobilized on glutathione sepharose beads in 1 ml of modified radioimmunoprecipitation buffer (mRIPA, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA) plus 2 mM dithiothreitol (DTT). After incubation for 1 h at 4°C with gentle inversion, the beads were washed five times (1 ml each) with mRIPA plus DTT, resuspended in 15 μl 5× sodium dodecyl sulfate (SDS) loading buffer, boiled, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Lsm10, SmD1, and SmD1ΔRG, as well as full-length and truncated versions of Lsm11 were made in reticulocyte lysate in the presence of ^{35}S-methionine and mixed with equimolar amounts of GST or GST–coilin fragments (~0.25 nmol each). The mixture was incubated for 2 h at 4°C in binding buffer [1× phosphate-buffered saline (PBS)+0.01% NP-40] with gentle inversion, followed by two washes using binding buffer. The beads were boiled in loading buffer and run on a SDS-PAGE, which was then dried and exposed to PhosphorImager. GST or GST-C214 beads (~0.25 nmol each) were also incubated with a total protein preparation from salt-disrupted spliceosomal snRNPs containing high concentrations of all Sm proteins (Sumpter et al. 1992) for 2 h at 4°C with inversion. The beads were then washed five times with binding buffer and then transferred to a new tube in the last washing step. The beads were boiled in SDS-PAGE loading buffer, loaded on a 12% gel and subjected to Western analysis using the anti-Sm antibody Y12, which recognizes primarily sDMA-modified Sm B/B′, D1, and D3 (Brahms et al. 2001). Such binding experiments were also carried out with 1.9 nmol each of purified, bacterially produced heterodimers of SmB lacking the RG-rich tail with SmD3, or SmD1 with SmD2 (Kambach et al. 1999b), or with similarly produced Sm F/E/G heterotrimers. In this case, the Western blots were probed with anti-Sm antibody Y12 or with antibodies specific for Sm D2, D3, G, or F (gifts of C.L. Will and R. Lührmann).

Purification of Sm protein subcomplexes

Sm protein heterodimers SmD1/D2 and SmD3/B as well as SmE/F/G heterohexamers (dimer of trimers) were produced in *E. coli*, as described in Kambach et al. (1999b). Briefly, the Sm complementary deoxyribonucleic acids (cDNAs) were subcloned as di- or tricistronic expression cassettes in T5 or T7 expression vectors. The first cistron contains a His6 tag, the other proteins are copurified via complex formation. Gel filtration results show that Sm proteins D1/D2 and D3/B are present as heterodimers (no higher order complexes were detected), whereas the F/E/G heterotrimers are actually present as a dimer of heterotrimers. The F/E/G proteins form hexameric ring-like structures under the electron microscope of similar size to native $(F/E/G)_2$ complexes obtained from HeLa cell nuclear extracts (Zaric et al. 2005). No monomer subunits have been detected in any of our assays.

Pulldown/RT-PCR assay

HeLa cells were harvested using trypsin, and nuclear and cytoplasmic extracts were made using the Pierce NE-PER nuclear and cytoplasmic extraction reagent. The nuclear extracts were incubated with immobilized GST, GST–coilin fragments, and GST-SMN proteins for 1 h as described above. The beads were then washed and split into halves. One half of the beads were run on 12% SDS-PAGE and subjected to Western blotting and Ponceau S staining. Antibodies used include anti-Sm Y12 (Lab Vision Inc., Fremont, CA), anti-U2B″ (Helen Salz, Case Western Reserve University, Cleveland, OH), and an antibody to the 60 kD protein associated with the U4/U6 snRNP (Nottrott et al. 2002). The other half of the reaction beads were resuspended in 100 μl DEPC-treated water and extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase of the solution was supplemented with 1/3 volume of 3 M sodium acetate and 20 μg glycogen and precipitated with 1.5 volume of prechilled isopropanol at −20°C overnight. The precipitated RNAs were spun down and the pellets were resuspended in 10 μl DEPC water. Reverse transcriptions (RT) were performed using the Invitrogen ThermoScript RT-polymerase chain reaction (PCR) system and corresponding snRNA antisense primers at 50°C for 45 min. The resultant cDNAs were PCR amplified using a variety of annealing temperatures with a 30-s extension time at 68°C for 20 cycles with Invitrogen Platinum Taq and corresponding snRNA primers. Primers used: U1 sense: 5′-ATACTTACCTGGCAGGGGAGATACCATGATCACG-3′; U1 antisense: CAGGGGAAAGCGCGAACGCAGTC CCCCACTACC-3′; U2 sense: 5′-ATCGCTTCTCGGCCT TTTGGCTAAGATCAAG-3′; U2 antisense: 5′-GTGCAC CGTTCCTGGAGGTACTGCAATACC-3′; U4 sense: 5′-AGCTTTGCGCAGTGGCAGTATCGTGCCAATGA-3′; U4 antisense: 5′-CCAGTCTCCGTAGAGACTGTCAAA

AATTGCC-3′; U5 sense: 5′-ATACTCTGGTTTCTCTTC AG-3′; U5 antisense: 5′-AATTGGGTTAAGACTCAGAG TTG-3′; U6 sense: 5′-GTGCTCGCTTCGGCAGCAC-3′; U6 antisense: 5′-ATATGGAACGCTTCACGAATTTGC G-3′; U7 sense: 5′-GCATAAGCTTAGTGTTACAGCTCT TTTAGAATTTGTC-3′; U7 antisense: 5′-CGTAGAATTC AGGGGCTTTCCGGTAAAAAGCCAG-3′.

Immunoprecipitation

HeLa cells transfected with GFP, GFP-SmD1, and GFP–coilin fragments (GFP-C156+NLS and GFP-C214) were harvested, and nuclear extracts were made using the NE-PER nuclear-cytoplasmic extraction kit from Pierce (Rockford, IL). The nuclear extract was diluted into 1 ml using modified RIPA buffer and precipitated with 2 μg of living color GFP full-length antibody (BD Science Clontech, Palo Alto, CA) and 30 μl protein A sepharose beads (Amersham Bioscience, Piscataway, NJ). Precipitated proteins were subjected to Western blotting. The resultant membrane was probed with antibodies against U1–70K protein (Research Diagnostics Inc., Flanders, NJ), U2B″ (Helen Salz, Case Western Reserve University) and GFP (Roche, Indianapolis, IN).

Results

Coilin binds to the Sm-fold regions of Sm and Sm-like proteins

Because CBs contain the highest concentration of snRNPs in the nucleus (Matera and Ward 1993), we set out to identify CB factors that may account for this accumulation. Since the CBs that are devoid of SMN maintain high concentrations of snRNPs (Liu and Dreyfuss 1996; Hebert et al. 2002), SMN is unlikely to be this factor. Previously, we showed that the C-terminal 214 amino acid (aa) residues of coilin are sufficient for interaction with SmB′, making coilin a candidate (Hebert et al. 2001). Since we had not previously defined the region of SmB′ that interacts with coilin, we were specifically interested in assessing if coilin binds SmB′ at a location distinct from SMN, which predominantly binds to the RG-rich tails of Sm proteins (Brahms et al. 2001; Friesen and Dreyfuss 2000; Selenko et al. 2001). We therefore generated two His-T7-tagged SmB′ truncations; one truncation deletes all of the RG dipeptides found in the C-terminal tail, whereas the other construct leaves approximately half of them. The Sm domain, composed of the two Sm motifs and a short linker, is intact in both of these constructs (Fig. 1). Incubation of His-T7-SmB′Δ73 and His-T7-SmB′Δ148 with GST-C214 revealed that the coilin fragment binds not only full-length SmB′ but both truncation mutants, albeit with somewhat reduced affinity (Fig. 2a, lanes 4–6, top panel). In contrast, no significant levels of SmB′ or mutants thereof were recovered after incubation with GST-only beads (Fig. 2a, lanes 1–3, top panel). Reprobing of the same blot with anti-GST verified that equal amounts of GST-C214 and GST were used in the reactions (Fig. 2a, bottom panel). Thus, the RG-rich tail of SmB′ is not required for interaction with the coilin subfragment.

To generalize these results, we tested whether coilin could interact with other Sm and Sm-like proteins. As shown in Fig. 2b and c, coilin binds to full-length SmD1, SmD3, and Lsm4. Similarly, removal of the C-terminal RG tails did not abolish coilin interaction (Fig. 2c, lane 9, and data not shown). Since Bellini and Gall (1998) showed that coilin interacts weakly with the U7 snRNP, we also decided to test if Lsm10, a U7-specific Sm protein that does not contain RG repeats (Fig. 1; Pillai et al. 2001), would bind to coilin. Lsm10 was translated in vitro and tested for interaction with either GST-C214 or GST alone. As shown in Fig. 2c (compare lane 4 to lane 7), Lsm10 interacts with the coilin C214 fragment but not with GST alone.

To corroborate our previous result that coilin binds the Sm domain of SmB′, we tested whether coilin-C214 could bind various fragments of Lsm11, another U7-specific Sm protein (Pillai et al. 2003). The Lsm11 constructs either contained or lacked the Sm motifs (Fig. 3a). As seen in Fig. 3b, full-length Lsm11 (Lsm11-FL) is recovered by GST-C214, but not by GST alone (compare lane 9 to lane 5). Truncation mutants of Lsm11 that contain the Sm motifs (designated as Lsm11-ΔN104 and Lsm11–ΔN140) are also recovered specifically by the coilin fragment and not by GST (compare lanes 10–11 to 6–7). However, an N-terminal fragment of Lsm11 that does not contain the Sm domain (designated Lsm11-N136) fails to bind coilin (lane 12), confirming that coilin binds the Sm-fold regions of Sm and Sm-like proteins.

The coilin C-terminus was also able to bind stable Sm protein heterodimers and heterotrimers. Purified recombinant heterodimers of SmB lacking the RG-rich tail with SmD3 (Fig. 4, top panel) or SmD1 with SmD2 (Fig. 4, middle panel) could be precipitated by immobilized GST-C214, but not by GST-only beads. Note that these heterodimer preparations are identical to those used previously

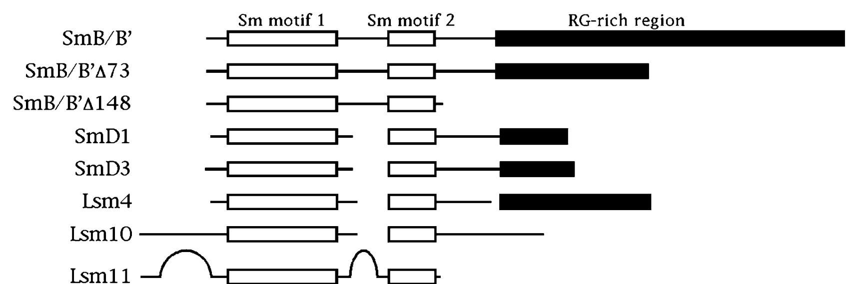

Fig. 1 Schematic presentation of the Sm and Lsm proteins and their mutants studied in this report. The Sm motifs are shown as *open boxes* and the glycine- and arginine-rich regions as *black boxes*. The rest of the linker regions of these proteins are shown as *straight* and *curved lines*. All parts have been drawn to scale

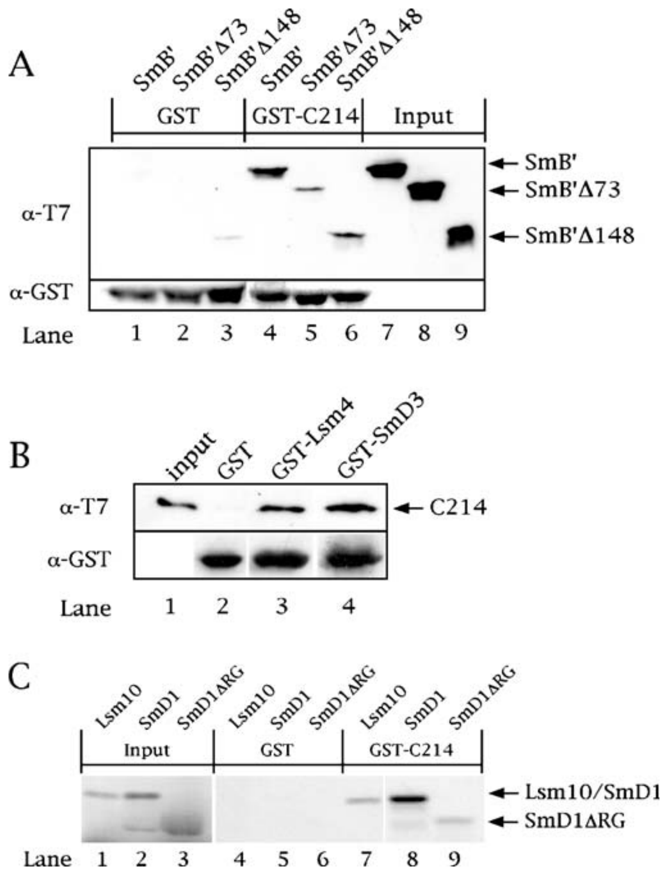

Fig. 2 Coilin interaction with Sm proteins is not mediated by the Sm RG tails. **a** The Sm-fold region of SmB′ interacts with coilin. Equal amounts of GST or the C-terminal coilin fragment fused to GST (*GST-C214*), immobilized on glutathione beads, were incubated with bacterially generated full-length T7-tagged SmB′ or SmB′, in which the C-terminal 73 (*SmB′Δ73*) or 148 residues (*SmB′Δ148*) have been deleted. After incubation and washing, the bound materials were subjected to Western analysis with antibodies to the T7 tag. The *bottom panel* shows the same blot reprobed with an antibody to GST, demonstrating that equal amounts of beads were used. The input lanes correspond to 20% of the amounts used in the binding reactions. **b** Coilin interacts with Lsm4 and SmD3. Equivalent amounts of immobilized GST, GST-Lsm4, or GST-SmD3 were incubated with recombinant T7-tagged coilin fragment (C214) and subjected to Western analysis using antibodies to the T7 tag (*top panel*) or GST (*bottom panel*). **c** Coilin interacts with the Sm-fold regions of SmD1 and Lsm10. Lsm10, SmD1, and SmD1 in which the RG box has been deleted (SmD1ΔRG) were generated in reticulocyte lysate, followed by incubation with equal amounts of immobilized GST or GST-C214. The ^{35}S-labeled proteins were detected on a PhosphorImager. The input lanes represent 10% of the amount used in the binding reactions

for X-ray structural analysis (Kambach et al. 1999b). Additionally, a similar preparation of recombinant SmF/E/G trimer was also precipitated by GST-C214 (Fig. 4, bottom panel). Although these complexes are extremely stable and we have been unable to detect the presence of monomers in gel filtration assays (C.K., unpublished data), we cannot entirely rule out the possibility that part of the observed binding activity could be due to disassembled subunits. Nevertheless, these results are compatible with the notion that coilin can bind Sm protein subcomplexes. Moreover, the SmF/E/G subcomplexes are known to form very stable hexameric ringlike structures (Plessel et al. 1997; Zaric et al. 2005), where the intersubunit surfaces are presumably not accessible to coilin. Thus, it is conceivable that coilin does not require intersubunit interfaces for binding, but rather recognizes structures that are exposed on the surface of fully assembled Sm core structures.

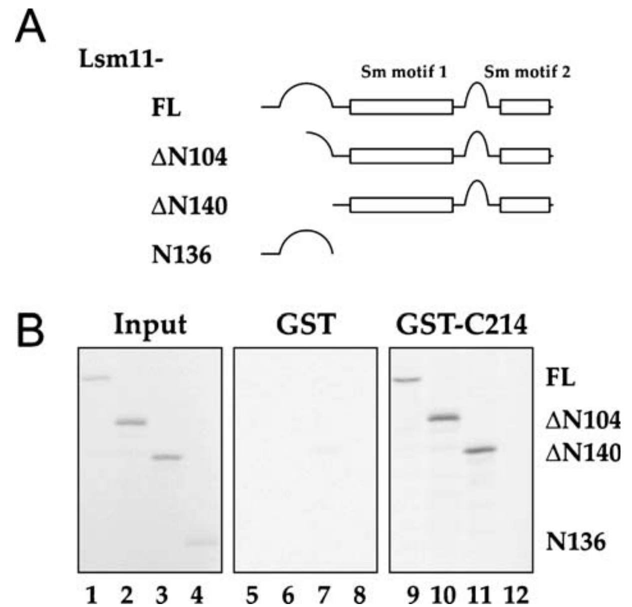

Fig. 3 Coilin interacts with the Sm motifs but not the N-terminal region of Lsm11. **a** The full-length (*FL*) murine Lsm11 protein and its mutants used in **b**. Lsm11-ΔN104 and Lsm11-ΔN140 are N-terminal truncations lacking the indicated number of amino acids. They retain both Sm motifs. Lsm11-N136 is an N-terminal fragment containing just the first 136 amino acids and therefore lacking the Sm-fold region. **b** The Sm-fold region of Lsm11 is necessary for the coilin interaction. The ^{35}S-labeled Lsm11 and its mutants (generated in reticulocyte lysate) were incubated with equal amounts of GST or GST-coilin fragment (GST-C214). The bound ^{35}S-labeled proteins were detected on a PhosphorImager

Coilin interacts with Sm proteins, irrespective of their methylation status

Because the interaction of SMN with both coilin and Sm proteins was greatly enhanced by the presence of symmetrical dimethylarginine (sDMA) residues (Brahms et al. 2001; Friesen et al. 2001; Hebert et al. 2002), we were interested to know whether coilin could bind to sDMA-modified Sm proteins. In the above experiments, the in vitro translated SmD1 (Fig. 2c) should have been sDMA-modified by factors in the reticulocyte lysate (Brahms et al. 2001). In contrast, the SmD1ΔRG protein that lacks an RG tail (Fig. 2c) as well as the recombinant proteins SmB′ (Fig. 2a), Lsm4, SmD3 (Fig. 2b), or the recombinant heterodimers and heterotrimers (Fig. 4) were unmodified. Therefore, purified Sm proteins from HeLa snRNPs (Raker et al. 1996; Sumpter et al. 1992) were used as a known source of sDMA-modified binding substrates. GST-pull-down assays demonstrated that SmB/B′, SmD3, and SmD1 were detected by monoclonal antibody Y12, which

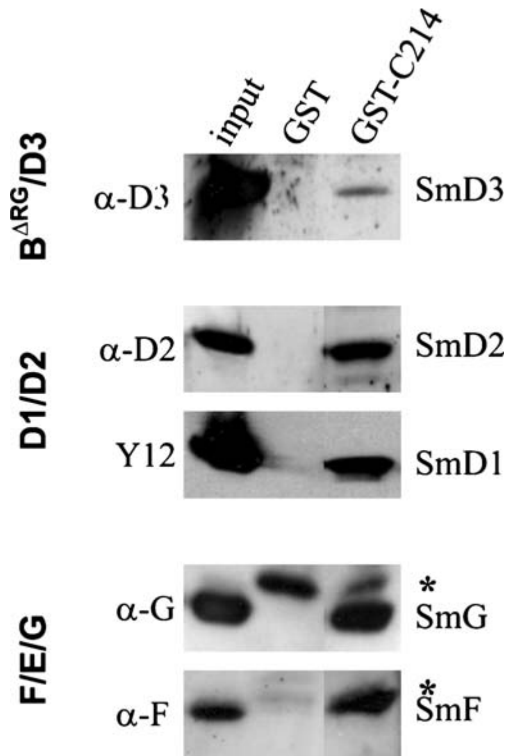

Fig. 4 Coilin interacts with Sm protein heterodimers and heterotrimers. Equal amounts of immobilized GST or GST-C214 were incubated with purified, bacterially produced heterodimers of SmB lacking the RG-rich tail with SmD3 (*top panel*), or SmD1 with SmD2 (*middle panel*), or with similarly produced Sm F/E/G heterotrimers (*bottom panel*). Western blots of the bound materials were probed with the antibodies indicated on the left of each panel. The input lanes correspond to 10% of the amounts used in the binding reactions. Nonspecific bands migrating slightly slower than SmG and SmF are indicated by *asterisks*

recognizes sDMA-modified substrates (Brahms et al. 2001; Hebert et al. 2002). We found that Sm proteins bound to the GST–coilin subfragment, but not to GST alone (Fig. 5). In summary, these results show that neither the C-terminal RG motifs of the Sm proteins nor their sDMA modifications are required for binding. Furthermore, the modifications do not interfere with binding to coilin.

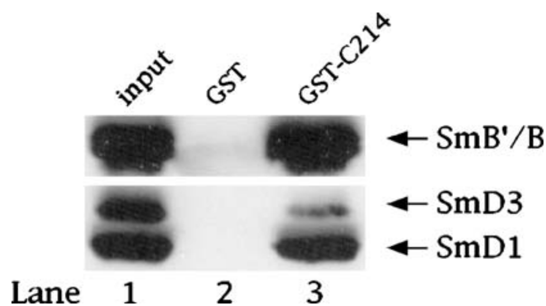

Fig. 5 Coilin interacts with Sm proteins purified from snRNPs. Equal amounts of immobilized GST or GST-C214 were incubated with an Sm protein mix from spliceosomal snRNPs purified from HeLa cells (Sumpter et al. 1992), followed by washing and Western analysis with anti-Sm antibody Y12. The input lanes represent 10% of the amounts used in the binding reactions

The SMN and Sm protein-binding sites on coilin are distinct

Having defined the Sm protein domain to which coilin binds, we next were interested in delimiting the corresponding binding region within coilin. Specifically, we wanted to know if Sm proteins bind to coilin at a site that is distinct from the RG box that binds SMN. We previously showed that SmB′ and SMN directly interact with the coilin-C214 fragment and that the binding sites for these proteins are in close proximity and possibly overlap (Hebert et al. 2001). In order to test whether SmB′ and SMN have separate coilin-binding sites, we set up GST-pulldowns using soluble, T7-tagged SmB′ or SMN- and GST-tagged coilin fragments. The coilin constructs were designed to separate the binding sites for SmB′ and SMN on coilin. Whereas C214 (aa 362–576) binds to both proteins (Hebert et al. 2001; this work), C156 (aa 420–576) lacks the RG box and therefore is not expected to bind SMN. In contrast, coilin residues 362–482 contain the RG box and are expected to bind SMN. As shown in Fig. 6a and b, the binding sites on coilin for SmB′ and SMN are indeed distinct. Notably, SMN binds to GST-C214 and GST-(362–482), both of which contain the RG box, but does not substantially interact with GST-C156, which lacks the RG box (Fig. 6a, compare lanes 3 and 5 to lane 4, top panel). Conversely, SmB′ binds to both GST-C214 and GST-C156, but does not interact efficiently with aa 362–482 of coilin (Fig. 6b). In fact, the C156 fragment of coilin bound greater amounts of SmB′ compared to C214 (Fig. 6b, compare lanes 3 and 4 in the top panel to lanes 3 and 4 of the bottom panel). Taken together, these data reveal that SmB′ binds distal to the SMN-interacting RG box of coilin (Fig. 6c).

Coilin fragments associate with intact snRNPs

We next were interested in determining whether coilin binds to fully assembled snRNPs. Work from the Gall Laboratory has shown that, in *Xenopus* egg extracts, immunodepletion of coilin depletes the levels of snRNPs within CBs (Bauer and Gall 1997). Moreover, coilin weakly associates with the U7, but not with the U1 or U2 snRNPs (Bellini and Gall 1998). To explore these properties in mammalian cells, we tested whether GST–coilin fragments interact with intact snRNPs. As shown in Fig. 7a, GST–coilin fragments C156 and C214 recover significant amounts of SmB/B′ (monitored by the anti-Sm antibody Y12) following incubation with HeLa nuclear extract (top panel). GST and GST-SMN proteins were used as negative and positive controls, respectively (Note that the GST-SMN reaction contained ten times less lysate.). The recovery of SmB/B′ by GST–coilin fragments indicates that coilin binds to intact snRNPs and/or that there is a pool of free (unassembled) Sm proteins in the nucleus.

To determine if the Sm proteins bound to coilin were part of intact snRNPs or represented free Sm proteins, we monitored the amount of U2B″, a U2 snRNP-specific protein, recovered in the pulldown. The presence of U2B″

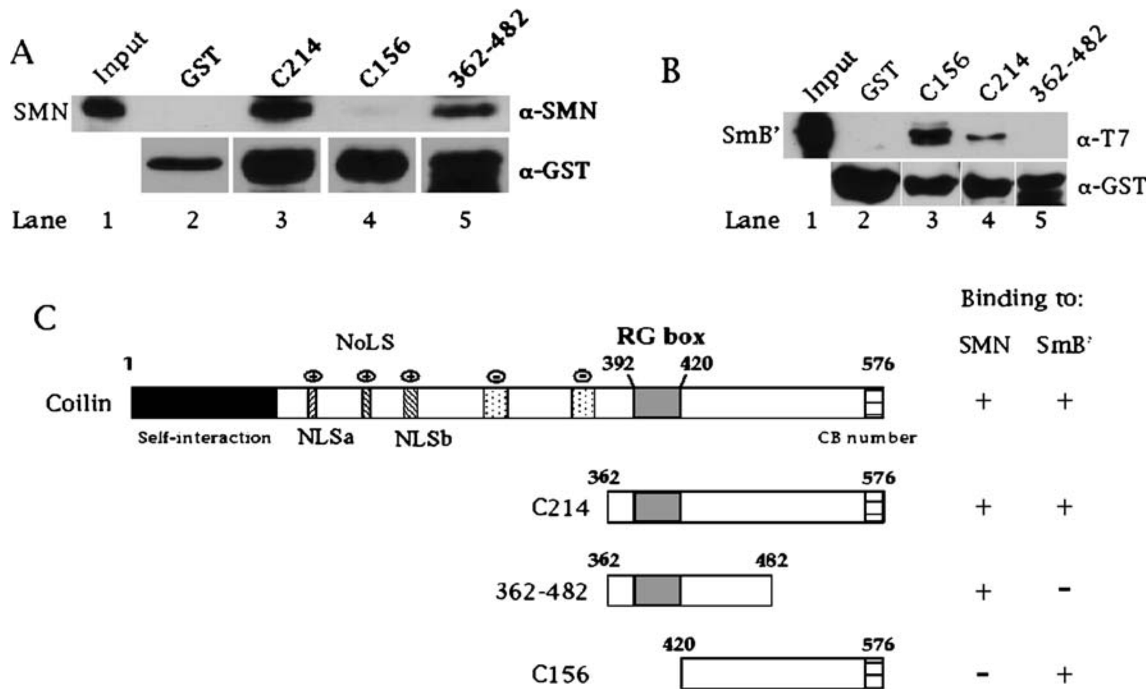

Fig. 6 SMN and Sm proteins bind to different sites on coilin. **a** SMN interacts with coilin through the RG box. Purified His-T7-tagged SMN was incubated with immobilized GST and GST-coilin fragments (GST-C156, GST-C216, and GST-362–482) followed by washing and Western analysis. The membrane was probed with antibodies against SMN and GST. The input lane represents 10% of the amounts used in the binding reactions. **b** SmB′ can bind to the coilin fragment lacking the RG box. In vitro binding assays were carried out using His-T7-tagged SmB′ and immobilized GST and GST-coilin fragments as described in **a**. The membrane was probed with antibodies against T7 tag and GST. The input lane represents 5% of the amount used in the binding reactions. **c** Schematic presentation of the binding abilities of different coilin motifs to SMN and SmB′

would indicate that coilin interacts with intact snRNPs rather than free SmB/B′. Probing the pulldown reactions with anti-U2B″ antibodies demonstrated that the coilin fragments and SMN, but not GST alone, recovered U2B″, suggesting an interaction with the U2 snRNP (Fig. 7a). We also tested whether the coilin fragments would recover a 60 kDa protein known to associate with the U4/U6 snRNPs (Nottrott et al. 2002). Whereas no signal was observed in the GST and GST-C156 lanes, both the larger coilin fragment (GST-C214) and GST-SMN recovered this protein (Fig. 7a). To prove that coilin interacts with U2 and U4/U6 snRNPs, RNA was isolated from the pulldown reactions and RT-PCR was used to detect the various snRNAs. Whereas no U2 snRNA was observed in the GST-only reaction, this RNA was present in the reactions containing the coilin fragments as well as SMN (Fig. 7b). Analysis of other snRNAs revealed that coilin fragment C214, by this assay, binds U4, U5, and U6, but not U1 and U7. Interestingly, the small coilin fragment (C156) recovered only the U2 and U5 snRNAs, and in reduced levels compared to the larger coilin fragment (C214), corroborating the protein-binding data. As expected, SMN bound all of the snRNAs tested.

We also tested for an in vivo interaction between coilin fragments and snRNP-specific proteins. In this assay, HeLa cells were transfected with GFP vector (negative control), GFP-C156 with a nuclear localization signal, GFP-C214 or GFP-SmD1 (positive control). Lysates were generated and immunoprecipitated with anti-GFP antibodies, followed by SDS-PAGE and Western blotting with antibodies specific to U1–70K or U2B″. Consistent with the lack of U1 snRNA recovered by the coilin fragments in the GST-pulldown reactions, only SmD1 recovered the U1 snRNP-specific 70K protein (Fig. 7c, top panel). In contrast, U2B″ is in a complex with both coilin fragments, albeit in amounts reduced compared to those found in the SmD1 reaction (Fig. 7c, middle panel). Probing of the blot with anti-GFP antibodies demonstrated that a large amount of the GFP control was recovered, but only background amounts of U2B″ were coprecipitated (Fig. 7c, bottom panel).

Given that coilin can interact with Sm proteins, it was possible that overexpression of coilin subfragments might disrupt snRNP localization. In order to analyze their subcellular distribution patterns, we cloned the C156 and C214 coilin subfragments into GFP expression vectors with or without an exogenous nuclear localization signal (NLS). As shown in Fig. 8, the constructs lacking NLS were distributed throughout the cell, whereas the fragments bearing NLS were restricted to the nucleoplasm. We did not observe any disruption of snRNP localization (visualized by anti-Sm antibodies) upon expression of any of these constructs. Although GFP-C156+NLS appeared to be enriched in nuclear speckles, the localization pattern was indistinguishable from that of the GFP+NLS control construct (Fig. 8). Thus, overexpression of coilin C-terminal fragments does not disrupt the overall speckled pattern. Similarly, overexpression of full-length coilin does not disrupt nuclear speckles, although it does have an effect on

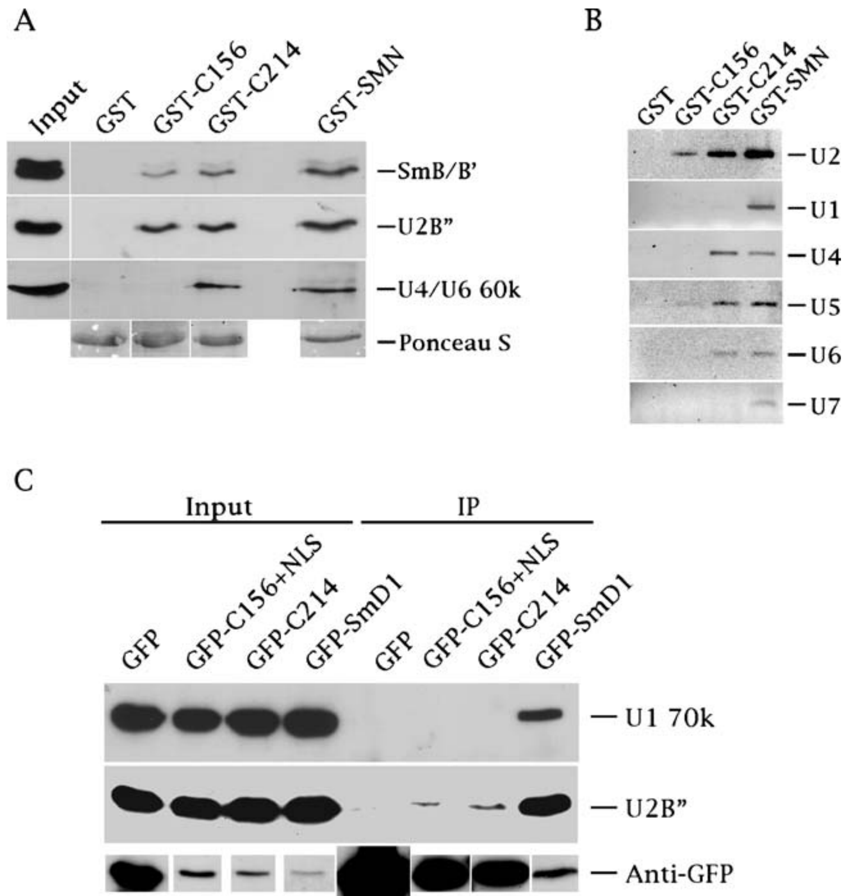

Fig. 7 Coilin binds to certain assembled snRNPs. **a** Coilin binds to complexes that contain Sm proteins, the U2 snRNP-specific U2B″ protein, as well as the U4/U6 60 kDa protein. HeLa cell nuclear extracts were incubated with GST, GST-C156, GST-C214, and GST-SMN immobilized on glutathione–sepharose beads. Western blots of the bound material were probed with Y12 anti-Sm antibody and by an antibody against the U2 snRNP-specific protein U2B″. A separate reaction was probed with antibodies to the 60 kDa protein associated with the U4/U6 snRNP. The input represents 5% of the amount used in the binding reactions with GST, GST-C156, and GST-C214. The amount of extract used in the reaction with GST-SMN was only 10% of that used in the other three reactions. The amounts of the GST and GST fusion proteins were monitored by Ponceau S staining. **b** The protein complexes binding to coilin include certain species of snRNAs. From half of the incubated beads used in **a**, total RNA was isolated and subjected to RT-PCR for U2, U1, U4, U5, U6, and U7 snRNAs using corresponding snRNA-specific primers. **c** Coilin in vivo forms complexes containing the U2-specific protein. HeLa cells were transfected with GFP, GFP-C156+NLS, GFP-C156, and GFP-SmD1. Nuclear extracts were made and immunoprecipitated with anti-GFP polyclonal antibody. Western blotting was performed on the coprecipitated proteins. The resultant membrane was probed with antibodies against the U1 and U2 snRNP-specific proteins U1–70K and U2B″. The expression and immunoprecipitation of the GFP and GFP fusion proteins were monitored by probing with anti-GFP antibodies

CBs themselves (Hebert and Matera 2000). In conclusion, the results show that expression of the coilin C-terminal fragments are not dominant negative for snRNP localization.

Discussion

Upon entering the nucleus, newly assembled snRNPs (visualized by GFP-tagged Sm proteins) first accumulate in CBs before moving on to speckles (Sleeman and Lamond 1999). Moreover, recent work has implicated the CB as a site of snRNA modification (Jady et al. 2003) and assembly of snRNP-specific proteins (Nesic et al. 2004; Tanackovic and Kramer 2005). These results raise several mechanistic questions. For example, how are nascent snRNPs targeted to the CB for modification and how are they tethered there during their stay? Given that coilin directly interacts with SMN, SmB/B′, SmD1, SmD3, Lsm4, Lsm10, and Lsm11, (Hebert et al. 2001; this work) and that SMN likely enters the nucleus bound to newly assembled snRNPs (Narayanan et al. 2002, 2004), it is possible that locally high concentrations of coilin within the CB play a role in snRNP targeting and/or in separating the snRNPs from the SMN complex. The previously identified interaction between coilin and SMN (Hebert et al. 2001) may attract SMN-bound snRNPs to CBs, where a takeover by coilin might take place. Notable in this regard is our finding that coilin binds to the Sm-fold regions of Sm and Sm-like proteins (Figs. 2, 3, 4). The Sm-fold region is the most highly conserved region in the Sm/Lsm protein family (reviewed in Kambach et al. 1999a). Unlike coilin, SMN binds predominantly to the RG tails of Sm proteins, especially when the arginines in this region are symmetrically dimethylated (Brahms et al. 2001; Friesen et al. 2001).

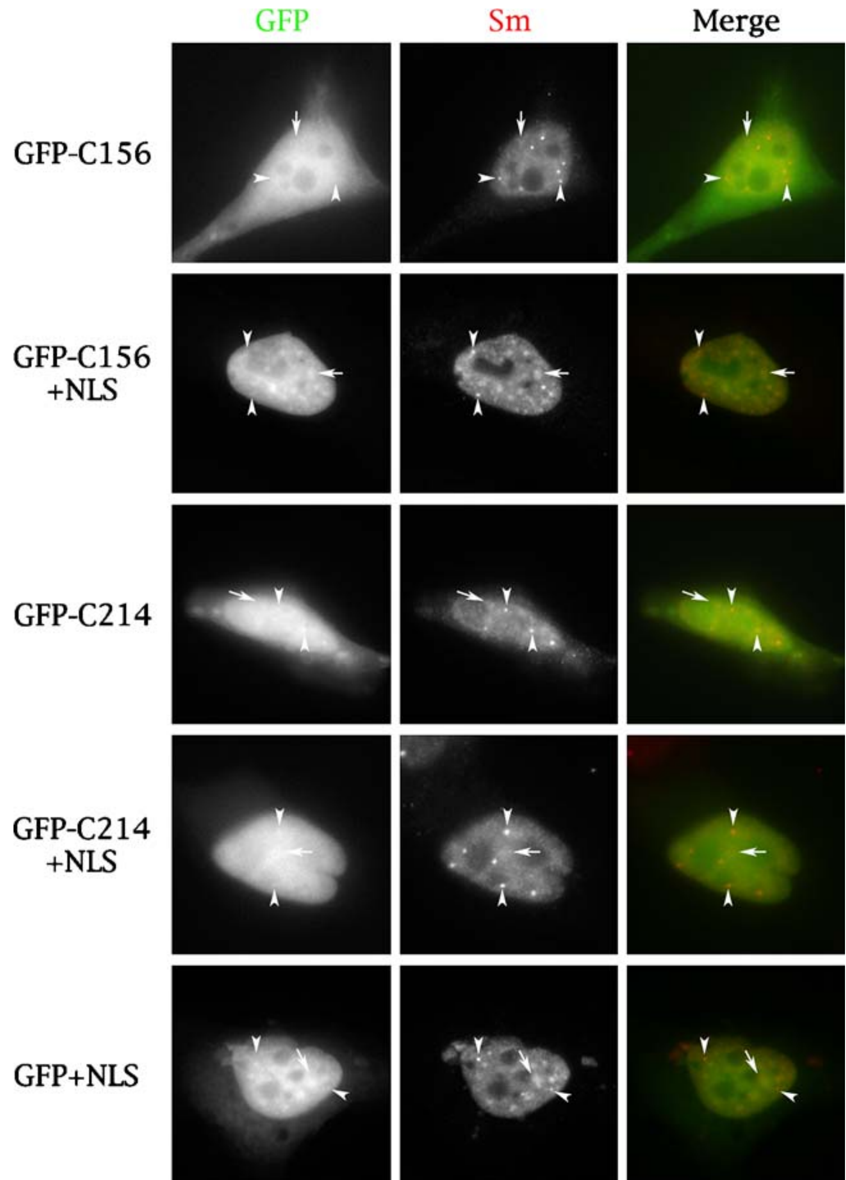

Fig. 8 Expression of coilin C-terminal fragments does not alter snRNP localization. HeLa cells were transfected with GFP-tagged coilin C214 or C156 with and without an NLS (inserted between the GFP and the coilin sequences), followed by staining with monoclonal antibody Y12 to detect snRNPs (*Sm*). The signals for GFP (*left column*) and Sm (*center column*) are shown, along with the merged image (*right column*). Note that none of the GFP-coilin fragments displayed significant accumulation within speckles (*arrows*) or CBs (*arrowheads*). Although the GFP-C156 construct was slightly enriched in speckles, a similar localization pattern was observed for the GFP+NLS control (*bottom row*)

Thus, many Sm proteins have the capacity to bind both SMN and coilin simultaneously. Moreover, we find that coilin has separate binding sites for SMN and Sm proteins (Fig. 6). Potentially, SMN, snRNPs, and coilin could all bind to each other in a single complex, which might favor the release of SMN and the transient interaction of the snRNPs with coilin.

However, some caveats for this scheme of events are appropriate. The fact that a coilin subfragment can bind to Sm-folds does not automatically imply that it can also bind to assembled snRNPs, as much of the Sm-fold is involved in interactions with other Sm and Lsm proteins (Kambach et al. 1999a,b). Hence, the binding could be due to intersubunit interfaces that are hidden in assembled Sm cores. Thus, it will be important to know which features of the Sm-fold structure are recognized by coilin and whether the full-length protein is capable of these interactions. Even our finding that coilin binds to Sm protein heterodimers and heterotrimers (Fig. 4) cannot be construed to mean that coilin binds to exposed surfaces within a heptameric Sm/Lsm protein ring, since these subcomplexes still have available intersubunit contact surfaces.

Therefore, a key question was whether or not coilin could bind to intact snRNPs. Here, we have clearly shown that this is the case (Fig. 7). Moreover, the U2 and U5 snRNPs were precipitated by the same minimal coilin fragment (C156) that also binds individual Sm proteins. However, the (limited) spectrum of snRNPs that can be bound by coilin is not fully compatible with a function of this interaction in the targeting or tethering of nascent snRNPs to CBs. U7 snRNPs are strongly associated with CBs (Frey and Matera 1995; Pillai et al. 2001), and although U1 snRNPs do not accumulate prominently in CBs at steady state, they likely transit through them (Sleeman and Lamond 1999).

Work from the Gall Laboratory showed that although *Xenopus* coilin binds weakly to the U7 snRNP, no association with the U1 or U2 snRNPs was detected (Bellini

and Gall 1998). For their assay, ^{32}P-labeled U1, U2, or U7 snRNA was injected into *Xenopus* oocytes followed by immunoprecipitation with anticoilin antibodies and quantification of the bound RNA compared to that recovered by the anti-Sm (Y12) antibody. For our assay, we conducted semiquantitative RT-PCR on RNA isolated from GST-pulldown reactions using coilin subfragments and found that U2, U4, U5, and U6 snRNAs are recovered, but U1 and U7 snRNAs were not (Fig. 7b). Thus, our findings are in partial contradiction to those of Bellini and Gall (1998). Some of the disparity between these two studies may be due to the different biological systems analyzed, *Xenopus* oocytes vs. mammalian tissue culture cells and extracts. The difference might also have to do with our use of coilin fragments (full-length human coilin is completely insoluble when expressed in bacteria), rather than full-length coilin, in the GST-pulldown experiments. On the other hand, the use of coilin subfragments allows us to monitor the Sm and snRNP interaction potential of coilin in the absence of any other known coilin-binding proteins.

One such coilin-binding protein is SMN, which interacts with the RG motif of coilin found between aa 392–420. Since the SMN complex can be associated with mature snRNPs, associations between SMN and coilin may also recover snRNPs in pulldown assays. Therefore, we cannot rule out the possibility that the U4 and U6 snRNAs, which were only precipitated by coilin fragment C214, but not by C156, were precipitated by virtue of their interaction with SMN. However, in this case, we would also have expected to find U1 and U7 snRNAs in the C214 precipitates. Certainly, for the U2 and U5 snRNAs that were precipitated by GST-C156, we can rule out that this precipitation was mediated by SMN. Nevertheless, certain snRNPs, such as U4 and U6, may interact directly with coilin-C214 as well as indirectly via SMN bound to the coilin RG motif. To conclusively prove this point, future investigations should monitor recovery of snRNAs from extracts depleted of SMN.

The interactions between coilin and snRNPs are likely to be transient, as evidenced by the fact that ten times more lysate is needed in the coilin fragment reactions compared to the SMN reactions in order to recover approximately the same amount of a given U snRNA (Fig. 7b). Additionally, we have found that coilin is not recovered upon isolation of U2 snRNPs from HeLa nuclear extract by precipitation with a biotinylated anti-U2 oligonucleotide coupled to magnetic streptavidin beads (data not shown). However, overexpression of GFP-tagged coilin is sufficient to recover at least some of the U2 snRNP in vivo, as evidenced by the presence of coimmunoprecipitating U2B″ (Fig. 7c). The amount of U2B″ recovered in the coilin fragment immunoprecipitation reactions is far less than that recovered by SmD1, again suggesting that the interaction between coilin and snRNPs is weak and/or transient.

If the spectrum of snRNAs that can be bound by coilin is not fully compatible with a function in targeting nascent snRNPs to CBs (see above), it may hint at another possible function of coilin or CBs: snRNP recycling after a splicing reaction has taken place. It is noteworthy that the U2, U5, and U6 snRNPs are all parts of the active spliceosome and that U4 has a major role in the U6 (and U5) cycle. Even if U4 and U6 are dismissed because of their possible co-precipitation along with SMN (see above), the remaining U2 and U5 snRNPs are two of the most central components of the spliceosome. Could coilin (and CBs) therefore be involved in spliceosome disassembly and/or the recycling of spliceosomal components? In this context, it is noteworthy that one Cajal body protein, hPrp24/SART3/p110, has been implicated in U4/U6 recycling (Bell et al. 2002; Stanek et al. 2003). Similarly, SMN and its associated proteins may also help to ensure that the Sm core is properly reformed onto the snRNA following splicing (Matera 1999; Pellizzoni et al. 1998).

A third function for the observed interaction between coilin and Sm/Lsm proteins could be the reclamation of disassembled snRNP components in the nucleus. In this scheme, the free coilin present in the nucleoplasm (~70% of the total pool) could associate with individual Sm/Lsm proteins and bring them back to CBs. There a limited amount of snRNP reassembly or even de novo assembly could take place, as the SMN complex is also present and several snRNA genes are frequently associated with CBs (Frey et al. 1999; Frey and Matera 1995, 2001; Jacobs et al. 1999; Schul et al. 1998; Smith et al. 1995). Alternatively, SMN could take over such reclaimed Sm/Lsm proteins at the CBs and return them to the cytoplasm where they could be reused in the assembly of new snRNPs. Currently, there is scant evidence supporting the existence of free Sm/Lsm proteins in mammalian nuclei. However, recent work has shown that plants contain a nuclear structure which contains Sm proteins but lacks snRNAs (Niedojadlo and Gorska-Brylass 2003). Consequently, it is possible that under certain circumstances, snRNPs may indeed disassemble, generating free Sm proteins and snRNA in the nucleus.

Despite the fact that our current state of knowledge does not allow us to distinguish which of these discussed functions (and perhaps others are possible as well) is the correct one, the fact that coilin contains a specific binding site for Sm proteins and at least some fully assembled snRNPs is significant. It indicates that coilin must play a direct role in snRNP metabolism above and beyond its ability to interact with SMN and its colocalization with scaRNPs and other snRNPs. Our data expand the complexity of the protein interactions thought to take place in the Cajal body. Furthermore, our finding that coilin interacts with a subset of spliceosomal snRNPs suggests that coilin may be involved in snRNP recycling. Finally, coilin might also help reclaim dissociated Sm/Lsm proteins from broken snRNAs. Experiments are underway to investigate these distinct, but related pathways.

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