Methylation of Sm proteins by a complex containing PRMT5 and the putative U snRNP assembly factor pICln

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Seven Sm proteins, termed B/B′, D1, D2, D3, E, F, and G, assemble in an ordered manner onto U snRNAs to form the Sm core of the spliceosomal snRNPs U1, U2, U4/U6, and U5 [1–4]. The survival of motor neuron (SMN) protein binds to Sm proteins and mediates in the context of a macromolecular (SMN-) complex the assembly of the Sm core [5–9]. Binding of SMN to Sm proteins is enhanced by modification of specific arginine residues in the Sm proteins D1 and D3 to symmetrical dimethylarginines (sDMAs), suggesting that assembly might be regulated at the posttranslational level [10–12]. Here we provide evidence that the previously described pICln-complex [13], consisting of Sm proteins, the methyltransferase PRMT5, pICln, and two novel factors, catalyzes the sDMA modification of Sm proteins. In vitro studies further revealed that the pICln complex inhibits the spontaneous assembly of Sm proteins onto a U snRNA. This effect is mediated by pICln via its binding to the Sm fold of Sm proteins, thereby preventing specific interactions between Sm proteins required for the formation of the Sm core. Our data suggest that the pICln complex regulates an early step in the assembly of U snRNPs, possibly the transfer of Sm proteins to the SMN-complex.

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Received: **18 September 2001**
Revised: **22 October 2001**
Accepted: **7 November 2001**

Published: **11 December 2001**

Current Biology 2001, 11:1990–1994

0960-9822/01/$ – see front matter
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Results and discussion

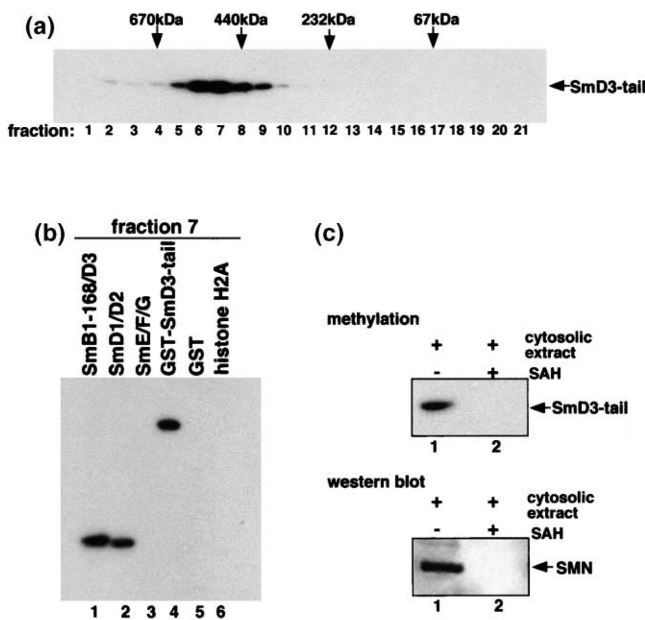
Identification of a sDMA-modifying activity for Sm proteins in HeLa cytosol

To identify the factor(s) that catalyze the formation of sDMAs in Sm proteins, we initially fractionated HeLa cytosolic extract by size exclusion chromatography and incubated individual fractions with recombinant GST-D3-tail (containing the RG-dipeptide motif of D3) in the presence of [^{3}H]S-adenosylmethionine (SAM). Protein methylation was subsequently analyzed by SDS-PAGE and fluorography. As shown in Figure 1a, the GST-D3-tail was efficiently methylated in a peak that corresponded to a molecular mass of roughly 500 kDa (lanes 5 through 9), whereas GST alone was not modified (data not shown). To test whether full-length Sm proteins were also methylated, we incubated the peak fraction with recombinant heterooligomeric complexes composed of Sm proteins D1/D2, B(1-168)/D3, and E/F/G and assayed for methyltransferase activity as described above (Figure 1b). Note that, due to their generation in *E. coli*, none of the recombinant Sm proteins contained sDMA residues. As shown in Figure 1b, the RG-containing Sm proteins (i.e., D1 and D3, lanes 1 and 2) but not the Sm proteins that either contain a shortened RG tail [i.e., B(1-168); lane 1] or lack an RG-tail (i.e., D2, E, F, and G; lanes 1 through 3) were efficiently modified. Importantly, methylation in the peak fractions was specific for Sm proteins, as neither GST nor histone 2A (an often-used model substrate for methyltransferases [14]) were modified (lanes 5 and 6). To analyze whether the Sm proteins received a symmetrical (s) or asymmetrical (a) DMA modification, we took advantage of the previous observation that only sDMA-modified Sm proteins can bind to SMN [10]. Thus, endogenous SMN should copurify with an Sm protein subsequent to incubation in a cellular extract only if the critical arginine residues were symmetrically methylated. Unmodified GST-D3-tail was incubated with cytosolic extract to allow methylation in vitro (Figure 1c, lane 1, upper panel). The GST-D3-tail protein was subsequently isolated with glutathione Sepharose beads, and copurification of cytosolic SMN was monitored by Western blotting using anti-SMN antibodies. As shown in Figure 1c, SMN was readily detected in the eluate, indicating that the GST-D3-tail had received a sDMA modification (lane1, lower panel). In contrast, blocking of the methyltransferase activity with S-adenosylhomocysteine (SAH) prevented the interaction between SMN and GST-D3-tail (lane 2, compare upper and lower panel). Similar results were obtained with other Sm proteins (data not shown). Taken together, these data show that HeLa cytosol contains a methyltransferase activity that specifically introduces sDMA residues into the RG-rich tails of Sm proteins.

The pICln complex catalyzes the formation of sDMAs in Sm proteins

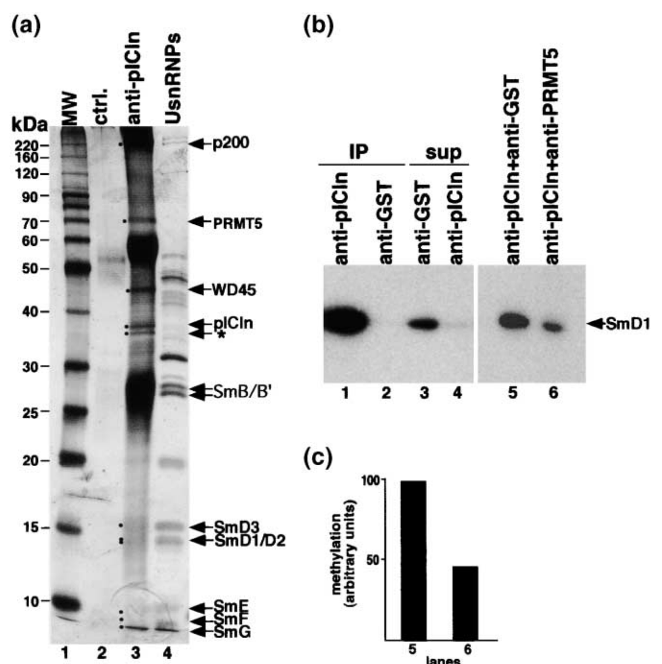
pICln has previously been shown to be associated with Sm proteins and the type II (sDMA-generating) arginine

Figure 1



A macromolecular complex catalyzes the sDMA modification of SmD1 and SmD3. **(a)** HeLa cytosolic extract was fractionated by size exclusion chromatography. To monitor methyltransferase activity, each fraction was incubated with recombinant GST-D3-tail in the presence of [^{3}H]-SAM, separated by SDS-PAGE, and analyzed by fluorography. Positions of size marker proteins are given at the top of the gel. **(b)** The peak fraction (fraction 7) of the experiment shown in (a) was incubated with either SmB(1-168)/D3 (lane 1), SmD1/D2 (lane 2), SmE/F/G (lane 3), GST-SmD3-tail (lane 4), GST-tag (lane 5), or histone H2A (lane 6) in the presence of [^{3}H]-SAM. The modified proteins were analyzed as in (a). **(c)** sDMA modification of GST-SmD3-tail in cytosolic extract. GST-SmD3-tail was incubated with cytosolic extract, [^{3}H]-SAM and either water (lane 1) or SAH (lane 2). After incubation at 37°C, GST-SmD3-tail was isolated by binding to glutathione Sepharose beads. Coisolated SMN was detected by Western blotting with anti-SMN monoclonal antibody 7B10 (lower panel), and GST-D3-tail methylation was analyzed by fluorography (upper panel).

Figure 2

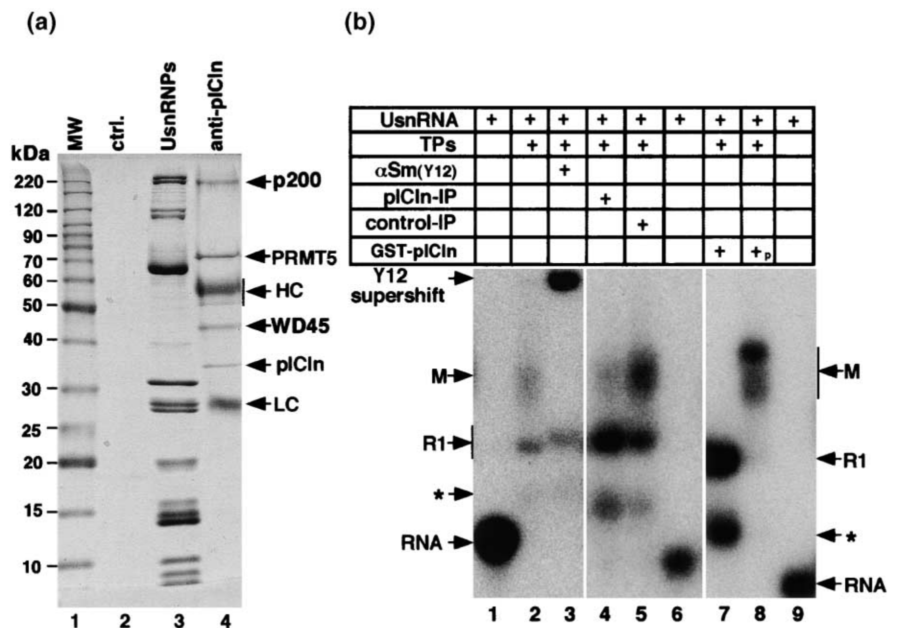


Affinity-purified pICln-complex catalyzes the formation of sDMAs in Sm proteins. **(a)** Protein elution profile of an anti-pICln affinity column (lane 3) and an anti-GST control column (lane 2). Proteins were separated by SDS-PAGE alongside a protein marker (lane 1) and affinity-purified UsnRNP proteins (lane 4) and were visualized by silver staining. Specific proteins of the pICln elution are indicated. The band indicated by * is a degradation product of pICln. **(b)** Anti-pICln antibodies and anti-GST antibodies were coupled to protein G Sepharose beads and incubated with HeLa cytosolic extract. The immunoprecipitates (lanes 1 and 2) and the corresponding supernatants (lanes 3 and 4) were assayed for methyltransferase activity, using SmD1/D2 complex as substrate. The methylation assays shown in lanes 5 and 6 were preincubated with affinity-purified anti-GST antibodies or anti-PRMT5 antibodies, respectively. **(c)** Quantification of the antibody inhibition shown in Figure 2b, lanes 5 and 6.

N-methyltransferase PRMT5 [13, 15, 16]. It was therefore tempting to speculate that this complex accounted for the methyltransferase activity observed in our assay (see Figure 1). To test for this possibility, we initially isolated the pICln complex by anti-pICln immunoaffinity chromatography and identified the proteins by MALDI-TOF and Western blotting. Consistent with previous reports [13], this complex contains pICln, PRMT5, and all Sm proteins (i.e., B/B′, D1, D2, D3, E, F, and G; Figure 2a, lane 3). In addition, two proteins with apparent molecular masses of 45 kDa and 200 kDa also coeluted specifically from the column. The 45 kDa component (termed "WD45") was identified as a WD motif protein of unknown function that shares pronounced similarity with β-transducin (see the Supplementary material available with this article online). This protein is likely to be identical to a factor termed "IBP42," which had previously been identified [13]. Peptide masses that unequivocally identify the 200 kDa component have not yet been obtained.

Next, the pICln complex was purified as described above and tested for methyltransferase activity. As shown in Figure 2b, SmD1 was strongly modified by the affinity-purified pICln complex (lane 1), whereas the flow through of the column was inactive (lane 4). In contrast, no methylation of SmD1 could be detected in a control purification using a nonrelated antibody (lanes 2 and 3). In the view of these data, we conclude that the pICln complex generates sDMA residues in Sm proteins. Importantly, antibodies directed against PRMT5 reduced significantly the methylation activity of the pICln complex (Figure 2b,c). This observation together with the previous finding that isolated PRMT5 catalyzes the formation of sDMA in a variety of substrates in vitro suggests that PRMT5 is the

Figure 3



Isolated pICln complex that lacks Sm proteins inhibits U snRNP assembly in vitro. **(a)** HeLa cytosolic extract prepared under low salt conditions was passed over an anti-pICln column (lane 4) or an anti-GST control column (lane 2). Eluted proteins from both columns were resolved by SDS-PAGE lane by lane with a U snRNP protein marker (lane 3) and a molecular weight marker (lane 1). All indicated proteins except for p200 were identified by MALDI-TOF. **(b)** ^{32}P-labeled U1 snRNA was incubated with either TPs (lane 2) or TPs supplemented with anti-Sm antibody Y12 (lane 3), purified pICln(ΔSm) complex (lane 4), a control eluate of an anti-GST column (lane 5), or recombinant GST-pICln either prior to (lane 7) or after (lane 8; indicated with p = post) the assembly reaction. Lanes 1, 6, and 9 show the U1 snRNA in the absence of TPs. Complex formation was analyzed by native gel electrophoresis.

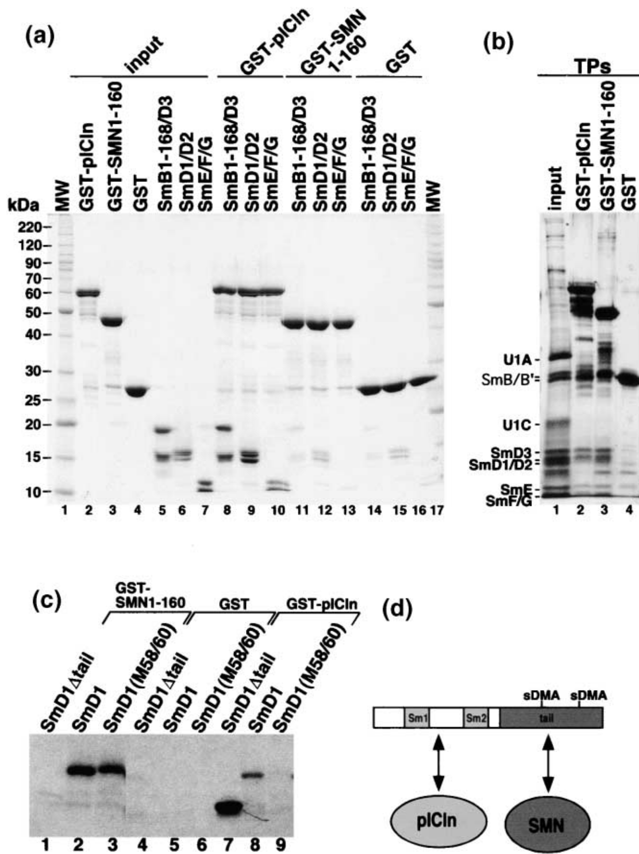
catalytic component of the pICln complex that generates sDMA in Sm proteins in vivo.

The pICln complex inhibits assembly of U snRNPs in vitro by binding to the Sm domain of Sm proteins

We next tested whether the pICln complex fulfills, apart from its methyltransferase activity, an additional role in the biogenesis of U snRNPs as suggested previously [13]. For this purpose, native U snRNP proteins, including all sDMA-modified Sm proteins (termed "TPs"), were prepared from affinity-purified nuclear U snRNPs and incubated with in vitro transcribed and ^{32}P-labeled U1 snRNA. U snRNP complex formation was subsequently analyzed by native gel electrophoresis. In agreement with previous findings, the U1 snRNA was shifted to two complexes, termed "R1" and "M" (Figure 3b, lane 2). Anti-Sm antibodies supershifted complex M (lane 3), indicating that it contains the Sm core assembled on U1 snRNA. Complex R1 does not contain Sm proteins and corresponds to U1 snRNA bound to the U1A protein (lane 3, see also [9] for a detailed characterization of these complexes). To test whether the pICln complex can interfere with U snRNP assembly, we isolated a pICln-containing complex under conditions that allowed for the coisolation of all previously described components except for the Sm proteins (i.e., pICln, PMRT5, WD45, and p200; Figure 3a, lane 4). The pICln(ΔSm) complex was then incubated with TPs, and assembly was analyzed as described above. Strikingly, when this complex was added to an assembly reaction, the formation of the U1 snRNP was severely impaired as compared to a control reconstitution lacking purified pICln(ΔSm) complex (Figure 3b, compare lanes 4 and 5). In contrast, binding of the U1 snRNP-specific protein A was not affected (lanes 4 and 5). Next, we tested whether the observed inhibition of assembly could be attributed to pICln alone, as a previous study had suggested [13]. Indeed, when snRNP proteins were preincubated with recombinant pICln, formation of complex M was abolished (Figure 3b, compare lane 7 with lanes 2 and 5). Importantly, an inhibition of assembly was strongly dependent on preincubation of pICln with Sm proteins, and no effect was observed when pICln was added after the reconstitution reaction had been completed (compare lanes 7 and 8). Thus, pICln is more likely to prevent binding of Sm proteins onto U snRNA than to disassemble the formed U snRNP.

To analyze the underlying mechanism of pICln's function, we tested whether pICln binds directly to Sm proteins and which domains are involved. Recombinant GST-pICln was immobilized on glutathione Sepharose beads and subsequently incubated with the bacterially expressed and hence unmethylated Sm complexes D1/D2, E/F/G, and B(1-168)/D3. As shown in Figure 4a, each Sm complex bound efficiently to pICln, whereas only marginal binding to GST was observed (compare lanes 8 through 10 with 14 through 16). None of the Sm complexes bound to a significant amount to the GST-SMN1-160 fragment, confirming that this interaction is enhanced upon sDMA modification of Sm proteins (lanes 11 through 13) [10]. In contrast, sDMA-modified Sm proteins obtained from isolated HeLa-U snRNPs bound equally well to pICln and SMN (Figure 4b, compare lanes 2 and 3). These results show that pICln interacts directly

Figure 4



pICln binds directly to the Sm domain of Sm proteins. **(a)** Recombinant GST-pICln (lanes 8 through 10), GST-SMN1-160 (lanes 11 through 13), and GST (lanes 14 through 16) were immobilized on glutathione Sepharose beads and incubated with recombinant heterooligomeric Sm complexes B(1-168)/D3 (lane 8, 11, and 14), D1/D2 (lane 9, 12, and 15), and E/F/G (lane 10, 13, and 16). Bound proteins were eluted with protein sample buffer, resolved by SDS-PAGE, and visualized by Coomassie staining. Lanes 2 through 7 show the proteins used for the binding studies; lanes 1 and 17, protein size markers. **(b)** GST-pICln (lane 2), GST-SMN1-160 (lane 3), and GST (lane 4) were immobilized on glutathione Sepharose beads and incubated with TPs obtained from affinity-purified U snRNPs. Lane 1 shows the TPs used for the binding reaction. Proteins were separated by SDS-PAGE and visualized by silver staining. **(c)** GST-pICln (lanes 7 through 9), GST-SMN1-160 (lanes 1 through 3), and GST (lanes 4 through 6) were immobilized on glutathione Sepharose beads and incubated with in vitro translated and ^{35}S-labeled D1Δtail (lanes 1, 4, and 7), D1 (lanes 2, 5, and 8), and D1(M58/60) (lanes 3, 6, and 9). Bound proteins were analyzed by SDS-PAGE and fluorography. **(d)** A schematic representation of the data shown in Figure 4a–c.

with Sm proteins but, unlike SMN, does not require the presence of sDMAs for binding.

The binding of pICln to all Sm heterooligomeric complexes suggested that this interaction involves the Sm domain, i.e., the N-terminal sequence that is common to Sm proteins and required for the formation of the Sm core. Indeed, a radiolabeled SmD1 translate that lacks the tail domain and hence consists of the Sm domain only bound efficiently to immobilized GST-pICln (Figure 4c, lane 7). In control reactions, no interaction of this mutant was observed with GST-SMN1-160 (which requires the sDMA-modified tails for interaction) or GST alone (lanes 1 and 4). Moreover, a double point mutation within the Sm domain (L58 was substituted with K, and I60 was substituted with R) abolished binding of SmD1 to pICln but not to SMD3 (compare lanes 3, 6, and 9). Similar results were obtained with SmB (data not shown). These observations provide strong support for a direct binding of pICln onto the Sm domain of Sm proteins (see Figure 4d for a model). Moreover, the mode of interaction between both proteins implies that pICln inhibits UsnRNP assembly by preventing contacts among Sm proteins required for the formation of the heptameric ring in the Sm core.

Friesen et al. [10] have recently reported that the presence of symmetrical dimethylarginines (sDMAs) in the Sm proteins D1 and D3 is a prerequisite for binding of these proteins to SMN and hence for the assembly onto U snRNA. Here we have shown that the macromolecular pICln complex mediates the formation of sDMAs in SmD1 and SmD3. Consistently, this complex contains as the catalytic subunit the type II methyltransferase PRMT5 [14]. It is interesting to note that isolated PRMT5 methylates a variety of different proteins, including Sm proteins, histones, myelin basic protein, and fibrillarin [14]. In striking contrast, the 500 kDa pICln complex efficiently modifies Sm proteins but not histones or GST in the same assay. Hence, it is likely that PRMT5-interacting factors such as pICln, WD45, and/or p200 confer substrate specificity toward Sm proteins in vivo.

The earlier observation that the PRMT5-interacting protein pICln interfered with the assembly of the Sm core when injected into *Xenopus laevis* oocytes lead to the proposal that pICln might act as a negative regulator of assembly [13]. In support of this notion, we have shown that pICln as part of a multiprotein complex directly interacts with Sm proteins and inhibits the assembly onto U snRNA in an in vitro reconstitution assay. Importantly, pICln binds to the Sm domain, i.e., the common structure found in all Sm proteins. As Sm proteins interact via this Sm domain to form the heptameric ring of the Sm core, it is likely that pICln inhibits assembly by preventing the formation of specific contacts among Sm proteins.

Our data are consistent with a dual function of the pICln complex at an early stage of U snRNP assembly. We propose that newly synthesized Sm proteins initially bind to the pICln complex where B/B′, D1, and D3 receive the sDMA-modification. The pICln complex may then control the transfer of the Sm proteins onto the SMN

complex to allow their subsequent assembly onto a U snRNA.

Supplementary material

Supplementary material including materials and methods and the amino acid sequence of a novel WD domain-containing protein is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgements

We are indebted to B. Laggerbauer and R. Lührmann for critically reading the manuscript; and G. Sowa and E. Keidel for technical support. This work was supported by the Boehringer Ingelheim Fonds (D.B.), the German Science Foundation (Fi-573/2-2), and the Max-Planck Society.

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