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Homodimerization of the human U1 snRNP-specific protein C

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ABSTRACT

The U1 snRNP-specific protein C contains an N-terminal zinc finger-like CH motif which is required for the binding of the U1C protein to the U1 snRNP particle. Recently a similar motif was reported to be essential for *in vivo* homodimerization of the yeast splicing factor PRP9. In the present study we demonstrate that the human U1C protein is able to form homodimers as well. U1C homodimers are found when (i) the human U1C protein is expressed in *Escherichia coli*, (ii) immunoprecipitations with anti-U1C antibodies are performed on *in vitro* translated U1C, and when (iii) the yeast two hybrid system is used. Analyses of mutant U1C proteins in an *in vitro* dimerization assay and the yeast two hybrid system revealed that amino acids within the CH motif, i.e. between positions 22 and 30, are required for homodimerization.

INTRODUCTION

During the process of pre-mRNA splicing, the U1 snRNP particle binds to the 5′ splice site of the pre-mRNA resulting in the formation of a commitment complex (1). In addition to the so-called common or Sm proteins (2) which are also present in the U2, U4 and U5 snRNPs, U1 snRNP contains three specific proteins denoted U1-70K, U1A and U1C (2,3). For its activity in splicing both the U1 RNA and protein, components are known to be needed but the precise function in the splicing process of the latter is only poorly understood (1–5). Recently it was shown by *in vitro* studies that the U1-70K protein plays an essential role in splicing, a role in which the phosphorylation state of the protein appears to be crucial (6).

With regard to the U1C protein, Heinrichs and co-workers (7) performed a functional study in which the pre-mRNA binding capacity of U1 snRNP particles containing or lacking the U1C protein was compared in an *in vitro* assay. U1 snRNPs lacking the U1C protein bound to the pre-mRNA 5′ splice site with only 40% efficiency as compared with complete U1 snRNPs. Binding efficiency could be restored to wild-type levels by adding purified HeLa U1C protein. These results suggested that the U1C protein is involved in the binding of the U1 snRNP particle to the 5′ splice site.

In contrast with the U1-70K and U1A protein, the U1C protein does not contain an RNP-80 or RNA recognition motif (8). Lacking such a motif, the U1C protein can only bind to the U1 snRNP particle when the U1-70K and common proteins are already associated with the U1 RNA (9). The N-terminal region of U1C, containing a zinc finger-like sequence which resembles the CC-HH zinc fingers of the TFIIIa type, is required and sufficient for this interaction (10). Similar CH motifs have been observed in the yeast splicing proteins PRP6, PRP9 and PRP11 (11,12). Neither one of the proteins U1C, PRP6, PRP9 or PRP11, however, has actually been shown to bind zinc. Recently, Legrain and co-workers (13) demonstrated in the yeast two hybrid system that the two CH motifs in the PRP9 protein are required for the formation of PRP9 homodimers.

In the present study we show that the human U1C protein is able to form homodimers as well. U1C dimers were detected in preparations of human U1C protein expressed in *Escherichia coli* and dimerization could be induced with *in vitro* translated U1C. In both an *in vitro* dimerization assay and the yeast two hybrid system it could be demonstrated that amino acid residues between positions 22 and 30 are required for dimerization.

MATERIALS AND METHODS

Expression and purification of $(his)_6$-tagged human U1C protein in *E.coli*

Using site directed mutagenesis an *Nde*I site was introduced at the translational start codon of the human U1C cDNA, which was cloned into pGEM-3Zf(+). By PCR the translational stop codon of the (*Nde*I)U1C cDNA was replaced by the codons of six histidine residues, followed by a stop codon and a *Bam*HI site. Subsequently the U1C$(his)_6$ cDNA was recloned as an *Nde*I–*Bam*HI fragment into the expression vector pET-3b (14) and with this construct *E.coli* strain BL21(DE3)pLysS (15) was transformed. The human U1C$(his)_6$ protein was overexpressed (16), followed by freeze–thawing and sonication of the cells in 10 ml PBS with 0.5 mM PMSF, 10 mM $MgCl_2$ and 0.5 mg DNAse I. After centrifugation all U1C$(his)_6$ was present in the non-soluble fraction which could be dissolved in 8 M urea. In the text the solubilized U1C$(his)_6$ fraction is referred to as U1C$(his)_6$ bacterial

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extract. A control extract was prepared from BL21(DE3)pLysS transformed with pET-3b lacking the U1C cDNA.

The U1C(his)$_6$ protein was purified from the bacterial extract following the procedures described by Janknecht *et al.* (17) and Schmitt *et al.* (18). U1C(his)$_6$ bacterial extract was incubated batchwise at 20°C with Ni–NTA–agarose beads (Qiagen) and IPP$_{100}$ (10 mM Tris–HCl pH 8.0; 100 mM NaCl; 0.05% NP-40). Proteins bound to the Ni-groups were eluted batchwise by incubating the beads two times during 5 min with elution buffer D [10 mM HEPES pH 7.9; 5 mM $MgCl_2$; 0.1 mM EDTA; 50 mM NaCl; 17% glycerol (v/v); 1 mM DTE; 1 mM PMSF; 10 mM NaF] containing increasing concentrations of imidazole: 40 mM, 60 mM, 200 mM, 500 mM. When the purification was performed under non-reducing conditions, DTE was omitted from the elution buffer.

Site directed mutagenesis

Single-stranded DNA of the U1C cDNA cloned into pGEM-3Zf(+) was produced with the helper phage M13K07. Point mutations were introduced into the cDNA using the oligo-directed mutagenesis system kit from Amersham and checked by sequencing.

In vitro transcription and translation

To produce T7-U1C mRNA for translation, 1 µg of linearized template was transcribed in essentially the same manner as described by Scherly *et al.* (19). The cDNA encoding U1C was recloned in pGEM–3Zf(+)(Promega) lacking a functional *Bam*HI site, as described previously (10). To produce ^{35}S-labeled U1C protein or derivatives thereof, 200 ng (2 µl) of the corresponding T7-mRNA were incubated with wheat germ extract (Amersham) and ^{35}S-methionine (Amersham) in essentially the same manner as described by Scherly *et al.* (19).

Truncated C proteins

To produce templates for the truncated human U1C proteins containing amino acids 23–159 and 30–159, the codons for residues 21/22 or 28/29 in the U1C cDNA were replaced by a *Bam*HI site (contains codons for Gly and Ser) using the site directed mutagenesis technique. The U1C sequences coding for residues 23–159 and 30–159 respectively, were subcloned as *Bam*HI–*Hin*dIII fragments directly behind the start codon of the mutant U1A (2/3) construct, in which codons for residues 2 and 3 were replaced by a *Bam*HI site (20). Except for the U1A start codon the new U1C constructs do not contain any other U1A codons.

In vitro U1C dimerization assay

U1C(his)$_6$ containing bacterial extract (15 µl) was incubated for 1 h at 20°C with 20 µl Ni–NTA–agarose (50% slurry) and 30 µl IPP$_{150}$. The beads were washed three times with 0.5 ml IPP$_{150}$, resuspended in 20 µl IPP$_{150}$ and incubated for 1 h at 20°C with 2 µl *in vitro* translated ^{35}S-labeled U1C protein, or mutants thereof. Non-bound protein was removed by extensive washing with IPP$_{150}$. All ^{35}S-labeled U1C associated with U1C(his)$_6$ protein bound to Ni-groups was eluted by incubating the beads with 20 µl elution buffer D containing 200 mM imidazole, but lacking DTE. The U1C(his)$_6$ was present in excess over the ^{35}S-labeled U1C protein, because when more ^{35}S-labeled U1C was added to the assay also more ^{35}S-labeled U1C could be precipitated (data not shown). The elution fractions were separated on an SDS–polyacrylamide gel. When separation was performed under non-reducing conditions the β-mercaptoethanol (5%) was omitted from the sample buffer.

Immunodetection of U1 snRNP proteins on Western blot

U1 snRNP proteins transferred to nitrocellulose were detected by immunostaining with monoclonal (mouse) and monospecific (rabbit) antibodies specifically directed against U1 snRNP proteins, and with phosphatase-conjugated second antibodies as described by Lehmeier *et al.* (21). The monoclonal mouse antibodies used were directed against the U1-70K (H111) (22), U1A (9A9) (23), B/B′ (KSm5) and D1 (KSm2) (24) proteins. Monospecific rabbit antibodies specifically raised against a peptide or recombinant fragment of the respective antigen, were used to detect the U1C, D3, D2, F and G (25) protein. Anti-(his)$_6$ antibodies were raised by immunizing a rabbit with (his)$_6$ coupled to BSA (Euro-Diagnostica B.V., Apeldoorn, The Netherlands). The anti-(his)$_6$-tag antibodies do recognize U1C(his)$_6$ protein but not U1C protein lacking the (his)$_6$-tag.

Detection of U1C homodimers using the yeast two hybrid system

Cloning of the U1C fusion proteins. A cloned human U1C cDNA (26) was used as a template for PCR amplification with different primers. The resulting DNA fragments encode the wild-type U1C protein or various N-terminal deletion mutants [corresponding to U1C(63–159), U1C(23–159) and U1C(30–159); see Fig. 4A]. These DNAs were cloned in the pAS2 plasmid, allowing synthesis of corresponding fusion proteins with the Gal4 DNA binding domain (Gal4BD). The wild-type amplified DNA fragment was also cloned in the pACTII plasmid for production of fusion protein with the Gal4 activator domain (Gal4AD; 27,28). Sequences of PCR amplified fragments were verified.

Yeast manipulations. Yeast cells were grown and transformed according to standard procedures. The Y526 yeast strain (*MATa, his3$^-$, ade2$^-$, lys2$^-$, trp1$^-$, leu2$^-$, canR, gal4$^-$, gal80$^-$, URA::GAL1-lacZ*) was transformed with the two plasmids and transformants were selected on minimal medium lacking leucine and tryptophan and assayed for β-galactosidase activity using a filter assay with X-gal. Several colonies were streaked and cells were grown in liquid cultures to perform quantitative β-galactosidase assays (13).

RESULTS

Expression and functionality of recombinant U1C(his)$_6$ protein

To investigate whether U1C is able to form homodimers, the human U1C protein with a C-terminal tag of six histidine residues, was expressed in *E.coli*. The expressed U1C(his)$_6$ protein was detected on a Coommassie Brilliant Blue stained SDS–polyacrylamide gel as a prominent 21 kD band, which was not present in a control extract of bacteria lacking U1C(his)$_6$. This 21 kD band corresponds with the expected mobility of the U1C protein from HeLa cells (26) and is immunologically recognized on a Western blot by anti-U1C monospecific antibodies directed to the CH

motif of the protein and by anti-$(his)_6$ polyclonal antibodies (data not shown). Both antibodies did not crossreact with bacterial proteins. Interestingly, a band of ~42 kD was also detected with both antibodies. The characterization of this band will be discussed below.

The U1C$(his)_6$ protein was purified from the extract with Ni–NTA–agarose. Proteins bound to the Ni–NTA-groups were specifically eluted with increasing imidazole concentrations. The U1C$(his)_6$ protein was eluted most efficiently at a concentration of 200 mM imidazole (data not shown). The purified U1C$(his)_6$ protein seems to be functional since it is capable of competing with *in vitro* translated ^{35}S-labeled U1C protein for binding to U1 snRNPs (data not shown) in an S100 reconstitution assay as described previously (10).

Recombinant human U1C protein is able to form homodimers

Based on the immunostaining of a 42 kD band as described above, we hypothesized that the 42 kD complex might represent a U1C$(his)_6$ homodimer. To detect the putative U1C$(his)_6$ dimer band on a Coomassie stained gel as well, the purification with Ni–NTA–agarose was repeated under non-reducing conditions, i.e. without DTE in the bacterial extract and elution buffers. Subsequently, samples of the elution fractions were separated under non-reducing conditions, i.e. β-mercaptoethanol was omitted from the sample buffer, on an SDS–polyacrylamide gel (Fig. 1A). Following this procedure ~90% of the U1C$(his)_6$ in the bacterial extract was retained on the Ni–NTA–agarose beads (compare lanes 2 and 3). Most of the eluted U1C$(his)_6$ is present in the 200 mM imidazole elution fraction (lane 6) but at 500 mM imidazole a considerable amount of U1C$(his)_6$ was eluted as well (lane 7). The bands which migrate at ~12 and 16 kD most likely represent distinct U1C degradation products. Figure 1A (lanes 5–7) demonstrates that under non-reducing conditions the putative U1C$(his)_6$ homodimer of 42 kD indeed can be detected on a Coomassie stained gel. The finding that this band is only present in the imidazole eluted protein preparation under non-reducing conditions suggests that the 42 kD band corresponds to a complex of proteins held together by one or more disulfide bridges.

To provide more evidence for the presence of the U1C$(his)_6$ protein in the 42 kD complex, U1C$(his)_6$ bacterial extract was separated on an SDS–polyacrylamide gel under non-reducing conditions. As a control, bacterial extract lacking U1C$(his)_6$ was separated on the same gel. With reference to the markers a gel piece from the lanes of both extracts was cut out of the gel at the position of the 42 kD band and extracted with standard reducing SDS-sample buffer. Both samples were separated on an SDS–polyacrylamide gel and Western blotted. Figure 1B shows the Western blot immunostained with monospecific antibodies directed to the U1C protein. With antibodies directed to the $(his)_6$-tag similar results were obtained (not shown). In the control lane (lane 1) no proteins were detected with anti-U1C antibodies. The 42 kD complex from the U1C$(his)_6$ bacterial extract, however, could partly be reduced to U1C$(his)_6$ monomers, migrating at 21 kD (Fig. 1B, lane 2). A similar result was obtained when purified, non-reduced U1C$(his)_6$ (200 mM imidazole elution fraction; Fig. 1A, lane 6) was pre-incubated with 25 mM DTE and separated by SDS–PAGE in the presence of DTE (data not shown). These results indicate that the U1C$(his)_6$ protein expressed in *E.coli* indeed is able to form homodimers, which might be stabilized by one ore more disulfide bridges. Apparently not all of the U1C$(his)_6$ dimers were reduced in the experiment of Figure 1B since the 42 kD band still could be detected. This may be due to the relatively high concentration of the recombinant U1C protein or limited accessibility of a disulfide bridge.

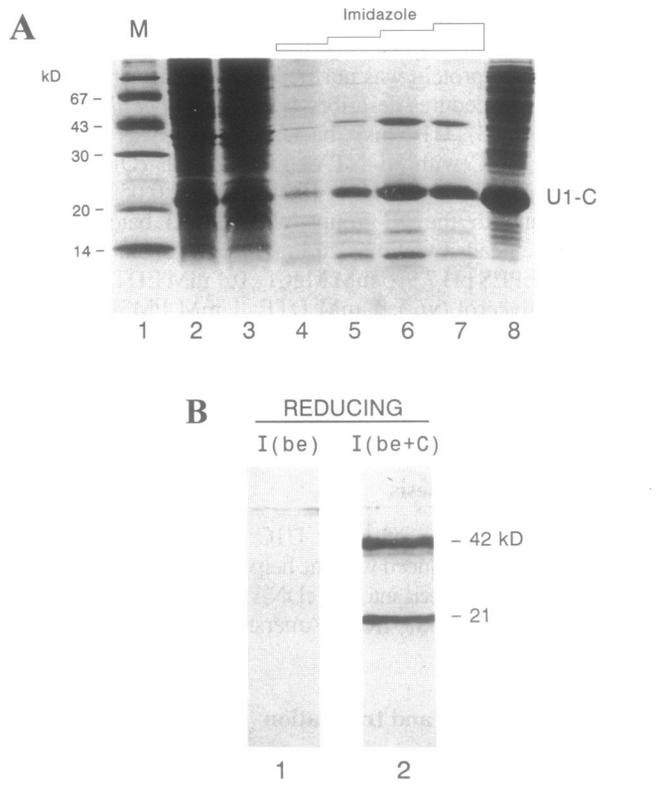

Figure 1. Detection of U1C$(his)_6$ homodimers. (**A**) Purification under non-reducing conditions of recombinant U1C$(his)_6$. Coomassie Brilliant Blue stained 10% SDS–polyacrylamide gel: lane 1: protein molecular weight markers; lane 2: 1 µl U1C$(his)_6$ bacterial extract, of which 2.5 ml was used for purification of U1C$(his)_6$; lane 3: 3 µl of the supernatant containing the proteins which did not bind to the Ni–NTA–agarose. The U1C$(his)_6$ protein was eluted batchwise with increasing concentration of imidazole. Of the elution fractions (2 ml in volume each) 10 µl was separated on gel; lane 4: 40 mM imidazole fraction; lane 5: 60 mM imidazole fraction; lane 6: 200 mM imidazole fraction; lane 7: 500 mM imidazole fraction; lane 8: 1% of the proteins which were still associated with the Ni–NTA–agarose beads after the elution procedure, dissolved in SDS-sample buffer. (**B**) Immunostaining of Western blot with the excised, non-reduced 42 kD band (band I) after separation under reducing conditions. U1C$(his)_6$ containing bacterial extract (20 µl) and bacterial extract lacking U1C$(his)_6$ (20 µl) were separated by non-reducing SDS–PAGE. From the lanes of both extracts a gel piece was excised at the position of 42 kD (band I) which was crushed and analyzed on a reducing 15% SDS–polyacrylamide gel. Proteins were transferred to nitrocellulose and immunostained with monospecific anti-U1C antibodies. Lane 1: proteins from bacterial extract lacking U1C$(his)_6$ at a position that corresponds with band I [I(be)]; lane 2: band I from bacterial extract containing U1C$(his)_6$ [I(be+C)].

In vitro translated U1C forms homodimers as well

In order to test whether U1C homodimers also can be detected when the protein is synthesized *in vitro* we immunoprecipitated ^{35}S-labeled U1C translated in wheat germ extract using polyclonal anti-U1C antibodies. Figure 2, lane 3 indicates that indeed a complex at the dimer position is precipitated. As a control the U1C dimer obtained in our *in vitro* dimerization assay (see below)

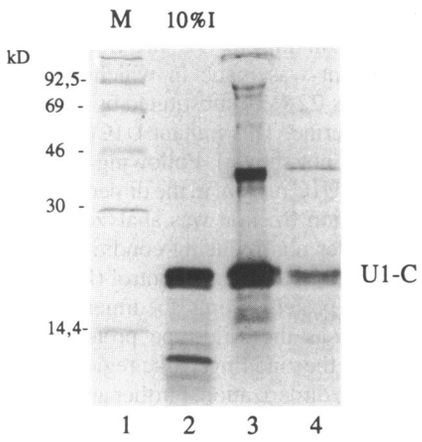

Figure 2. Immunoprecipitation of *in vitro* translated U1C. *In vitro* translated U1C (2 µl) was incubated for 90 min at 20°C with polyclonal anti-U1C antibodies coupled to protein-A agarose beads (Biozym) in a final volume of 40 µl IPP_{150}. The beads were washed three times with IPP_{150} and resuspended in non-reducing SDS sample buffer. The supernatants were separated on a 10% SDS–polyacrylamide gel. Lane 1: Protein molecular weight markers; lane 2: 10% of the input of *in vitro* translated ^{35}S-labeled U1C; lane 3: immunoprecipitation of 2 µl *in vitro* translated U1C via polyclonal anti-U1C antibodies; lane 4: control lane with the dimer obtained from the *in vitro* dimerization assay (see Fig. 3).

was used (Fig. 2, lane 4). The dimer from the *in vitro* U1C translation migrates somewhat faster than the dimer generated in the dimerization assay (Fig. 2, compare lanes 3 and 4) because the *in vitro* translated protein lacks a $(his)_6$-tag. No dimers were observed when the U1C translation mixture was separated by non-reducing SDS–PAGE (Fig. 2, lane 2).

The dimerization assay

An *in vitro* dimerization assay was developed in which the dimerization between U1C$(his)_6$ expressed in *E.coli* and *in vitro* translated ^{35}S-labeled U1C could be analyzed in more detail (Fig. 3A).

In this assay, U1C$(his)_6$ from a bacterial extract was coupled to Ni–NTA–agarose beads. All non-bound proteins were removed by extensive washing of the beads. Based on the results obtained by SDS–PAGE analyses shown above we can assume that U1C$(his)_6$ is present on the Ni-beads as a mixture of monomers and dimers. The U1C$(his)_6$–Ni-beads were then incubated with *in vitro* translated ^{35}S-labeled human U1C protein lacking a $(his)_6$-tag. Following several wash steps the bound U1C$(his)_6$ and the ^{35}S-labeled U1C associated with it, were specifically eluted by 200 mM imidazole. As a negative control the ^{35}S-labeled U1C was added to Ni–NTA–agarose beads pre-incubated with bacterial extract lacking U1C$(his)_6$.

One sample of each elution fraction was analyzed on a standard reducing SDS–polyacrylamide gel, another sample on a non-reducing gel, i.e. in the absence of β-mercaptoethanol. Figure 3B, lane 2 demonstrates that a ^{35}S-labeled protein(complex) of ~41 kD is eluted in the absence of a reducing agent. Since the 41 kD band was never observed in the control assays (lanes 3 and 5) it is most likely to conclude that the 41 kD complex corresponds to a dimer formed by U1C$(his)_6$ and ^{35}S-labeled U1C [this dimer thus migrates somewhat faster than the 42 kD U1C$(his)_6$ homodimer]. As expected the ^{35}S-labeled 41 kD complex is destabilized under reducing SDS–PAGE conditions

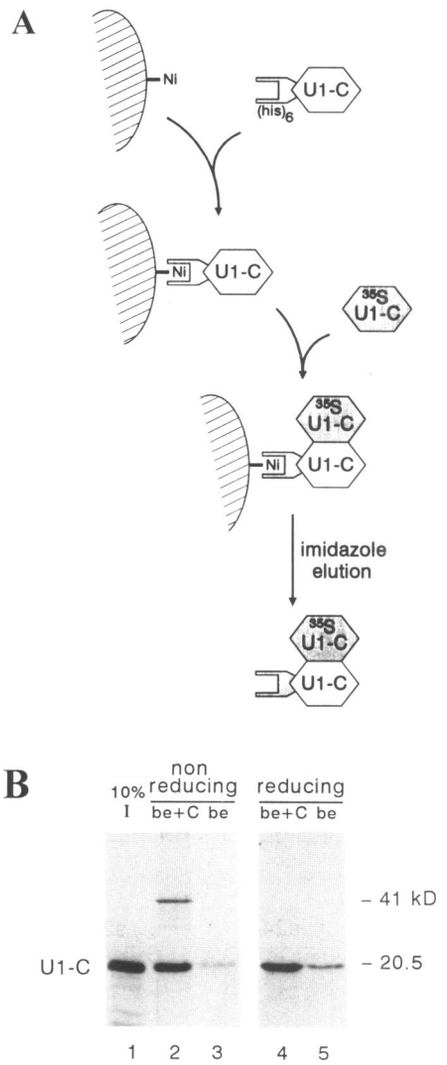

Figure 3. The dimerization assay. (**A**) Scheme of the *in vitro* U1C dimerization assay. U1C$(his)_6$ from bacterial extract was coupled to Ni–NTA–agarose beads. The U1C$(his)_6$-beads were incubated with *in vitro* translated ^{35}S-labeled U1C protein or mutants thereof and precipitated ^{35}S-labeled protein was eluted from the beads with 200 mM imidazole containing elution buffers. The eluted ^{35}S-labeled protein was visualized by SDS–PAGE followed by autoradiography. (**B**) Dimerization of ^{35}S-labeled U1C and recombinant U1C$(his)_6$. Lane 1: 10% of the input of *in vitro* translated ^{35}S-labeled U1C protein; lanes 2 and 3: non-reductive separation of ^{35}S-labeled U1C eluted from beads containing or lacking U1C$(his)_6$, respectively; lanes 4 and 5: as lanes 2 and 3, but analyzed by SDS–PAGE with sample buffer containing 5% β-mercaptoethanol.

and only U1C monomers can be detected (lane 4). The eluted ^{35}S-labeled U1C monomers seen in lanes 2 and 4 are probably generated by background binding of ^{35}S-labeled U1C to Ni–agarose associated bacterial proteins (see background lanes 3 and 5) and by destabilization of eluted dimers.

Amino acids between positions 22 and 30 are required for homodimerization

To determine which part of the U1C protein is involved in dimer formation ^{35}S-labeled U1C mutant proteins (Fig. 4A) were tested in the *in vitro* dimerization assay. The mutant U1C(1–60) is

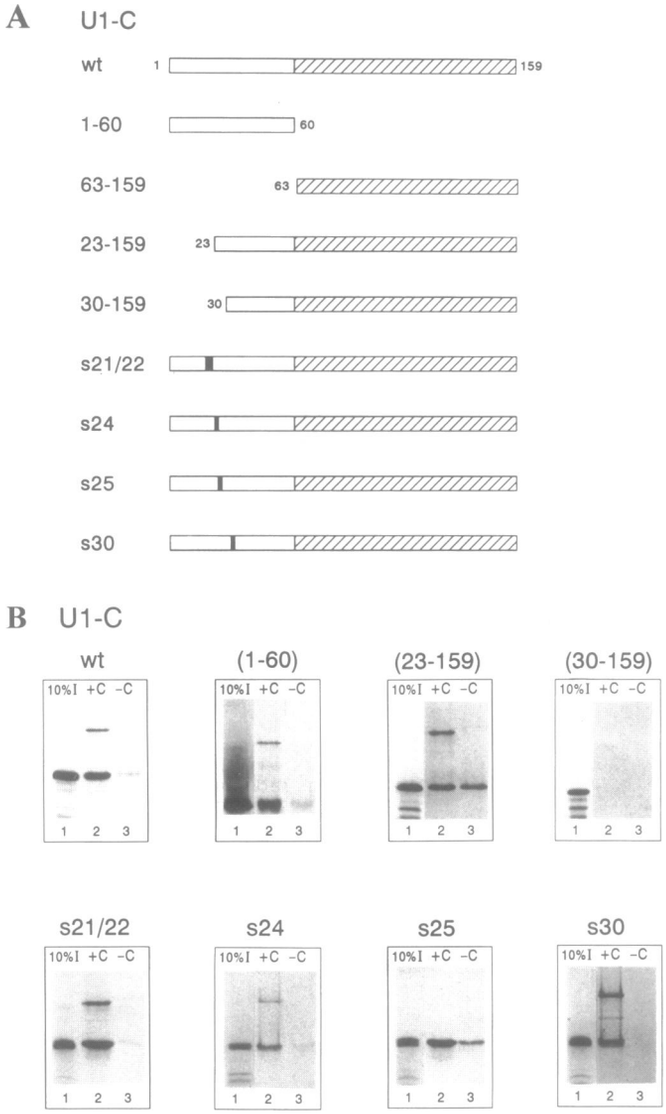

Figure 4. *In vitro* dimerization of ^{35}S-labeled U1C or mutants thereof and U1C(his)$_6$ immobilized on Ni–NTA–agarose. As a control the ^{35}S-labeled proteins were tested with Ni-beads which were preincubated with bacterial extract lacking U1C(his)$_6$. (**A**) Schematic representation of the mutant proteins derived from the wild-type human U1C protein. The amino acid numbers are indicated. The shaded box represents the proline and methionine rich C-terminal domain, starting at residue 61. Dark-grey boxes indicate the position at which an amino acid is substituted by another residue [U1C mutants (s25): substitution of cysteine by serine; (s24) and (s30): substitution of histidine by glutamine; (s21/22): substitution of arginine/lysine by glycine/serine]. (**B**) 200 mM imidazole elution fractions of ^{35}S-labeled U1C (wild-type) and U1C mutants tested in the dimerization assay and separated by non-reducing SDS–PAGE. Lanes 1: 10% of the 2 µl input of *in vitro* translated ^{35}S-labeled U1C protein (wild-type or mutant); lanes 2 and 3: ^{35}S-labeled U1C (wild-type or mutant) eluted from beads containing (+C) or lacking U1C(his)$_6$ (–C) respectively. The assay was performed with the mutant proteins U1C(1–60), U1C(23–159), U1C(30–159), U1C(s21/22), U1C(s24), U1C(s25) and U1C(s30) respectively.

known to bind to U1 snRNP with comparable efficiency as the wild-type protein (9). The U1C substitution mutants (s24) and (s30), in which a histidine residue is substituted by a glutamine, were previously reported to have lost their ability to bind to U1 snRNP. In contrast, substitution of the Cys-25 by Ser-25 (mutant s25) did not affect binding to U1 snRNP (10). Additionally, a double point mutant was made in which the codons for the residues Arg-21/Lys-22 were substituted by a *Bam*HI site, which codes for glycine/serine. This mutant U1C(s21/22) cannot bind to U1 snRNP (data not shown). Following the incubation of an ^{35}S-labeled mutant U1C protein in the dimerization assay the 200 mM imidazole elution fraction was analyzed on an SDS–polyacrylamide gel under non-reducing conditions. Wild-type U1C protein was included as a positive control (Fig. 4B).

The U1C(1–60) protein seems to dimerize with U1C(his)$_6$ almost as efficient as the wild-type protein. The N-terminal domain, containing the zinc finger-like region thus appears to be sufficient for *in vitro* dimerization. Further analyses of N-terminal deletion mutants show that the first 22 amino acids, which encompass the two conserved cysteines of the zinc finger-like region, are not essential for dimerization. The mutant protein U1C(23–159) still is able to dimerize with U1C(his)$_6$ whereas U1C(30–159) has completely lost its ability to interact with U1C(his)$_6$ [Fig. 4B, lanes 2 of (23–159) and (30–159)]. All the mutations which previously were shown to abolish binding to U1 snRNP hardly or only moderately interfered with dimerization in this assay [Fig. 4B, lanes 2 of (s21/22), (s24), (s30)]. When Cys-25 is substituted, no stable dimers can be detected after SDS–PAGE (Fig. 4B, lane 2 of s25), indicating that Cys-25 is most likely the residue responsible for disulfide bridge formation between two U1C proteins. However, as for all dimerizing U1C derivates, incubation of mutant s25 leads to a specific monomeric signal (Fig. 4B, lanes 2). These results suggest that s25 is able to form homodimers purely as a result of non-covalent interactions which are disrupted during SDS–PAGE.

The N-terminal deletion mutant U1C(23–159) for some reason gives a higher monomer background in the dimerization assay. At the moment we have no explanation for this phenomenon.

Similar results were obtained when *in vitro* translated ^{35}S-labeled mutant U1C proteins were immunoprecipitated via polyclonal anti-U1C antibodies, as in the experiment of Figure 2 (data not shown).

U1C homodimers are detected in the yeast two hybrid assay

To verify the results of our *in vitro* assays, we used the two hybrid assay in yeast which allows the detection of protein–protein interactions *in vivo* (29). When the two U1C wild-type fusion proteins were expressed in yeast, β-galactosidase activity was detected using a qualitative X-gal conversion assay (data not shown). This was specific since the production of any of the two fusion proteins in combination with an irrelevant protein did not induce β-galactosidase synthesis. However, transformants grew slowly and, when primary transformants were streaked on plates, fast growing variants were often recovered which were systematically negative in an X-gal conversion assay.

To analyze the formation of U1C protein homodimers in more detail, several versions of the U1C protein fused to the Gal4 DNA binding domain were assayed in combination with the wild-type U1C partner fused to the Gal4 activation domain. In Figure 5 it is shown that dimers are formed between wild-type and U1C(23–159) proteins and resulting β-galactosidase activities are comparable or even higher than those detected for the wild-type homodimer. The various clones exhibited different activities,

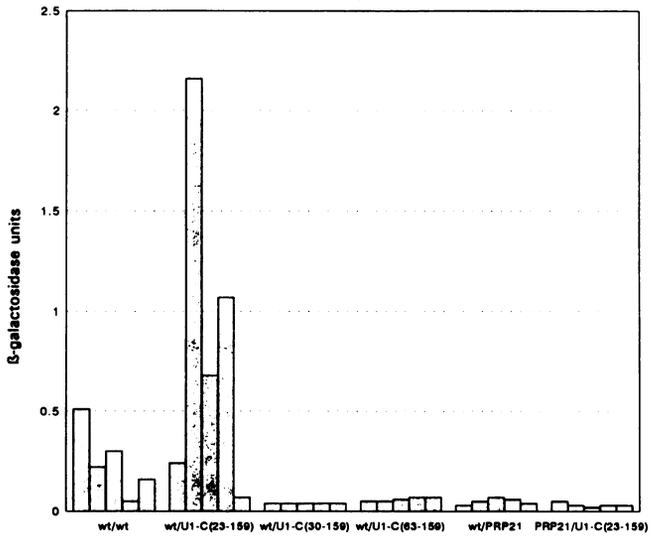

Figure 5. Analysis of U1C dimers in the yeast two hybrid assay. Fusion proteins expressed by the cells are indicated below the histograms (Gal4AD fusion protein/Gal4BD fusion protein). Five independent transformants were streaked for each experiment. PRP21 is a yeast splicing factor (28). Assays were done in duplicate for each transformant and the values were within a 10% variation range.

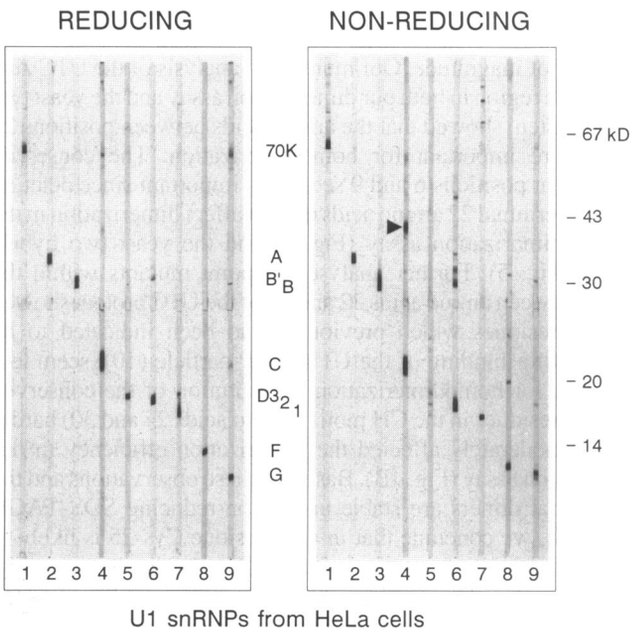

Figure 6. Detection of U1C dimers in U1 snRNPs by immunostaining. Native U1 snRNPs were isolated from HeLa cells by immunoaffinity chromatography followed by Mono Q chromatography as described by Bach *et al.* (30) (generous gift of C. Will and R. Lührmann, Marburg). U1 snRNPs (~50 μg; protein weight) were separated on a 10% SDS/high TEMED–polyacrylamide gel under reducing or non-reducing conditions and transferred to nitrocellulose. The nitrocellulose lanes were cut into strips and each strip was immunostained with a monoclonal or monospecific antibody directed to the U1 snRNP proteins U1-70K (lanes 1), U1A (lanes 2), Sm-B/B′ (lanes 3), U1C (lanes 4), Sm-D3 (lanes 5), Sm-D2 (lanes 6), Sm-D1 (lanes 7), Sm-F (lanes 8) and Sm-G (lanes 9) respectively. Left panel: U1 snRNP proteins separated under reducing conditions; right panel: U1 snRNP proteins separated under non-reducing conditions. The arrowhead indicates the position of the U1C homodimer.

probably reflecting a variable amount of negative variants arising during the liquid culture. When the wild-type U1C fused to the GAL4 activation domain was replaced by an irrelevant protein such as PRP21, no β-galactosidase activity was observed indicating that the activity measured for the combination of the wild-type U1C and the U1C(23–159) proteins very likely reflects the formation of a dimer. Similarly, the co-expression of the wild-type U1C protein in combination with U1C(30–159), U1C(63–159) or an irrelevant protein did not lead to any detectable β-galactosidase activity. A number of U1C point mutants (s6, s9, s24, s25 and s30) were tested in the yeast two hybrid assay as well. These mutants also showed dimer formation *in vivo* (data not shown). Our results suggest that in the yeast two hybrid system non-covalent interactions are sufficient for U1C dimerization. We conclude that in the two hybrid assay U1C homodimers are detected and that their assembly points to a functional region between the amino acid residues 22 and 30, which is in agreement with the results obtained with our dimerization assay.

Are U1C homodimers present in HeLa U1 snRNPs?

Assuming that dimerization of the human U1C protein has functional implications, it would be interesting to investigate whether U1C dimers are present in U1 snRNP particles as well. For the detection of U1C homodimers in U1 snRNPs, U1 particles were purified from HeLa cells as described by Bach *et al.* (30) and its protein components were separated on a reducing and a non-reducing SDS–polyacrylamide gel followed by Western blotting (Fig. 6). Individual U1 snRNP proteins were visualized on the blot by immunostaining with monoclonal or monospecific antibodies specifically raised against particular U1 snRNP proteins, except for Sm-E, as described previously (9). Under reducing conditions the U1C protein was detected as a monomer migrating at a position of 21 kD. However, under non-reducing conditions not only U1C monomers were detected with monospecific anti-U1C antibodies, but also a band of ~42 kD which corresponds to the expected molecular weight of U1C homodimers (Fig. 6, lane 4). Since none of the other antibodies stained a protein(complex) at the same position, these results suggest that U1C homodimers indeed are present in HeLa U1 snRNPs. Because the experiment was performed under conditions which are not favourable to interparticle interactions (22) the possibility of such interactions involving the U1C protein seems unlikely. We were not able to detect dimers in U1 snRNP particles purified from HeLa S100 extract using sucrose gradient centrifugation (data not shown). We do not know whether U1C dimers are not present in this U1 snRNP population or that dimers have already been reduced to monomers due to the isolation and separation procedures.

DISCUSSION

Our results demonstrate that the human U1 snRNP-specific protein C is able to form homodimers. The finding that the residues between positions 22 and 30 within the CH-motif are necessary for dimerization, as shown in an *in vitro* dimerization assay and in the yeast two hybrid system, is in principle in agreement with the reported involvement of the two zinc finger-like motifs in the homodimerization of the yeast splicing factor PRP9 (13). Legrain and co-workers observed in the yeast two hybrid system that

homodimerization of PRP9, mutated in the conserved cysteine and histidine residues of the CH motif, was reduced with at least one order of magnitude. Our mutational analysis of the U1C zinc finger-like region in both our dimerization assay and the yeast two hybrid system showed that the amino acids between positions 22 and 30 are important for homodimerization. The conserved cysteines at positions 6 and 9 seem less important since deletion of the N-terminal 22 amino acids did not affect dimerization in the *in vitro* dimerization assay (Fig. 4) and the yeast two hybrid system (Fig. 5). Further analyses of point mutants within the region between amino acids 22 and 30 of the U1C protein showed that the residues which previously had been indicated to be important for binding of the U1 snRNP particle (10), seem less important for homodimerization. Substitution of the conserved histidine residues in the CH motif (amino acids 24 and 30) hardly or only moderately affected the dimerization efficiency in our dimerization assay (Fig. 4B). Based on these observations and the finding that dimers are stable under non-reducing SDS–PAGE conditions, we conclude that *in vitro* residue Cys-25 is likely to be involved in the formation of a disulfide bridge between two U1C molecules. This conclusion is in concert with data reported by Benezra (31) who showed that E2A proteins, a subfamily of the basic helix–loop–helix family of DNA-binding proteins, form homodimers that are linked via a disulfide bond. However, the appearance of relatively high monomer signals in our dimerization assay (Fig. 4B) do indicate that non-covalent interactions are involved in dimer formation as well.

We were also able to show the formation of U1C dimers with *in vitro* translated U1C. When a U1C translation mixture was separated by non-reducing SDS–PAGE no dimers were observed (Fig. 2, lane 2). However, U1C dimers could be detected after immunoprecipitation with anti-U1C antibodies (Fig. 2, lane 3). Since formation of dimers most likely depends on the concentration of the monomer protein, dimerization might be facilitated by binding of U1C to the bivalent antibodies. Alternatively, the binding of the antibodies could stimulate the U1C protein to adopt its native structure. Such antibody assisted protein folding has been demonstrated by Carlson and Yarmush for the ribonuclease A S-protein (32). A third possibility is that the cell-free translation system contains a component that prevents dimer formation, as indeed has been suggested by Benezra (31). By immunoprecipitation of U1C this component might be separated efficiently from the U1C protein.

Our data indicate that the U1C protein can form homodimers *in vivo* as well. This was shown in the yeast two hybrid assay (Fig. 5). Using this system the importance of the amino acids 23–29 of the U1C protein for dimer formation was corroborated. Indeed, the absolute values of β-galactosidase activity are low compared with those found for other homodimers detected with the yeast two hybrid assay, such as PRP9 (40 units, ref. 28). Nevertheless, the positive results with the U1C protein were reproducibly found. It should be stressed that in the case of homodimerization, an inhibition of productive interactions may occur due to the formation of true homodimers between identical fusion proteins. The exact nature of the link between the two U1C monomers *in vivo* is not precisely known. In our *in vitro* experiments the formation of a disulfide bridge in addition to non-covalent interactions is necessary for the detection of U1C dimers after SDS–PAGE, as has been discussed above. In the yeast two hybrid system probably only non-covalent interactions between the two monomers exist as has been suggested by the positive results obtained with the U1C point mutants. But also *in vivo* such non-covalent interactions are primarily mediated by amino acid residues between positions 22 and 30.

The data shown in Figure 6 suggest that at least a considerable portion of the U1C protein from purified HeLa U1 snRNPs is present in dimers stabilized by a disulfide bridge. Part of the putative U1C dimers from HeLa U1 snRNPs may have been reduced during the preparation of the particles for which DTT-containing buffers were used (30) which may lead to an underrepresentation of dimers. We do not know whether the disulfide bridges are actually present in U1 snRNP particles or that they are formed during the snRNP isolation procedure. It is likely that the dimers are formed by non-covalent interactions which subsequently might facilitate the formation of a disulfide bridge. Our data are in agreement with the estimated stoichiometry for U1 snRNPs as proposed by Feeney *et al.* (33) who predicted the presence of two U1C molecules per U1 snRNP particle.

Since the U1C homodimer might be present in U1 snRNP particles *in vivo*, as suggested by the results of Figure 6, an important question concerns the function of these U1C dimers. Data reported by Heinrichs *et al.* (7) indicated that the U1C protein is involved in the binding of the U1 snRNP particle to the pre-mRNA 5′ splice site. Possibly, stable binding to the 5′ splice site requires the presence of a U1C dimer on the U1 snRNP particle. Within such a model it can be speculated that U1C dimers are responsible for a direct contact with the pre-mRNA. Our present studies are directed to test this hypothesis.

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