Zinc finger-like structure in U1-specific protein C is essential for specific binding to U1 snRNP

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ABSTRACT

The U1 small nuclear ribonucleoprotein (snRNP) contains three specific proteins denoted 70K, A and C, in addition to the common proteins. Specific functions of these proteins are not known although recently protein C was shown to be involved in the binding of U1 snRNP to the 5′ splice site of a pre-mRNA. Unlike proteins A and 70K, U1-C lacks an RNA binding domain (RNP-80 motif) and does not appear to bind directly to U1 snRNA. However, at the amino terminal end protein C contains a zinc finger-like structure of the CC-HH type found in transcription factor TF IIIA. Several lines of evidence indicate that the zinc finger-like structure is essential for the binding of protein C to U1 snRNP particles: i) deletion analysis of protein C showed that the N-terminal 45 amino acids are sufficient for binding to U1 snRNPs, ii) modification of the cysteine residues in the N-terminal domain with N-ethylmaleimide and iii) single point mutations of the cysteines and histidines contributing to the putative zinc finger abolished binding of protein C to U1 snRNPs. Interestingly, unlike the proteins U1-A and U1-70K the U1-C protein is unable to bind to naked U1 snRNA. On the other hand it is shown that protein C does not bind to the known protein constituents of the U1 particle without the U1 snRNA being present. These data indicate that the binding of protein C to U1 snRNP is dependent on the presence of both the U1 snRNA and one or more of the U1 snRNP proteins.

INTRODUCTION

The U1 snRNP particle is essential for the splicing of pre-mRNA. It is the most abundant of the major snRNPs (U1−U6) (1−3) and is a complex of U1 snRNA and many different proteins of which the proteins B, B′, D, D′, E, F and G (the so-called Sm proteins) are present in all major snRNPs whereas proteins 70K, A and C are specific for U1 (4). The U1 snRNP functions in the first step of the splicing process which is initiated by binding of U1 snRNP to the 5′ splice site of the pre-mRNA (5−9). Although base pairing between the 5′ end of U1 snRNA and the 5′ splice site is essential for complex formation (10, 11), U1 snRNP proteins are necessary as well (5, 12). Recently it was shown by Heinrichs *et al.* (13), that the U1-C protein is needed for the binding of U1 snRNP to pre-mRNA. Binding of U1 particles lacking the C protein to the 5′ splice site of rabbit β-globin pre-mRNA was reduced by 50% as compared to native U1 particles. The binding activity could be restored by adding purified HeLa C protein (13). Whether protein C augments interaction between the 5′ end of U1 RNA and a 5′ splice site indirectly via long-range interactions or by direct contact with the mRNA-U1 RNA hybrid remains to be elucidated.

Another intriguing question is the mode of interaction of U1-C with U1 snRNP. It is known that in the formation of U1 snRNP the U1-specific proteins A and 70K bind directly to the U1 RNA. A conserved 80 amino acids domain, referred to as the RNP-80 motif, is essential for the interaction of both proteins with U1 RNA (14, 15). Protein C does not contain such a motif (16) and unlike the proteins A and 70K the C protein does not appear to bind naked U1 snRNA (see Results, Interaction of protein C with U1 snRNP constituents).

Yet, the 159 amino acids long human C protein reveals two other interesting structural domains. The carboxy terminal two-thirds of the protein is unusually rich in proline and methionine residues, some of which occur in repeating motifs. In contrast, the amino-terminal 60 amino acids long domain lacks proline and contains several cysteines, histidines and aromatic residues (16). On close inspection of the latter sequence we noticed that some of these cysteines and histidines could be arranged to form a zinc finger-like structure of the CC-HH type as found in TF IIIA (Figure 1) (17, 18). Zinc fingers have previously been shown to be involved in protein-nucleic acid or protein-protein interactions (19). In order to gain information as to whether the zinc finger-like motif contributes to the function of the C protein we have investigated the minimal region of the C protein needed for stable interaction with U1 snRNP. In this paper we show that an amino-terminal 45 residues long region of protein C, encompassing the putative zinc finger is sufficient for binding to U1 snRNP. In further experiments we demonstrate that the

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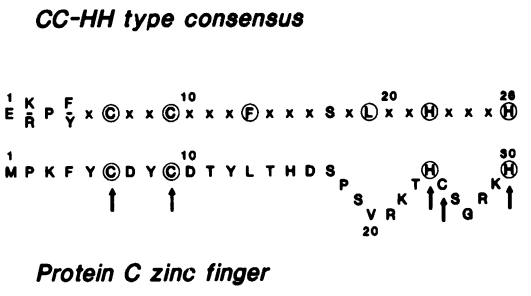

Figure 1. Comparison of the putative zinc finger motif of the U1-C protein with the CC-HH-type zinc finger consensus sequence. The residues which are more than 90% conserved are circled, whereas the non-circled letters in the consensus sequence indicate more than 50% representation (18). X, any amino acid. The arrows point to the amino acids in protein C which were changed by site directed mutagenesis.

residues, which might form a zinc finger, are crucial for the activity of this binding region.

The interaction of protein C within the U1 particle is, however, rather complex. Our data show that the C protein is unable to bind to naked U1 snRNA nor does it bind to the known protein constituents of U1 snRNP without intact U1 snRNA being present.

MATERIALS AND METHODS

In vitro transcription

To produce T7-U1 C mRNA or T7-U1 70K mRNA for translation, 1 μg of linearized (*Hin*dIII) template was incubated in essentially the same way as described by Scherly *et al.* (15). Protein U1-C cDNA (16) was recloned as *Eco*RI-*Eco*RI fragment into the vector pGEM-3Zf(+) from Promega. Our U1-70K cDNA, of which the sequence is fully contained within the FL70K sequence described by Theissen *et al.* (20), was mutated at the translation initiation codon and inserted into pGEM-3Zf(+) as described by Query *et al.* (14). U1 snRNA transcripts (cDNA cloned in pGEM-3Zf(+)) were produced and biotin labeled as described previously (15).

In vitro translation

To produce ^{35}S-labelled U1-C protein or derivatives thereof 200 ng (2μl) of the corresponding T7-mRNA was incubated with wheat germ extract (Promega) and ^{35}S-methionine (Amersham) in essentially the same way as described by Scherly *et al.* (15).

Isolation of native and Δ-U1 snRNPs

The native U1 snRNPs, ΔC U1 snRNPs and Δ[A,C] U1 snRNPs were isolated as described by Heinrichs *et al.*, (13) and Bach *et al.*, (21).

Reconstitution assay

In the reconstitution assay, 1μl of a standard *in vitro* translation reaction driven by protein C T7-mRNA was incubated for 30 min at 4°C in buffer A (20 mM Hepes-KOH pH 7.9, 25 mM KCl, 1 mM MgCl2, 5% glycerine, 0.5 mM DTE, 0.5 mM PMSF) in the presence of 200 fmol native U1 snRNPs or Δ-particles (final volume 30μl). U1 snRNPs were immunoprecipitated by adding 20μl of protein A-Sepharose coupled anti-m3G polyclonal antibodies (2mg of antibodies coupled to 1 ml of protein A-Sepharose pellet), 30 min incubation at 4°C and 30 s centrifugation. The pellets were washed twice with IPP_{100} (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.1% NP40), resuspended in 20μl SDS-PAGE sample buffer and boiled for 5 min. After short centrifugation, the supernatant was loaded on a 15% SDS-polyacrylamide gel. After electrophoresis, the gel was treated with Amplify (Amersham), dried for 1 h at 80°C and exposed to Kodak XOMAT film at −80°C for 1 day. The amount of bound protein C was calculated by comparing the blackening on the film with the counts (cpm) of a TCA-precipitate of input protein C.

Truncated C proteins

To produce ^{35}S-labelled truncated C proteins C-Del 1 to 6, the U1-C cDNA was linearized within the coding sequence by the appropriate restriction enzymes (respectively *Taq*I, *Bgl*I, *Nco*I, *Hae*III, *Ava*II, *Dde*I) prior to transcription and translation. In order to obtain truncated C proteins C-Del 7 and 8 the U1-C cDNA was cloned in the multicloning site of vector pT7-7 (22) after being 5′ shortened by respectively *Mae*III and *Dde*I digestion. Transcription (*Hin*dIII linearization) and translation of C-Del 7 and 8 templates resulted in truncated C proteins with an amino-terminal fusion peptide of 7 (C-Del 7) or 9 (C-Del 8) amino acids.

NEM treatment of in vitro made protein C

One microliter of a standard protein C translate was incubated with 1 mM N-ethyl maleimide (NEM) (final volume 5μl) for 30 min at 25°C. After incubation, unbound NEM was inactivated by adding 10 mM DTT (final volume 10μl) followed by 5 min incubation at 25°C.

Site directed mutagenesis

Single stranded DNA of the U1-C 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. Of each mutated cDNA the mutated area was checked by sequencing.

Preparation of S100 extract and S100-reconstitution-assay with protein U1-C

HeLa S100 extract was prepared as described previously (23) with minor modifications. After washing the cells with PBS buffer, they were resuspended in 25 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.25 mM DTT, 0.5 mM PMSC, 0.1 M KCl, 0.01% NP40, 20% glycerol. The S100 extract contained 3.2 mg protein/ml (24). RNase treated S100 was prepared by incubating the extract with RNase A (Sigma) (40 μg/ml) for 1 h at 20°C.

In the S100-reconstitution assay 1μl of protein C translate was incubated for 1.5 h at 20°C in buffer B (20 mM Hepes-KOH pH7.9, 100 mM KCl, 1 mM $MgCl_2$, 0.05% NP40) in the presence of 6μl S100 or RNase treated S100 (final volume 30μl). U1 RNA associated proteins were immunoprecipitated by adding 10μl of protein A-Sepharose coupled monoclonal antibodies directed against proteins U1-70K, U1-A or the core proteins B′, B and D (Sm complex) (25). The monoclonal antibodies used were 2.73 (26), 9A9 (27) and Y12 (28), respectively.

RESULTS

Binding of in vitro made C protein to native and protein C lacking U1 snRNP

To study the importance of distinct domains of protein C for the binding to U1 snRNP, a reconstitution assay was carried out in which *in vitro* translated radioactively labeled C protein or

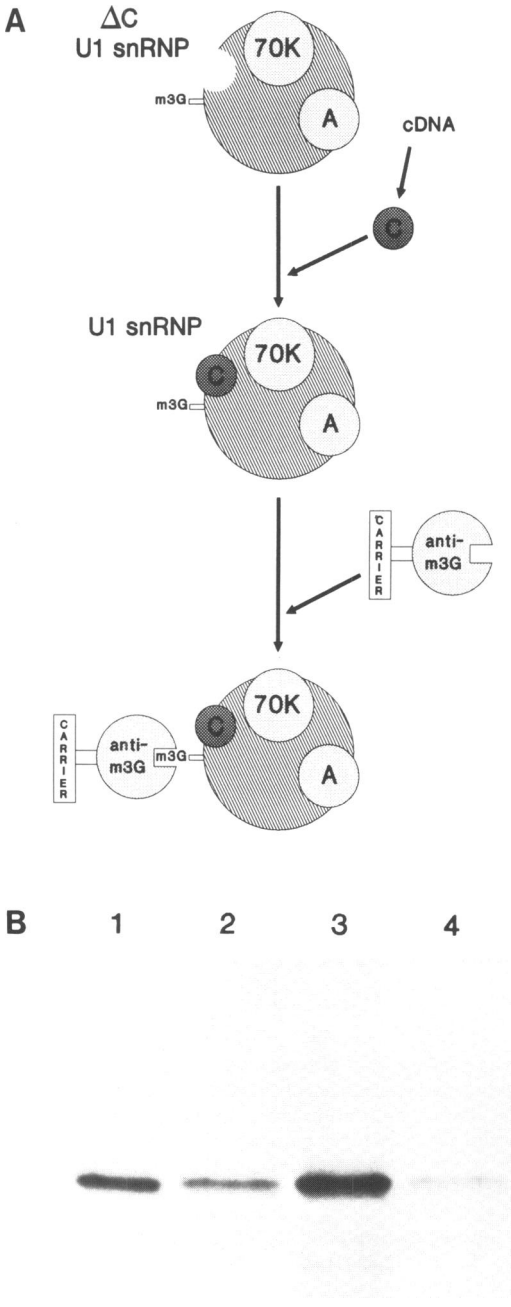

Figure 2. **(A)** Scheme of the reconstitution assay in which recombinant protein C is offered to ΔC U1 snRNPs in order to assemble native U1 snRNPs. Incorporation of methionine labeled C protein was determined by immunoprecipitating the newly formed particles with anti-m3G cap antibodies (25). **(B)** Reconstitution of ΔC U1 snRNP with *in vitro* translated C protein. The figure shows an autoradiogram of an SDS-polyacrylamide gel containing: **lane 1**: 20% of the standard input amount of C protein labeled with ^{35}S-methionine; total of ^{35}S-methionine labeled C protein incorporated into an snRNP particle when 200 fmol native U1 snRNPs (**lane 2**), 200 fmol ΔC-U1 snRNPs (**lane 3**) or no snRNPs (**lane 4**) were added to the reconstitution assay.

derivates thereof were allowed to bind to U1 snRNPs which had been selectively depleted of their C protein (ΔC U1 snRNPs) or both A and C proteins (Δ[A,C] U1 snRNPs) by Mono Q chromatography (13, 21). Binding of protein C to the ΔC or Δ[A,C] U1 particles was detected by immunoprecipitation of the

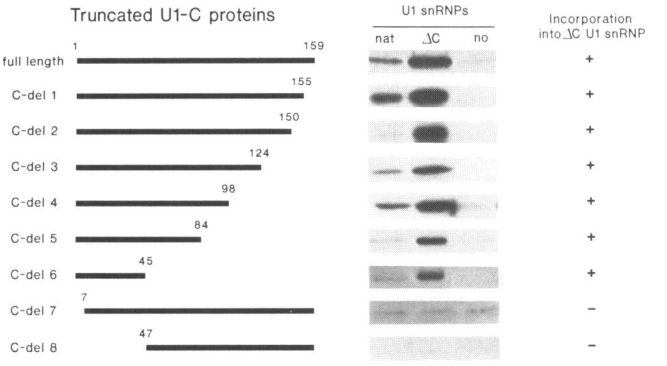

Figure 3. Truncated protein C mutants were tested for their ability to reconstitute ΔC or Δ[A,C] U1 snRNP particles in a reconstitution assay. The amino acid numbers are indicated. The shortened ^{35}S-methionine labeled mutant proteins (C-del 1 to C-del 6) needed longer exposure times in order to obtain signals as shown for wild type protein C. Whether or not a truncated protein is capable of reconstituting ΔC U1 snRNPs is indicated with respectively a (+) or (−) sign.

U1 snRNPs with anti-m3G cap antibodies (29) linked to protein A Sepharose beads (Figure 2A), followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Figure 2B shows a typical example of such a reconstitution assay. Using ΔC U1 snRNPs (200 fmol) in excess over *in vitro* translated protein C, about 40 to 50% of full length protein C (about 60 fmol) is specifically incorporated into these particles, whereas only 10 to 15% of the protein was bound when incubated with native U1 snRNPs (i.e. U1 snRNPs containing their full complement of U1-specific proteins) (Figure 2B, lanes 2 and 3). The binding of protein C to native U1 particles is probably due to exchange of the native C protein with the offered *in vitro* synthesized protein. In the absence of added U1 snRNPs the background amount of immunoprecipitated protein C was about 2% or less (Figure 2B, lane 4).

Essentially the same binding results were obtained when reconstitution was performed with Δ[A,C] instead of ΔC U1 snRNPs (not shown). This indicates that incorporation of protein C in U1 snRNP is not dependent on the presence of protein A. Therefore both ΔC and Δ[A,C] U1 particles can be used for protein C binding studies.

The region of protein C required for binding to U1 snRNP

To define the minimal structure necessary for association of the C protein with a U1 snRNP particle, we tested six carboxy-terminal deletion mutants (C-del 1 to 6) and two amino-terminal deletion mutants (C-del 7 and 8) of the C protein for their ability to reconstitute ΔC U1 snRNPs (Figure 3). The results show that all six carboxy-terminal truncated protein C mutants are able to bind specifically to ΔC U1 particles with roughly equal efficiency. In contrast, deletion of amino acids from the amino-terminus (mutants C-del 7 and 8) completely abolished binding of the thus truncated C proteins to ΔC U1 snRNPs (Figure 3). From this we can conclude that the amino-terminal 45 amino acids are sufficient and essential for the binding of protein C to ΔC U1 snRNPs.

The zinc finger-like motif shown in Figure 1 is still encompassed in the 45 amino acids long U1 snRNP binding domain of protein C. Therefore, we next investigated the importance of the cysteines and histidines contributing to the putative zinc finger structure of the C protein for the U1 snRNP binding activity.

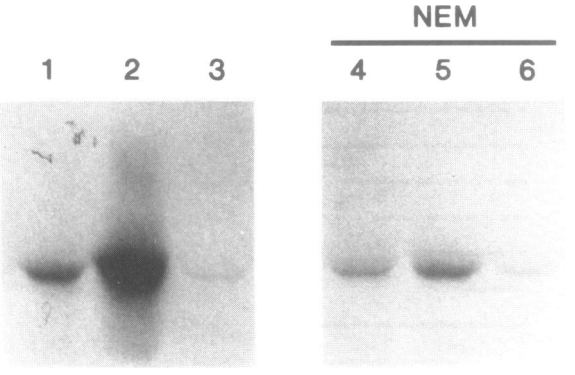

Figure 4. U1-reconstitution assays with NEM treated C protein. In the reconstitution assay 1μl of untreated C translate (**lane 1–3**) or 10μl NEM treated C translate (equivalent of 1μl untreated C translate) (**lane 4–6**) was incubated with respectively 200 fmol native U1 snRNPs, 200 fmol ΔC U1 snRNPs, or no snRNPs.

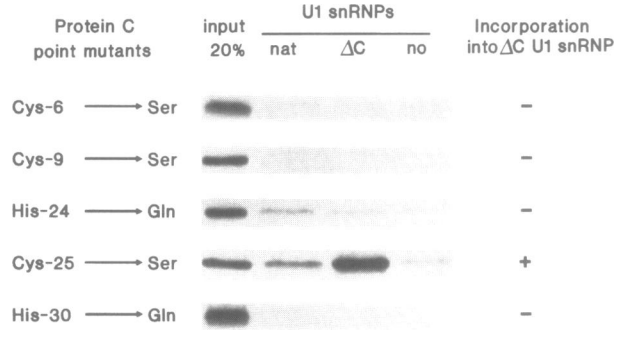

Figure 5. Reconstitution assays with mutated C protein. Each point mutated protein translate (1μl) was incubated with 200 fmol of native U1 snRNPs (**nat**), 200 fmol of ΔC or Δ[A,C] U1 snRNPs (ΔC) or no snRNPs (**no**). Assays were carried out as described in Methods. Whether or not a point mutated protein is capable of reconstituting ΔC or Δ[A,C] U1 snRNPs is indicated with respectively a (+) or (−) sign.

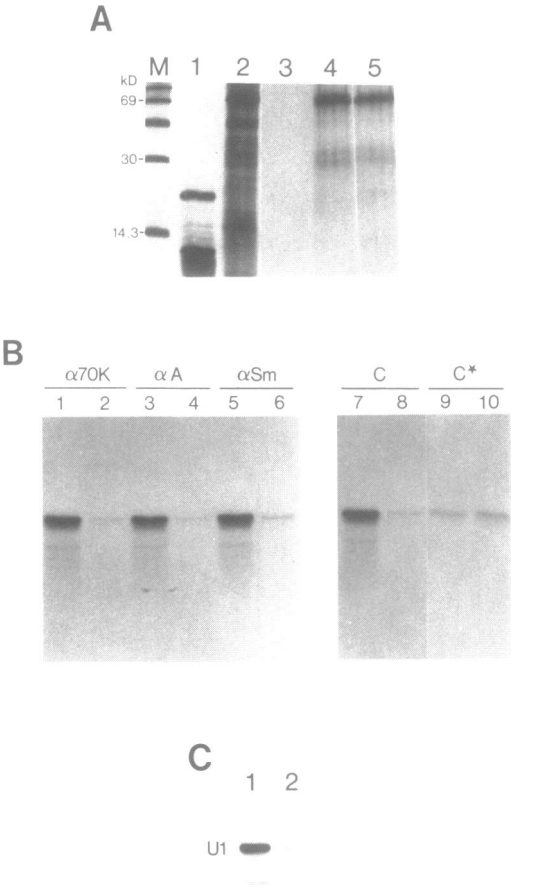

Figure 6. Binding of U1-C protein to other snRNP components in S100 extract. **(A)** Streptavidin-agarose precipitations of U1 snRNA incubated with proteins U1-C and/or U1-70K. The assay with biotin labeled U1 snRNA and streptavidin-agarose beads was carried out as described previously (15). **Lanes 1 and 2**: The input material of *in vitro* made ^{35}S-labeled proteins U1-C (1μl of standard translate) and U1-70K (2μl of standard translate), respectively, into the RNA binding assay. **Lanes 3 and 4**: 20 ng U1 snRNA incubated with C protein and 70K protein, respectively, followed by streptavidin-agarose precipitation and analysis of the bound proteins. **Lane 5**: 20 ng U1 snRNA incubated with a mixture of proteins C and 70K. **M**: Molecular weight protein markers. **(B)** Immunoprecipitations of ^{35}S-labeled *in vitro* made protein C incubated with S100 extract. **Lanes 1, 3 and 5**: C protein incubated in S100, immunoprecipitated by monoclonal antibodies directed against proteins U1-70K, U1-A and core proteins B′, B and D, respectively. **Lanes 2, 4 and 6**: C protein incubated in RNase A treated S100, immunoprecipitated by the same antibodies (for amount of input C protein: see Figure 6A, lane 1). Control experiment: **Lanes 7 and 8**: Protein C (C) incubated in respectively S100 and RNase A treated S100, immunoprecipitated by monoclonal antibody directed against U1-70K protein. **Lanes 9 and 10**: Protein C point mutant (Cys-9 mutated to Ser) (C*) incubated as described for lanes 7 and 8. **(C)** Hybridization of U1 snRNA isolated from the S100 extract by phenol/chloroform extraction on a Northern blot with a ^{32}P-labeled anti-sense U1 snRNA probe. The anti-sense probe was produced by transcribing U1 snRNA-pGEM-3Zf(+) with SP6 RNA polymerase. The blot was prepared by blotting a denaturing gel on which the RNA of 15μl S100 (**lane 1**) and the RNA of 15μl RNase A treated S100 (**lane 2**) was loaded.

Importance of the zinc finger-like structure of protein C for binding to U1 snRNP

To establish whether the cysteines are needed for binding of protein C to ΔC U1 particles, reconstitution experiments were performed in which the sulfhydryl groups of the cysteines in the wild type protein C were irreversibly blocked by treatment with 1 mM N-ethyl maleimide (NEM) (30). Before NEM-treated protein C was added to the assay, free NEM was inactivated by a ten-fold excess of dithiothreitol (DTT). The NEM treatment resulted in a dramatic decrease in the binding capacity of protein C to ΔC U1 particles (Figure 4). A control experiment in which NEM was inactivated with DTT before it was added to protein C gave in the reconstitution assay essentially the same results as with untreated protein C. These data indicate that cysteine residues are required for the U1 snRNP binding activity of the C protein.

To investigate to what extent the residues Cys-6, Cys-9, His-24 and His-30 (i.e. the ones which are thought to be involved in the putative zinc finger (Figure 1)) contribute to the U1 snRNP binding activity of protein C, single point mutations were introduced into the C cDNA via site directed mutagenesis. As a control Cys-25 was also mutated. To avoid structural disturbance of protein C as much as possible we substituted each cysteine by a serine and each histidine by a glutamine. *In vitro* products of the protein C point mutants were then tested in the ΔC U1 reconstitution assay (Figure 5).

Strikingly, a single point mutation at any of amino acid positions 6, 9, 24 and 30 of protein C completely abolished binding of C protein to ΔC U1 snRNPs, demonstrating that the cysteines and histidines at these positions are crucial for binding of protein C to U1 snRNP. On the other hand, conversion of cysteine-25 into a serine has no effect on the incorporation of the C protein into Δ-U1 particles (see autoradiogram Figure 5). From these results we conclude that the U1 snRNP-binding domain of protein C contains a zinc finger-like structure of the CC-HH type which is necessary for binding to U1 snRNP.

Interaction of protein C with U1 snRNP constituents

To study the kind of interactions of protein C within the U1 snRNP particle we first incubated *in vitro* made U1-C protein and biotin labeled U1 snRNA to see whether the C protein binds directly to the U1 RNA or not (Figure 6A). It is clear that *in vitro* made U1-70K protein is able to bind U1 snRNA directly (lane 4) in contrast to the non-binding behaviour of the C protein (lane 3). Varying the salt conditions from 25 mM to 100 mM and addition of $MgCl_2$ did not improve binding of protein C to naked U1 snRNA. As the results of Hamm *et al.* (31) suggested that binding of protein C to U1 snRNP might be dependent on the presence of protein 70K in the U1 particle we added a mixture of *in vitro* made proteins U1-C and U1-70K to biotin labeled U1 snRNA in the precipitation assay. Again no binding of protein C in an RNA-protein complex could be detected (lane 5).

We then approached the binding of protein C to U1 snRNP differently by adding ^{35}S-labeled *in vitro* made C protein to S100 extract. After incubation of C protein in S100 extract, the assay mixture was immunoprecipitated with monoclonal antibodies directed against proteins U1-70K, U1-A and core proteins B′, B and D (25) (monoclonal antibodies: 2.73 (26), 9A9 (27) and Y12 (28), respectively) (Figure 6B). With each type of monoclonal antibodies the same amount of protein C is precipitated (Figure 6B, lanes 1, 3 and 5). After RNase A treatment of the S100 extract (1 h at 20°C) no protein C could be precipitated by any of the monoclonal antibodies (lanes 2, 4 and 6) suggesting that the RNA component of U1 snRNP is essential for binding of the C protein. Immunoprecipitations of protein C incubated in S100 or RNase treated S100 with anti-m3G cap antibodies gave identical results as with the monoclonal antibodies (not shown). Incubation of S100 for 1 h at 20°C without RNase gave the same results as untreated S100 in all these precipitations. Figure 6C demonstrates the presence of U1 snRNA in untreated S100 extract (lane 1) and its absence in RNase treated S100 (lane 2). As a control one of the protein C point mutants (Cys-9 mutated to Ser) was also tested in the S100-reconstitution-assay (Figure 6B, lanes 9 and 10). The mutant protein could not be precipitated, a finding which is in complete agreement with the data of the ΔC U1 snRNP-experiments (see Figure 5). Apparently, the U1-C protein binds only to (partially assembled) U1 snRNP particles but not to any of the free U1 snRNP protein components once the U1 snRNA is degraded.

DISCUSSION

Our results point to three conclusions: **(a)** The N-terminal 45 amino acids of the U1-C protein are sufficient and essential for the incorporation of U1-C into U1 snRNP particles. **(b)** Within this amino terminal domain a zinc finger-like structure of the CC-HH type is contained, and mutation analysis of each cysteine or histidine of this structure showed that all these four residues are crucial for interaction with the U1 snRNP complex. **(c)** As to the identity of the U1 snRNP component with which this domain interacts our data strongly suggest that for binding of protein C to the U1 particle the U1 RNA is required in combination with one or more of the U1 snRNP proteins.

The protein C zinc finger shows a few differences when compared with the CC-HH consensus sequence derived from Gibson *et al.* (18) for example longer spacing between cysteines and histidines, lack of the conserved leucine (position 19) and substitution of the conserved phenylalanine (position 13) by another aromatic residue (tyrosine at position 12). However, such differences have also been observed in, for example, a few TF IIIA zinc fingers (32). While this work was in progress Legrain *et al.*, (33) elucidating the structure of the yeast proteins prp6 and prp9, noticed the presence of a zinc finger-like motif of the CC-HH type in these two proteins and pointed out that such a structure is shared by prp6, prp9, prp11 (34) and the human U1-specific protein C. One of the motifs in prp9 was shown to be essential for the function of this protein in yeast and all four proteins seem to be essential to the splicing machinery.

The experiments with biotin-labeled U1 RNA and *in vitro* made protein C have shown that the protein itself is not able to bind to naked U1 RNA. However, this does not exclude the possibility that there is an interaction between the U1 RNA and protein C in the intact particle. The data on the binding of protein C to U1 particles in S100 extract indicate that the C protein only interacts with (partially assembled) U1 particles. After degradation of the U1 RNA no protein C can be precipitated any more via proteins 70K, A, B′, B or D. It appears that the U1-C protein does not interact with just one of these free proteins, but only associates with a U1 RNA-protein complex. Apart from the possibility that there might be a direct interaction with the RNA in the U1 snRNP particle, indirect evidence suggests that protein C may be associated with the U1 snRNP via protein-protein interactions. Hamm and co-workers demonstrated that a mutation in the U1 RNA, which abolishes binding of U1-70K to the RNA *in vivo*, leads to loss of binding of protein C to the U1 particle (31). Our results, however, show that the U1 RNA-70K complex is not sufficient to allow stable interaction with the C protein (Figure 6A) and that interactions with other U1 RNA associated proteins might be important as well. The data on the NEM treatment and the point mutations of the C protein clearly show that the zinc finger-like structure in the C protein is involved in and essential for such interactions. The U1-A protein is probably not needed since particles lacking the A and C protein (Δ[A,C]) incorporate the C protein as efficient as ΔC particles which contain the A protein.

There are only a few examples in which an involvement of a zinc finger motif in protein-protein interaction has been shown to occur. The bacteriophage gene 32 protein contains a zinc binding structure which appears to be essential for formation of a dimeric version of the protein and one of the adenovirus E1A proteins contains a zinc finger that is thought to interact with proteins involved in transcription (19). It is interesting to note that as is the case with the U1-C protein, both the gene 32 product and E1A protein contain only one zinc finger motif.

Anyway, our data indicate that if protein C binds to U1 snRNP via protein-protein interaction indeed this only can occur on the RNA-backbone of the particle. Our current hypothesis therefore is that (one of) the U1 RNP core proteins together with the U1-70K protein and the U1 RNA backbone are necessary for a stable interaction of the C protein with the U1 snRNP particle.

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