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Analysis of U1snRNP-specific A protein cross-linked complexes

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The organization of the U1snRNP-specific A protein (34 kDa) has been analyzed by 12 and 16 Å thiol-reversible chemical cross-linking and Western blotting. A-containing cross-linked complexes had molecular masses of 43, 47, 56, 62, 67, 105 and 125 kDa. None of these complexes could be cross-linked following ribonuclease digestion, suggesting that UsnRNA may play important roles in the spatial organization of A and other proteins. Moreover, the data suggest that A is proximal to, and may have interactions with, UsnRNP-specific proteins C and 70 kDa as well as with UsnRNP-common proteins B, E and G.

UsnRNP; U1snRNP-specific protein; Cross-linking

1. INTRODUCTION

Uridine-rich, small, nuclear RNAs (UsnRNAs) are involved in heterogeneous nuclear RNA (hnRNA) processing [1,2]. U1, U2, U5 and U4/U6 are the major UsnRNAs involved in RNA splicing and are assembled as ribonucleoprotein particles (UsnRNPs) with at least 14 proteins. Proteins A (34 kDa), C (22 kDa) and 70 kDa are only found associated with U1snRNA whereas A′ [33] and B″ (28.5 kDa) interact specifically with U2snRNA. Proteins B′, B, D, D′, E, F and G (29, 28, 16, 15, 5, 13, 11 and 9 kDa, respectively) are common to all the major UsnRNPs [3].

Ribonuclease digestion analysis [4,5] and in vitro assembly of UsnRNPs using mutant snRNAs [6–12] have suggested that the common proteins bind to a conserved sequence motif (the Sm binding domain) within the 3′ and central domain of U1 and U2 snRNAs, respectively. These studies also revealed that the U1- and U2-specific proteins were bound to different domains of UsnRNA sequence and secondary structure than those involved in common UsnRNP protein binding.

The stoichiometries and potential interactions among UsnRNP proteins have been recently proposed from studies of the complexes generated by thiol-reversible chemical cross-linking [13,14]. The data suggested that the UsnRNP core of common proteins contained at least two asymmetrical copies of B′:B:D:D′:E:G with stoichiometries of 2:1:1:1:1:1 and 1:2:1:1:1:1. Though several ribonuclease-resistant protein–protein interactions were identified, the organization of 70 kDa and larger arrays of B′, B, E and G were dependent on UsnRNA.

In this report the thiol-reversible chemical cross-linking pattern of U1snRNP protein A is presented. The data suggest that the spatial organization of A relative to other UsnRNP proteins is dependent on UsnRNA. Interactions between UsnRNP-specific and the UsnRNP-common proteins are also suggested.

2. MATERIALS AND METHODS

Nuclear extracts [15] were prepared from spinner culture HeLa cells grown to mid log phase in Joklik's media containing 10% fetal calf serum [13,14]. Competency of the extracts in splicing was tested by in vitro RNA splicing reactions as described previously [13,14].

Splicing competent extracts were cross-linked with 6 mM of the 12 Å cross-linker dimethyl-3,3′-dithiobispropionimidate (DTBP) and 20 mM of the 16 Å cross-linker 2-iminothiolane (ITH) using optimized conditions for UsnRNPs [13,14].

The cross-linked extracts were resolved in the absence of reducing agents on 5–18% polyacrylamide gradient gels (the first dimension) [13,14,16]. Lanes were cut out and after treatment with reducing agents, proteins were resolved on 12% PAGE (the second dimension) [13,14,16]. In this diagonal 2D gel system, non-cross-linked proteins distributed along a diagonal according to their monomeric molecular weights but monomers released by chemical cleavage from cross-linked complexes, migrated to the right of the diagonal (off-diagonal). The distance from the diagonal was dependent on the composite molecular weight of the cross-linked complex in the first dimension prior its chemical cleavage [4,5,13,14]. Molecular weights of the cross-linked complexes were calculated from the migration of known molecular weight standard proteins as described previously [13,14].

Diagonal 2D gel patterns were electrotransferred to nitrocellulose (Western blotted) [13,14,17] and probed with 10 μg/ml of 9A9 monoclonal antibodies. This antibody recognizes mainly U1snRNP-specific protein A, but also the U2snRNP-specific B″ protein (Fig. 1). Immunoreactivity was detected on the blots with peroxidase-conjugated goat anti-mouse IgG followed by development with 3,3-diaminobenzidine as described previously [8,9].

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Where indicated, ribonuclease (RNase) digestion was performed before the cross-linking procedure by incubating the samples for 1 h at 37°C with RNase A plus RNase T1 (respectively 1–5 U and 0.6–3 U/μg of total protein in the sample).

3. RESULTS

Monoclonal antibodies 9A9 showed prominent reactivity with A (34 kDa) and B″ (28.5 kDa) monomers (Fig. 1). Cross-linking of extracts with DTBP followed by Western blotting of diagonal 2D gels resolved 4 distinct off-diagonal A protein monomers corresponding to cross-linked complexes with composite molecular masses of 45, 56, 67 and 105 kDa (Fig. 2A). Similarly, monomers of A protein were resolved from ITH cross-linked complexes having composite molecular masses of 45, 56, 67, 105 and 125 kDa (Fig. 2B). With both cross-linking agents, off-diagonal monomers resolved as nodes within an off-diagonal streak. Similar problems with resolution have also been encountered with the U1snRNP-specific 70 kDa protein [13,14]. These problems were proposed to arise from poor solubility of U1snRNP-specific protein cross-linked complexes during the first dimension electrophoresis (due to the absence of reducing agents) as well as the tendency for cross-linked complexes with similar molecular weights to blend together as one spot or streaks upon electrophoresis through the second dimensional gel [13,14]. This problem was partially overcome by Western blotting the first dimension gel without chemical cleavage (Fig. 3).

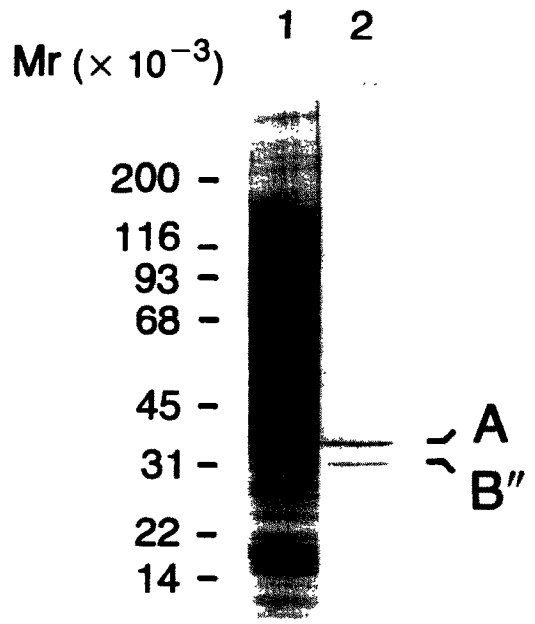

Fig. 1. Nuclear extracts were resolved on 5–18% gradient polyacrylamide gels and Coomassie blue stained (lane 1) or Western blotted and reacted with 9A9 antibodies (lane 2) as described in section 2. The relative mobility of molecular standards and A and B″ are shown to the left and right, respectively.

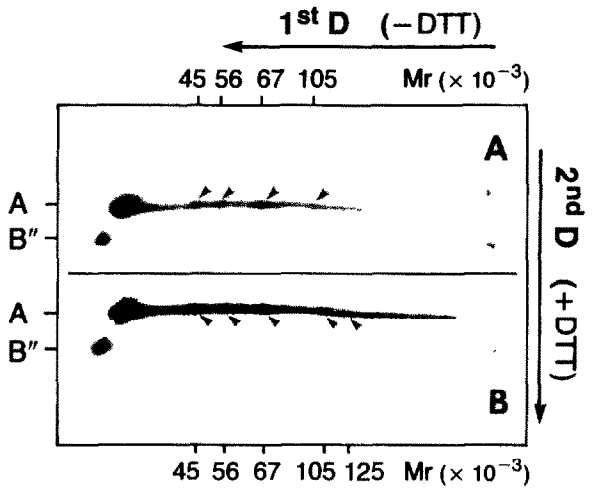

Fig. 2. Nuclear extract proteins cross-linked with DTBP were resolved on a diagonal 2D gel, electrotransferred and probed with 9A9 monoclonal antibody as described in section 2. The amount of protein loaded in (B) is twice the amount used in (A). Only the relevant region of the diagonal 2D gel blot is shown. First and second dimension gel coordinates are shown at the top, bottom and sides of the figure.

A protein-containing cross-linked complex appeared as a series of bands on the first dimensional 5–18% polyacrylamide gel having composite molecular masses of 43, 47, 56, 62, 67, 75 and 105, for both DTBP and ITH cross-linking (Fig. 3). These complexes have been designated Aa, Ab, Ac, Ad, Ac, Af and Ag, respectively (Fig. 3 and Table I). In addition, ITH yielded a 125 kDa cross-linked complex (Ah) and a 155 kDa complex (Ai).

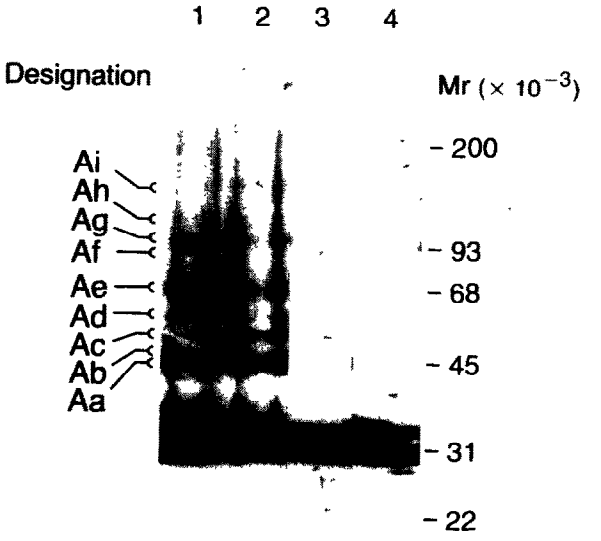

Fig. 3. Nuclear extract proteins, cross-linked with DTBP (1 and 3) and ITH (2 and 4), were resolved on 5–18% polyacrylamide gradient gels, blotted and reacted with 9A9 monoclonal antibodies as described in section 2. Samples (3) and (4) were digested with RNase A and RNase T1 prior to cross-linking as described in section 2.

Table I

Composite molecular mass of cross-linked complexes and their proposed composition

Designation	Composite molecular mass[a]	Proposed composition[b]	Proposed stoichiometry[b]
Aa	43 kDa	A:G	1:1
Ab	47 kDa	A:E	1:1
Ac	56 kDa	A:C	1:1
Ad	62 kDa	A:B	1:1
Ae	67 kDa	A:E:F:G or A:A′	1:1:1:1 or 1:1
Af	75 kDa	A:B:F	1:1:1
Ag	105 kDa	A:70 kDa	1:1
Ah (ITH)	125 kDa	A:C:70 kDa	1:1:1
Ai (ITH)	155 kDa	A:C:70:E:F:G	1:1:1:1:1:1

Extracts were cross-linked, resolved on gels, blotted and reacted with 9A9 monoclonal antibodies as described in section 2. [a]Molecular masses of the cross-linked complexes were calculated from the migration of molecular mass standards. [b]The proposed composition and stoichiometries were determined by subtracting the molecular mass of A protein monomer (34 kDa) from the composite molecular masses of the cross-linked complexes and comparing the difference to the molecular masses of known UsnRNP proteins

RNase A and T1 digestion of the extract prior to cross-linking abolished cross-linking of the A protein (Fig. 3, lanes 3 and 4). RNase digestion after cross-linking had no effect on A protein cross-linked complexes (data not shown and [13,14]).

4. DISCUSSION

Previous chemical cross-linking analyses have suggested that the ribonucleoprotein organization within UsnRNPs involves at least two types of protein–protein interactions; those dependent on RNA for positioning and those whose interactions do not depend on RNA [13,14]. U1snRNP-specific 70 kDa protein interactions with other proteins were proposed to be dependent on U1snRNA as was the organization of UsnRNP common proteins B′, B, E and G as a large protein core. Smaller complexes containing the UsnRNP common proteins (particularly those containing D and D′) were proposed to be organized through UsnRNA-independent interactions.

In this report, A protein-containing cross-linked complexes were shown to be best resolved by a single 5–18% polyacrylamide gel electrophoresis followed by Western blotting with A protein-specific monoclonal antibodies. Cross-reactivity of the antibody with U2snRNP-specific B″ protein did not interfere with the analysis because its titer and the concentration of B″ in the extracts were below the limit where cross-linked complexes could be clearly resolved (confirmed separately with B″-specific monoclonal antibodies, data not shown, and see also Fig. 1). All of the cross-linked complexes of the U1snRNP-specific A protein were sensitive to RNase digestion. Therefore like 70 kDa, organization of the A protein within U1snRNPs depends on intact U1snRNA for its positioning. A and 70 kDa were also similar in their tendency to resolve poorly in the second dimension and cross-linked into more numerous and better-defined complexes with 16 Å rather than 12 Å cross-linking reagents.

Based on the composite molecular weights of cross-linked complexes and the monomeric molecular weights of U1snRNP proteins, A is proposed to lie within 12–16 Å of both U1snRNP-specific C and 70 kDa proteins (Table I). Complexes Ac, Ag and Ah are proposed to result from cross-linking of A:C, A:70 kDa and A:C:70 kDa (respectively) with stoichiometries of 1:1, 1:1 and 1:1:1 (respectively). The higher yield of the Ah complex with ITH suggests that amine residues reactive in the cross-linking of A:C:70 kDa complexes are best approximated by 16 Å. Similar reasoning can also be applied to explain the identification of the Ai complexes (A:C:70:E:F:G, stoichiometry of 1:1:1:1:1:1) with ITH and not with DTBP.

These data resolve ambiguities in the earlier cross-linking analyses of the 70 kDa protein alone [13,14] and confirm data from other experimental approaches [3] suggesting that A, C and 70 kDa are organized proximal to one another. In terms of the morphology of U1snRNPs observed by electron microscopy [18] and the positioning of the 5′-end of the U1snRNA within the globular domain of this structure, the data suggest that cross-linking of A:C:70 kDa probably occurred within the globular domain of U1snRNPs and not within its opposing protuberances.

Several of the A protein-containing cross-linked complexes had composite molecular weights which presicely corresponded to complexes containing UsnRNP-common protein B, E, F and G. This property is dintinctly different from that of the U1snRNP-specific 70 kDa protein and further supports the possibility that A is part of the globular domain of U1snRNPs [18]. The 45 kDa complex, resolved by diagonal 2D gels, became apparent as two distinct complexes (Aa and Ab), upon

Western blotting of one-dimensional gels. Aa and Ab are proposed to contain cross-linked complexes of A:G and A:E (stiochiometries of 1:1 and 1:1, respectively). This proposal is supported by the identification of the Ae cross-linked complexes with both cross-linking reagents (A;E:F:G:; stoichiometry of 1:1:1:1:).

Alternatively, Ae might have resulted from cross-linking of A to the U2snRNP-specific A′ protein. Although this possibility is intriguing from the point of view of spliceosome assembly [19–22], inter-UsnRNP cross-linking is a lower yield chemical reaction than intra-UsnRNP cross-linking and is therefore considered less likely.

Cross-linked complexes Ae and Af had much lower yields than the others and were most apparent when ITH was used as a cross-linking reagent, again suggesting spatial relationships which are most optimally approximated with 16 Å cross-linking reagents. These complexes are proposed to contain A:B and A:B:F (respectively) with stoichiometries of 1:1 and 1:1:1 (respectively). Previous cross-linking analyses [13,14] suggested that B and B′ were organized in two asymmetrical copies of B′:B:E:G with stoichiometries of 2:1:1:1 and 1:2:1:1. The proposal that A cross-linked to B but not to B′ would be consistent with the proposed asymmetry and suggests that A might predominantly reside to one side of U1snRNPs; perhaps closer to the B′:B:E:G core with 1:2:1:1 stoichiometry.

It is also significant that F is not readily accounted for in cross-linked complexes unless B or E:G are also cross-linked to A (see Table I). We propose therefore, that F and A are not within 12 or 16 Å of each other but rather, F cross-links to B, E or G. The low yield of these complexes and the exclusive use of diagonal 2D gels in the earlier cross-linking studies, instead of one-dimensional gels, may account for why F was not detected [13,14].

In summary we propose that the stiochiometry of U1snRNP-specific proteins A, C and 70 kDa is 1:1:1 within U1snRNPs and that A is asymmetrically positioned within these particles such that it can only be cross-linked to a limited subset of the UsnRNP-common proteins consisting of B, E and G.

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