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Abstract
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Hierarchical assembly of the Alu domain of the mammalian signal recognition particle

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ABSTRACT

The mammalian signal recognition particle (SRP) catalytically promotes cotranslational translocation of signal sequence containing proteins across the endoplasmic reticulum membrane. While the S-domain of SRP binds the N-terminal signal sequence on the nascent polypeptide, the Alu domain of SRP temporarily interferes with the ribosomal elongation cycle until the translocation pore in the membrane is correctly engaged. Here we present biochemical and biophysical evidence for a hierarchical assembly pathway of the SRP Alu domain. The proteins SRP9 and SRP14 first heterodimerize and then initially bind to the Alu RNA 5’ domain. This creates the binding site for the Alu RNA 3’ domain. Alu RNA then undergoes a large conformational change with the flexibly linked 3’ domain folding back by 180° onto the 5’ domain complex to form the final compact Alu ribonucleoprotein particle (Alu RNP). We discuss the possible mechanistic consequences of the likely reversibility of this final step with reference to translational regulation by the SRP Alu domain and with reference to the structurally similar Alu RNP retroposition intermediates derived from Alu elements in genomic DNA.

Keywords: retroposition; RNA–protein recognition; RNP assembly; signal recognition particle; translational control

INTRODUCTION

The signal recognition particle (SRP; Walter & Blobel, 1980), like the ribosome, is a cytoplasmic ribonucleoprotein particle (RNP) of ancient evolutionary origin (Poritz et al., 1990; Bhuiyan et al., 2000). SRP has an intrinsic affinity for ribosomes (Walter et al., 1981) and its catalytic promotion of the cotranslational mode of protein translocation across membranes is well documented (Walter & Johnson, 1994; Lütcke, 1995). In mammals, SRP consists of the highly base-paired 300-nt-long SRP RNA and six proteins: SRP54, SRP19, and the heterodimers SRP68/72 and SRP9/14 (Fig. 1). SRP9/14 associates with the terminal sequences of SRP RNA, forming the enzymatically separable Alu domain of SRP (Gundelfinger et al., 1983), whereas the other proteins together with the central RNA sequence form the S-domain of SRP. High resolution crystal structures are now available for a number of SRP components: the NG- and M-domains of SRP54 (Freymann et al., 1997; Montoya et al., 1997; Keenan et al., 1998; Clemons et al., 1999) and the M-domain in complex with helix 8 of SRP RNA (Batey et al., 2000) as well as the free helices 6 (Wild et al., 1999) and 8 (Jovine et al., 2000) of SRP RNA, free SRP9/14 (Birse et al., 1997) and, most recently, the Alu domain with SRP9/14 clamping together in its concave beta-sheet the 5’ and 3’ domains of Alu RNA (Weichenrieder et al., 2000). In electron micrographs the particle appears as a flexible, tri-segmented rod of 60 Å by 260–280 Å with the two domains distinguishable at opposite ends (Andrews et al., 1985, 1987).

SRP selects ribosomes displaying the N-terminal signal sequence of nascent secretory and membrane proteins that first emerges at the exit pore on the large ribosomal subunit. The Alu domain of SRP is responsible for retarding the elongation of these proteins once their export signal sequence is bound by the S-domain of SRP and prior to engagement with the translocation machinery in the endoplasmic reticulum. Considering the apparent length of the particle, it has been pro-
posed that the Alu domain might reach the cleft between the two ribosomal subunits where the elongation factors bind (Andrews et al., 1987; Siegel & Walter, 1988), but the Alu RNP crystal structure does not support the hypothesis that elongation arrest is caused by a mechanism of mimicry-based active competition with elongation factors (Weichenrieder et al., 2000). Knowledge of the preferred orientation and the degree of flexibility of the Alu domain (and the crucial C-terminal tail of SRP14; Thomas et al., 1997; Mason et al., 2000) with respect to the signal sequence binding S-domain will therefore be of primary importance for an explanation of the mechanism of SRP-mediated elongation arrest. These features, which result from the proper assembly of the particle, are decisive in determining the physiologically relevant size and shape of SRP and they govern possible multivalent interactions of SRP with different regions of the ribosome.

The assembly of the S-domain of SRP has been found to be cooperative in vitro (Walter & Blobel, 1983) and to partially take place in the nucleolus and in the cytoplasm in vivo (Politz et al., 2000). This underlines the notion that the assembly of multicomponent particles like SRP or the ribosome in vivo has to be imagined in a stepwise, hierarchical, and controlled order with precise temporal and spatial separation of events. Concerning the assembly and function of the Alu domain of SRP it has been shown previously that SRP9/14 heterodimerization is a prerequisite for Alu RNA binding (Strub & Walter, 1990). We have also determined experimentally the minimal Alu RNA folding domain, SA86 (Weichenrieder et al., 1997), which consists of a distinct 5' domain (nt 1–47 of human SRP RNA) covalently linked to a 3' domain (nt 48–64 of SRP RNA base paired to nt 282–300; Fig. 1). Alu RNA constructs designed according to SA86 have been shown to be sufficient for accurate SRP RNA 3' end processing (Chen et al., 1998) and for efficient export of SRP RNA to the cytoplasm (Jacobson & Pederson, 1998). A construct corresponding to the SA86 5' domain strongly activates transcription by RNA polymerase III (Emde et al., 1997).

Structures with very similar RNA and protein components to the SRP Alu domain are found in other, non-SRP contexts (Fig. 1) and understanding the assembly and conformational state of these Alu RNPs will also be critical to understanding their function. Most interesting are the neuron-specific BC200 RNP, which migrate into dendrites to possibly regulate localized protein translation (Kremerskothen et al., 1998) and the Alu retroposition intermediates repeated in tandem, which are responsible for the creation of 10–12% of the human genome in the form of Alu elements (Mighell et al., 1997). Alu retroposition is an ongoing process that must have had a significant impact on the evolution of the human genome (Kazazian, 1998).

Here we delineate in vitro the detailed assembly pathway for the SRP Alu domain by studying the behavior in solution of variants of its RNA and protein components. We discuss the physiological implications of the results in the light of the crystal structures, which have been determined of two Alu RNP variants (Weichenrieder et al., 2000).
RESULTS

Preferential binding of SRP9/14 to the 5’ domain of Alu RNA as the primary recognition event

In gel filtration experiments, under conditions that would allow reconstitution of complete Alu RNPs with SA86, SRP9/14 binds only to 5’ domain RNA (SA47, blue in Fig. 1D) even if 3’ domain RNA (SA39, cyan in Fig. 1D) is present in trans in equimolar amounts (Fig. 2A). Specificity of the interaction of the 5’ domain with SRP9/14 is indicated by the comparison of RNase V1 digestion patterns of various Alu RNAs in the absence and presence of SRP9/14. The protection of nt G20 to C22 from cleavage (Fig. 2B,C) is typical and agrees well with hydroxyl-radical footprinting data on complete SRP RNA (Strub et al., 1991). The continued exposure and cleavage at A43 is characteristic as well, suggesting that the first and third stem stack in the complex. The slight enhancement of cleavage at this site in the presence of protein, which is even more pronounced in the context of longer Alu RNAs, suggests that SRP9/14 stabilizes the stacked conformation.

These results, obtained in solution, are entirely consistent with the crystal structure of a specific Alu RNP consisting of SRP9/14 bound to 5’ domain Alu RNA (SA50, Fig. 2B; Weichenrieder et al., 2000). In solution, the free SA50 is likely to be largely prefolded but with enhanced flexibility between the helical stacks to the extent that the tertiary interactions between the two loops (including three Watson–Crick base pairs) may be partially disrupted (Figs. 2C, 4B). Specific binding of SRP9/14 to the conserved U-turn of the 5’ domain rigidifies the structure and promotes formation of the tertiary interactions. Such flexibility between helical RNA stacks is of general importance for the process of RNA folding and might be functionally relevant in many cases (Batey & Doudna, 1998). In summary, the preferential binding of SRP9/14 to the Alu RNA 5’ domain is a novel observation and the structural rearrangements that occur in the RNA upon protein binding illustrate the role of proteins to assist large RNAs in adopting their physiologically relevant conformations.

Detection of a secondary recognition event in the context of a flexibly linked Alu RNA 3’ domain

To investigate further the role of the RNA 3’ domain in protein binding, we made use of two circularly permuted Alu RNAs, SA88 and SA91, in which the original 5’ and 3’ ends of SA86 are connected with linkers of one or four uridines, respectively. In SA88, the 3’ domain is designed to stack on the terminal stem of the 5’ domain, whereas in SA91, due to the bulge of three uridines, it has considerably more flexibility, mimicking wild-type RNA (Fig. 3A,B). We first compared the relative affinities of SRP9/14 for SA86, SA91, and SA88 in a pilot competition experiment based on nitrocellulose filter binding using radiolabeled SA86 and competing with increasing concentrations of unlabeled SA86, SA91, and SA88. The titration experiment (Fig. 3C,D) indicates SA88 to have about 50–100-fold lower affinity for SRP9/14 than SA86.

Based on these results, we then did gel retardation experiments with radiolabeled SA86, SA91, or SA88 and a 20-fold excess of each SA86, SA91, SA88, and SA50 as unlabeled competitor RNA. Under these conditions we can confirm that SA86 and SA91 have vir-
A compactly folded RNP resulting from a large-scale protein-induced \textit{Alu} RNA bending

To study the biophysical properties and the stoichiometry of free \textit{Alu} RNAs and \textit{Alu} RNPs in concentrated solution we analyzed and purified them by preparative gel filtration chromatography for use in small angle neutron scattering experiments (Fig. 5). Neutron small angle scattering allows the independent determination of the molecular weight and the radius of gyration of a particle. For the SA91 and SA88 RNPs (in H\textsubscript{2}O buffers) we measured values of relative molecular weight corresponding to 0.99 and 1.96 times the mass of monomeric SA86 RNP. The corresponding values for the radius of gyration of the SA86, SA91, and SA88 RNPs are 27.7 (±0.5), 28.2 (±0.5), and 37.0 (±1) Å. The results strongly suggest that under these conditions the SA88 RNP forms mainly dimers, whereas the SA86 and SA91 RNPs are monomers. In contrast, the free RNAs are monomeric in all three cases because the measured relative molecular weights for SA91 and SA88 RNA are 1.12 and 1.01 times the value of monomeric SA86 RNA. Similar results are found by preparative gel filtration of complexes (particle concentration above 100 μM): the SA88 RNP has an anomalously high hydrodynamic radius compared to the SA91 and the SA86 RNPs, whereas all three RNAs behave similarly. In the gel retardation assay, where particle concentrations are below 5 μM, dimerization of the SA88 RNPs is not detected, pointing to a rather weak interaction.

The observation that dimer formation of the SA88 RNP in solution is clearly a property of the entire ribonucleoprotein particle, but not of its individual compo-
The crystal structure of the SA88 RNP (Weichenrieder et al., 2000) explains the domain interactions observed in the SA88 RNP monomer. Dimeric interactions of the SA88 RNP arise from the interaction of the Alu RNA 3' domain of one SA88 RNP with the Alu RNP 5' domain of a second, crystallographically twofold related, SA88 RNP. This result, together with the protection of the Alu RNA 3’ domain from RNase V1 cleavage in the monomeric SA86 and SA91 RNP complexes, leads to a model of the compact Alu RNP monomer in which the 5’ domain RNA interacts with its own 3’ domain RNA (Weichenrieder et al., 2000; Fig. 7). The neutron scattering and gel filtration experiments suggest the free SA86, SA91, and SA88 RNAs all adopt similar conformations (probably resembling the extended conformation observed in the SA88 RNP monomer), the model of the compact Alu RNP monomer implies a large conformational change of Alu RNA upon complete SRP9/14 binding, turning the 3’ domain by 180°.

### Corroboration of the compact Alu RNP model by mutagenesis of conserved amino acids

The possibility to experimentally distinguish in the gel retardation assay between the strong interaction of SRP9/14 with the Alu RNA 5' domain and the weak interaction of the Alu RNP 5’ domain with the Alu RNA 3’ domain allowed us to further corroborate the sug-

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**FIGURE 4.** Experimental distinction between the primary (5’ domain) and secondary (3’ domain) binding sites of SRP9/14 on Alu RNA. A: Relative affinities of various Alu RNA constructs for SRP9/14 as revealed by an electrophoretic mobility shift assay. Direct competition experiments show SA86 and SA91 to have similar protein affinities, whereas SA88 can be competed out with isolated 5’ domain RNA (SA50). SS125: nonspecific SRP 5' domain RNA. B: Specific interaction of SRP9/14 with SA91 as revealed by enzymatic probing with double-strand-specific RNase V1 (V1). In the presence of SRP9/14, diagnostic cleavage sites at G20-C22 and at U60 and C61 get protected (green in Fig. 3A), whereas cleavage at A43 gets enhanced (red in Fig. 3A). L: nucleotide ladder; G: guanosine ladder. C: Protection of the Alu RNA 3’ domain from RNase V1 cleavage in SA91 Alu RNPs versus exposure in SA88 Alu RNPs.
gested compact Alu RNP model by mutation of conserved amino acids in the RNA protein interface. Our rationale was that mutations of amino acids involved in Alu RNA 5’ domain binding should weaken complexes with labeled SA91 but not change their resistance towards competition with cold SA50 competitor. In contrast, mutations of amino acids involved in the bending of the Alu RNA 3’ domain or in the stabilization of the bent RNA conformation should lead to more open, slower migrating complexes that are susceptible to cold SA50 competitor, very much like complexes of labeled SA88 with wild-type SRP9/14 (Fig. 6). We therefore tried to identify such a case in support of the compact Alu RNP model.

All of the single or double point mutations of SRP9/14 that we tested still form specific complexes with SA91 Alu RNA (Fig. 6A). Among these, the strongest RNA-binding defects are detected for the SRP9 K30A/R32A double mutant and the SRP14 R59A substitution, where side chains critical for the specific recognition of the 5’ domain have been replaced (Weichenrieder et al., 2000). The other single point mutants of SRP9 (C39S, C48S, E15A) or SRP14 (Y27A) do not behave significantly differently from wild-type protein. This is in agreement with the structural model (Weichenrieder et al., 2000), where the respective side chains are not part of the RNA–protein interface at all (SRP9 E15A), part of the interface but not within hydrogen bonding distance (SRP9 C39S), or potentially affecting only a single weak contact to the Alu RNA 5’ domain (SRP14 Y27A) or 3’ domain (SRP9 C48S).

The SRP9 K41A/C39A double mutant displays the most interesting phenotype (Figs. 6A and 6B). Its selective deficiency in Alu RNA 3’ domain binding (Fig. 6) is most probably due only to the substitution of SRP9 K41, because SRP9 C39 is located rather far away (>5.5 Å) from the RNA in the structure and its substitution with serine has no effect (Fig. 6A). The complex of SA91 with the mutant SRP9/14 is susceptible to SA50 competition and migrates slightly slower in the gel than the complex with wild-type SRP9/14. This indicates a more open conformation of this mutant RNP. The mutation does not seem to affect 5’ domain binding as reflected by the migration profiles of the respective wild-type and mutant complexes with the rigidified SA88 Alu RNA (Fig. 6B). SRP9 K41 is one of three potential domain RNA backbones (Fig. 6C), consistent with an important function in stabilizing the bent RNA conformation. The ε-amino nitrogen of K41 is close to the 5’ domain (O3’ of U23) and 3’ domain (O2’ of G58) nucleotides although the current resolution does not permit a precise definition of its interactions.

**FIGURE 5.** Dimerization of soluble SA88 Alu RNPs at high particle concentrations (SA86 (black), SA91 (green), SA88 (red); particle concentrations above 100 μM). A: Preparative gel filtration chromatography. Alu RNAs (thin lines) are of similar size and shape, whereas the SA88 Alu RNP has a considerably larger hydrodynamic radius than its counterparts (thick lines). V: Elution volume; OD: optical density; *: excess protein. B: Neutron small angle scattering of uncomplexed Alu RNAs. A Guinier plot of representative scattering curves normalized for concentration (~2.5 mg/mL) and for the molecular weight of SA86 Alu RNA reveals almost identical masses and radii of gyration. C: Neutron small angle scattering of Alu RNPs. The Guinier plot, normalized for concentration (~9.0 mg/mL) and the molecular weight of the SA86 Alu RNP clearly shows the SA88 Alu RNP to form dimers with a significantly larger radius of gyration. q: scattering vector; I(q): scattering intensity.
Hierarchical assembly of Alu RNPs in vitro

Taking into account the presented biochemical data and previous structural data on the SRP Alu domain (Weichenrieder et al., 2000), we deduce a sequential assembly pathway for the SRP Alu domain in vitro (Fig. 7). In the first step, SRP9 and SRP14 must heterodimerize, because neither of them alone binds Alu RNA (Strub & Walter, 1990). In the second step the SRP9/14 heterodimer associates with free Alu RNA, which is monomeric and likely to be in an extended conformation, because the measured radii of gyration of SA86, SA91, and SA88 are all similar. SRP9/14 initially interacts with the Alu RNA 5' domain, inducing and/or stabilizing the stacking of RNA helices H1.2 and H1.1 and possibly strengthening the tertiary interactions between loops L2 and L1.2. At this intermediate stage in assembly the particle might resemble the rigidified...
truly sequential order of the assembly process, as the formation of the 5' domain complex is a prerequisite for the binding of the RNA 3' domain as is SRP9/14 heterodimerization for Alu RNA 5' domain binding.

The dissociation constant of SRP9/14 and SRP RNA is smaller than 0.1 nM (Janiak et al., 1992), corresponding to a total free binding energy of more than 13.7 kcal/mol, and our preliminary estimates indicate that the closed conformation is only about 2.3–2.7 kcal/mol more stable than the open conformation. This raises the questions of how readily reversible is the conformational switch between the open ((46 Å × 25 Å) × 95 Å in length) and closed ((46 Å × 50 Å) × 59 Å in length) Alu RNA and whether the switch could be triggered in the physiological context of SRP interacting with other cellular components such as the ribosome. There is experimental support for the reversibility of 3' domain binding in the flexibly linked SA86 RNP, because purified compact monomers are found to crystallize as SA88 RNP-like domain-swap dimers of the open form (O. Weichenrieder and S. Cusack, unpubl. data), presumably favored by the high concentration. Also in the context of purified, complete SRP, there are experimental conditions under which the SRP9 cysteines are preferentially accessible to alkylation, indicating an opening of the Alu domain (Walter & Blobel, 1980).

**Physiological aspects of Alu RNP assembly and 5'-3' domain flexibility**

The assembly pathway of the Alu RNP in vivo is likely to be the same as in vitro but must be seen in the context of the complete SRP and in the context of the compartmentalization of the eukaryotic cell, which leads to a spatial separation of events.

Combining our results on the assembly of the Alu domain with previously published data and hypotheses, we suggest the following scenario for the assembly of SRP in vivo. SRP9 and SRP14 first heterodimerize in the cytoplasm and subsequently enter the nucleoplasm. There SRP9/14 binds cotranscriptionally to the Alu RNA 5' domain enhancing transcription in cis (Emde et al., 1997). After termination of transcription, the Alu RNA 3' domain bends into the closed conformation and exposes the 3' end for nuclear processing (Chen et al., 1998). Then SRP19 and SRP68/72 join the S-domain RNA in the nucleolus before the subparticle gets exported to the cytoplasm where SRP54 adds on in a final step (Politz et al., 2000). There is experimental evidence that Alu RNA contains all necessary elements for efficient nuclear export (Jacobson & Pederson, 1998). Although the presence of SRP9/14 in the nucleus and its cotranscriptional association with Alu RNA have not explicitly been demonstrated, we consider the scenario to be quite plausible. Recent data from the distantly related yeast (y)SRP (Ciufo & Brown,
altered to have additional mutations. The model presented above is expected to be equally likely to be valid for the assembly of a core particle together with ySRP21, ySRP68, ySRP72, and ySRP RNA (scR1). The model presented above is expected to be valid for the assembly, transport, and function of non-SRP Alu RNPs.

The potential flexibility of the Alu domain might be relevant for the elongation arrest function of SRP. It would allow SRP to exist in clearly different states during its functional cycle and it would pose the crucial C-terminal tail of SRP14 (Thomas et al., 1997; Mason et al., 2000) into alternating structural environments. In the context of the Alu retroposition intermediates repeated in tandem, the flexibility between the Alu RNP and 3′ domain combined with the high local particle concentration due to the covalent linkage could favor the formation of a compact particle that resembles the SA88 RNP dimer. Such a particle could be of functional importance at some stage in the retroposition cycle.

MATERIAL AND METHODS

Preparation of RNA and protein samples
RNA molecules with 3′ terminal hammerhead ribozymes were synthesized by in vitro transcription. RNA was purified as described (Weichenrieder et al., 1997). Plasmids pSA47H and pSA39H, coding for SA47 and SA39, were obtained by introducing synthetic oligonucleotides into plasmid pSA68H (Weichenrieder et al., 1997) digested with Avai/HindIII or EcoRI/XbaI, respectively. Human SRP9 (1–108) was obtained as described (Weichenrieder et al., 2000). Mutants thereof were made with the QuickChange (Stratagene) site-directed mutagenesis protocol using for SRP9 a derivative of pQE-70 (Quiagen) as the parent vector and Escherichia coli pREP4 (Quiagen) as the parent vector and Escherichia coli directed mutagenesis protocol thereof were made with the QuickChange (Stratagene) site-directed mutagenesis protocol thereof were made with the QuickChange (Stratagene) site-directed mutagenesis protocol thereof were made with the QuickChange (Stratagene) site-directed mutagenesis protocol thereof were made with the QuickChange (Stratagene).

RNA competition experiments and enzymatic probing of RNA structure

Competition experiments were done and quantitated as described previously (Weichenrieder et al., 1997). Samples (20 μL, 20,000 cpm) for the relative affinities of various Alu RNA constructs for SRP9/14 contained 500 nM of each SRP9/14 and 5′ 32P-labeled substrate RNA and 10 μM of unlabeled competitor RNA. RNAs were preannealed and complex formation was analyzed by RNA mobility shift in nondenaturing 8% polyacrylamide gels. For the screening of protein mutants, 200 nM labeled SA91 RNA was mixed with 2 μM total protein and challenged with 4 μM SA50 competitor RNA. The SRP9 C39A/K41A mutant was further analyzed in the context of 200 nM labeled substrate RNA, an equal amount of active protein, and 4 μM of the respective competitor RNA. Samples (25 μL, 100,000 cpm) for RNase V1 probing (Weichenrieder et al., 1997; 0.2–0.7 enzymatic units for 2–5 min at 37 °C) contained 500 nM 5′ 32P-labeled Alu RNA, 10 μM SRP9/14, and 0.2 mg/mL of Escherichia coli tRNA.

Gel filtration chromatography and neutron small angle scattering

For analytical purposes (Fig. 2), 1 mL of pre-annealed 6 μM RNA(s) and 4 μM SRP9/14 was analyzed on a HiLoad 16/60 Superdex 200 column (Pharmacia). For preparative purposes (Fig. 5), particles were purified from a starting mixture of 1 mL of 150 μM Alu RNA and 300 μM SRP9/14 and concentrated to 20 mg/mL. Neutron small angle scattering experiments were done on instrument D22 of the Institut Laue Langevin, Grenoble. Samples (2.3–22.2 mg/mL in buffers of various H2O/D2O ratios) were measured at a wavelength of 10 Å, a detector distance of 3 m and in cells with 0.1 or 0.2 cm path length (1 cm2 illuminated area). For each sample, the transmission, T, of the direct beam and the intensity I(q) of the scattered radiation as a function of the scattering vector, q, were determined. Data, normalized using scattering from pure H2O, was plotted according to the Guinier equation, ln I(q) = ln I(0) − (Rg2/3) q2, where Rg is the radius of gyration and I(0) the scattering intensity at zero angle that can be used to calculate the molecular weight as described (Jacrot & Zaccai, 1981).

Structure coordinates and figures

PDB entry codes are le80 for the SA50 RNP and le8s for the SA88 RNP (Weichenrieder et al., 2000). Figures were produced with BobScript (Esnouf, 1997)/Raster 3D (Merritt & Bacon, 1997).

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