The *Trypanosoma brucei* signal recognition particle lacks the Alu-domain-binding proteins: purification and functional analysis of its binding proteins by RNAi

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**Summary**

Trypanosomes are protozoan parasites that have a major impact on human health and that of livestock. These parasites represent a very early branch in the eukaryotic lineage, and possess unique RNA processing mechanisms. The trypanosome signal recognition particle (SRP) is also unusual in being the first signal recognition particle described in nature to be comprised of two RNA molecules, the 7SL RNA and a tRNA-like molecule. In this study, we further elucidated the unique properties of this particle. The genes encoding three SRP proteins (SRP19, SRP72 and SRP68) were identified by bioinformatics analysis. Silencing of these genes by RNAi suggests that the SRP-mediated protein translocation pathway is essential for growth. The depletion of SRP72 and SRP68 induced sudden death, most probably as a result of toxicity due to the accumulation of the pre-SRP in the nucleolus. Purification of the trypanosome particle to homogeneity, by TAP-tagging, identified four SRP proteins (SRP72, SRP68, SRP54 and SRP19), but no Alu-domain-binding protein homologs. This study highlights the unique features of the trypanosome SRP complex and further supports the hypothesis that the tRNA-like molecule present in this particle may replace the function of the Alu-domain-binding proteins present in many eukaryotic SRP complexes.

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Key words: Trypanosomes, SRP complex, Alu domain

**Introduction**

The signal recognition particle (SRP) is one of the few functional small RNP particles that is conserved in the three kingdoms of life (Köch et al., 2003). The SRP couples the synthesis of membrane and secretory proteins across or into the endoplasmic reticulum (ER) membrane in eukaryotes (Stroud and Walter, 1999; Walter et al., 2000; Keenan et al., 2001; Köch et al., 2003), as well as across the bacterial plasma membrane (Köch et al., 2003), and chloroplast thylakoid membranes (Dalbey and Kuhn, 2000). In vitro studies in mammalian systems demonstrated the binding of the SRP to the hydrophobic signal peptide at the N-terminal of the nascent chain as it emerges from the ribosome. Binding of the SRP leads to retardation of peptide elongation, known as 'elongation arrest' (Walter et al., 1981; Wolin and Walter, 1989; Egea et al., 2005). The SRP–ribosome–nascent-chain complex (RNC) then interacts with the SRP receptor (SR), which docks the complex to the membrane (Gilmore et al., 1982). The SRP–SR complex dissociates from the ribosome, and the SRP and SR dissociate from each other upon GTP hydrolysis (Egea et al., 2004). The RNC is transferred to the protein-conducting channel, the translocon. Following binding of the RNC to the translocon, the nascent chain is inserted into the channel, translation resumes and the protein is co-translationally translocated (Walter and Johnson, 1994; Meacock et al., 2000; Müller et al., 2001).

The most well studied eukaryotic SRPs are the mammalian ( canine) and the yeast complexes. The mammalian SRP is composed of a single RNA, the 7SL RNA, and six proteins (Walter and Blobel, 1981). SRP9 and SRP14 bind to the 5' end of the RNA (Alu-domain), which functions in translational arrest (Siegel and Walter, 1986; Siegel and Walter, 1988a). SRP54 is the signal-peptide-binding protein that is conserved from bacteria to humans (Bernstein et al., 1989; Römisch et al., 1989). This protein has an N-terminal four-helix bundle (N domain), a central GTPase domain (G domain) and a C-terminal methionine-rich domain (M domain). The M domain attaches SRP54 to helix 8 of 7SL RNA (Keenan et al., 2001; Rosenblad et al., 2004). The proteins SRP19, SRP68 and SRP72 interact with the S domain of the 7SL RNA (Siegel and Walter, 1988b). SRP19 was shown to significantly enhance SRP54 attachment to helix 8 of 7SL RNA. Binding of SRP19 leads to restructuring of both helix 6 and 8, causing local changes at the SRP54-binding site (Hainzl et al., 2002). SRP68 and SRP72 bind in the vicinity of the three-way junction of the 7SL RNA, independently of SRP19 and SRP54 (Siegel and Walter, 1988a; Siegel and Walter, 1988b). More recently, it was suggested that the brace-like localization of SRP68/72 enables communication...
between the S domain and the Alu domain. Binding of the signal-peptide to the ribosome may induce a conformational change in SRP68/72 that further induces the formation of a right angle SRP configuration, bringing the Alu-domain close and into the elongation-factor-binding site of the ribosome (Halic et al., 2004). In yeast, the SRP particle contains six proteins, Srp72p, Srp68p, Srp54p, Sec63p (homologous to Srp19), Srp14p and Srp21p (Brown et al., 1994). The yeast Alu domain, which is truncated compared with the mammalian RNA, binds a dimer of Srp14p (Strub et al., 1999). The yeast-specific Srp21p was recently suggested to be the homologue of mammalian SRP9 (Rosenblad et al., 2004).

In recent years, major progress was made in solving the crystal structure of SRP54 and SRP19 alone and together with the 7SL RNA or with the RNA domain bound by the protein (Keenan et al., 1998; Batey et al., 2000; Walter et al., 2000; Weichenrieder et al., 2000; Wild et al., 2001; Hainzl et al., 2002; Oubridge et al., 2002; Pool et al., 2002; Wild et al., 2002; Nagai et al., 2003). Most relevant to this study is the structure of human SRP19 bound to helix 6 of the 7SL RNA (Wild et al., 2001) and the structure of Methanococcus jannaschii bound to the entire S domain of human 7SL RNA (Oubridge et al., 2002). The crystal structure demonstrates that SRP19 binds to the tips of helices 6 and 8 and facilitates their interactions (Oubridge et al., 2002). More recently, the ribosome interaction with the SRP was resolved by examining the structure of the mammalian SRP bound to 80S wheat germ ribosomes using cryo-EM. The proposed structure is composed of two hinges; one of the two hinges separates the 160 Å long S domain of SRP, which is near the peptide exit site, from a linker connecting the Alu domain. The Alu domain is positioned in the subunit interface; SRP9/14 seems to bind the tRNA small subunit, whereas the Alu RNA is proposed to interact with both proteins and tRNA of the large ribosomal subunit. This study explains how elongation arrest is elicited by the particle, and suggests that binding of the SRP prevents the binding of elongation factors and thereby arrests translation (Halic et al., 2004; Halic and Beckmann, 2005).

The function of the SRP was elucidated mostly from in vitro studies using canine SRP (Walter et al., 1981), while the in vivo role of the particle in protein translocation was elucidated mainly in E. coli (Ulbrandt et al., 1997; Herskovits et al., 2000) and yeast (Hann and Walter, 1991). In E. coli, the SRP is composed of a single RNA (4.5S RNA) and a single protein, the signal-peptide-binding protein, Ffh (Poritz et al., 1990). The SRP pathway is essential in E. coli due to its role in polytopic membrane protein biogenesis (Ulbrandt et al., 1997; Köch et al., 1999). Surprisingly, however, signal-peptide-containing proteins are translocated in E. coli via the chaperone pathway (Lee and Bernstein, 2001). In yeast, the SRP pathway is essential for growth in Schizosaccharomyces pombe (Althoff et al., 1994), but not in Saccharomyces cerevisiae (Brown et al., 1994; Mutka and Walter, 2001). Nevertheless, the secretion of signal-peptide-containing proteins in both these yeast strains depends on the SRP pathway, and during its depletion these proteins are not properly translocated to the ER, suggesting that each protein has its dedicated pathway for translocation (Hann and Walter, 1991; Stirling and Hewitt, 1992; Brown et al., 1994).

Very little is known about the trypanosome SRP pathway. Trypanosomes diverged very early in the eukaryotic lineage and are an excellent system in which to study the evolution of RNA-mediated processes. The trypanosome 7SL RNA can be folded into the canonical structure of the eukaryotic 7SL RNA, except for the Alu domain, which is truncated (Ben Shlomo et al., 1997). Uniquely, the trypanosome SRP complex is composed of two RNA molecules: the 7SL RNA and a tRNA-like molecule, sRNA-76 in Trypanosoma brucei and sRNA-85 in Leptomonas collosoma (Bêja et al., 1993; Liu et al., 2003). RNAi was used to study the necessity of this pathway, and its role in protein translocation (Liu et al., 2002). Using this system, SRP depletion was shown to be lethal in trypanosomes. Surprisingly, signal-peptide-containing proteins are translocated to the ER, and properly processed, suggesting that an alternative protein translocation pathway must exist in trypanosomes. However, following translocation to the ER under SRP54 depletion, proteins are mislocalized in the cell (Liu et al., 2002).

In this study, the T. brucei SRP proteins (SRP19, SRP68 and SRP72) were identified by using a bioinformatic approach. Purification of the particle to homogeneity by TAP-tagging suggests that the particle is composed of only the S-domain-binding proteins and, most importantly, lacks the Alu-domain-binding proteins. RNAi-mediated depletion of SRP68 and SRP72 resulted in nucleolus accumulation of the 7SL RNA, suggesting that the particle assembles in the nucleolus. The lack of Alu-domain-binding proteins in the trypanosome genomes, as well as their absence in the pure T. brucei SRP complex, further supports our hypothesis that the tRNA-like molecule could functionally replace the Alu-domain.

Materials and Methods

Cell growth and transfection

Procyclic T. brucei strain 29-13 was grown in SDM-79 medium supplemented with 10% fetal calf serum in the presence of 50 μg/ml hygromycin and 15 μg/ml G418. To establish the cell lines expressing the dsRNA-constructs SRP68-hp-1 and SRP19-T7-1, transformants were selected on 5 μg/ml phleomycin. To establish the cell line with the dsRNA-expressing construct, SRP72-T7bl-1, transformants were selected with 10 μg/ml blasticidin. Cloning of the parasites was carried out as previously described (Mandelboim et al., 2003).

Immunofluorescence and confocal microscopy

The cells were washed with PBS, mounted on poly-L-lysine-coated slides, and fixed in 4% formaldehyde in PBS at room temperature for 30 minutes. Cells were incubated in PBS containing 1% FCS at room temperature for 30 minutes and then with the anti-EP monoclonal antibody (Cedarline) for 1 hour (diluted 1:500). Confocal microscopy and image analysis was performed using the Laser Sharp 3.0 application as previously described (Liu et al., 2002).

Northern analysis

Total RNA was prepared with Trizol reagent and 20 μg/lane was fractionated on a 1.2% agarose, 2.2 M formaldehyde gel. The RNA was visualized with ethidium bromide. The 7SL RNA, U3 and the mRNAs SRP68, SRP72 and SRP19 were detected with [α-32P]-random-labeled probe (Random Primer DNA Labeling Mix, Biological Industries). For analyzing small RNAs, total RNA or RNA from gradient fractions were fractionated on a 10% polyacrylamide gel containing 7 M urea. The RNA was transferred to a nylon membrane (Hybond; Amersham Biosciences) and probed with...
Western analysis
The whole cell extract (10^6 cell equivalents per lane) or the sucrose fractions were separated on a 12% SDS-PAGE gel, transferred to PROTRAN membrane (BioScience), and probed with appropriate antibodies. Anti-SRP19 antibodies (kindly provided by Katharina Strub, University of Geneva, Switzerland), and IgG antibodies (Sigma) were diluted 1:200 and 1:2000, respectively. The bound antibodies were detected with goat anti-rabbit IgG coupled to horseradish peroxidase, and visualized by ECL (Amersham Biosciences).

Preparation of nucleolar and cytoplasmic extracts
*T. brucei* procaryons (10^9) were harvested and washed with phosphate buffered saline (PBS). The cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 5 µg/ml leupeptine, and 0.1 U/ml RNase inhibitor (Promega). Next, the cells were broken by 20 strokes in a dounce homogenizer in the presence of 0.1% NP-40. Preparation of nuclear, nucleolar and cytoplasmic fractions was performed using sucrose cushions, as previously described (Liang et al., 2002). These fractions were extracted with phenol and the RNA was subjected to northern analysis.

In situ hybridization
PCR for preparing the digoxigenin-11 (DIG)-labeled DNA probe, was performed under standard conditions using the Expand high fidelity enzyme (Roche Molecular Biochemicals). The dNTP mixture contained 200 µM of each of the nucleotides except dTTP (130 µM) and DIG-dUTP (70 µM). Conditions for fixation and hybridization were similar to those published previously (Liu et al., 2003). For detection of the DIG-labeled probes, the slides were incubated for 60 minutes at room temperature with 1:200 diluted fluorescein isothiocyanate-conjugated goat anti-DIG (Roche). The cells were incubated with propidium iodide (10 µg/ml) for 10 minutes to stain the nucleus. Finally, the cells were visualized using the Laser-sharp 2000 Bio-Rad MRC 1024 upright confocal microscope, as previously described (Liu et al., 2003).

Extract preparation and sucrose gradient separation
*T. brucei* procaryons (5×10^9) were harvested and washed with PBS. The cell pellet was resuspended in buffer A (35 mM HEPES, pH 7.9, 10 mM MgCl2, 24 mM KCl, 5 mM β-mercaptoethanol, 5 µg/ml leupeptin). Next, the cells were broken by 20 strokes in a Dounce homogenizer in the presence of 0.1% NP-40. Preparation of nuclear, nucleolar and cytoplasmic fractions was performed using sucrose cushions, as previously described (Liang et al., 2002). These fractions were extracted with phenol and the RNA was subjected to northern analysis.

TAP-tag purification of the signal recognition particle
The *T. brucei* cell line, 29-13, co-expressing the Tet repressor and T7 RNA polymerase, was transfected with Ncol-linearized pLew79-SRP19TAP plasmid encoding the SRP19TAP tagged protein under the control of tetracycline, and carrying the phleomycin-resistance gene. Expression of SRP19TAP in the transgenic cell line was induced for 60 hours with tetracycline (0.1 µg/ml). Cells (1 litre, 10^9 cells/ml) were harvested, washed with PBS and resuspended in IPP150 buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Nonidet P-40, 1% BSA, 5 µg/ml leupeptine). Next, 1% Triton X-100 was added and the extract was incubated on ice for 15 minutes. The extract was then centrifuged at 10,000 g for 15 minutes, and the supernatant was subjected to affinity selection using IgG Sepharose beads for 2 hours. The beads were washed with IPP150 buffer and resuspended in TEV cleavage buffer (10 mM Tris-HCl, pH 8, 0.1% NP40, 0.5 mM EDTA, 1 mM DTT). TEV protease was purified from an *E. coli* strain carrying a His-tag TEV construct (kindly provided by Ulrich Göringer, Darmstadt Germany). TEV protease (0.4 µg) was added and the mixture was incubated for 3 hours. To remove the TEV and concentrate the SRP complex the TEV cleaved complex was bound to a DEAE column in buffer A containing 50 mM KCl. Elution was carried out with buffer A containing 400 mM KCl, and proteins were precipitated with acetone and fractionated on a 10% SDS-PAGE gel. Finally, the gel was stained with silver and proteins were excised and analyzed by mass spectrometry (Technion, Haifa).

**Results**

Identification of SRP19
As a first step towards elucidating the unique composition of the trypanosome SRP complex, the trypanosome databases were searched for genes homologous to those coding for SRP proteins present in the mammalian and yeast complexes. The SRP54 protein was identified and characterized in our previous studies (Liu et al., 2002). The searches identified SRP19, SRP68 and SRP72 homologs, but not the Alu-binding proteins, SRP9 and SRP14 (Fig. 1 and supplementary material Figs S1 and S2).

SRP19 (GeneDB Tb10.1560) was identified based on its relatedness to the human protein (29% identity and 56% similarity). The alignment of the trypanosome proteins from *Trypanosoma brucei, Trypanosoma cruzi* and *Leishmania major*, and its comparison to the human and archaeal *Methanococcus jannaschii* (M.j.) proteins is presented in Fig. 1. Based on the conservation observed in the specific protein domains, the trypanosome protein could potentially be folded into three-standard β-sheets and two helices with βαβα topology (Fig. 1A). The molecular weight of the *T. brucei* SRP19 homolog is ~17 kDa. Human SRP19 was shown to first bind to the tetraloop on both helices 6 and 8 of the 7SL RNA (Fig. 1B) and only later to bind SRP54 (Hainzl et al., 2002). Tetraloops are important structural elements that serve both as nucleation sites for RNA folding as well as for stabilizing RNA tertiary structures. Based on the tertiary structure of the mammalian protein, it was suggested that G147 and G150 of the amino acids that are conserved in both human and M.j. proteins is presented in Fig. 1.

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in the trypanosome proteins. For instance, lysine 51 in the \textit{M.j.} protein and lysine 66 in the human protein are not conserved in the trypanosome proteins and arginine in position 81 is cysteine in all the trypanosome species (indicated by blue squares in Fig. 1A).

**Identification of the trypanosome SRP68 and SRP72 proteins**

To further examine the existence of additional SRP proteins in the trypanosome genomes, we searched for SRP72 and SRP68 protein homologs. These proteins were identified in the three trypanosome species (see supplementary material Figs S1 and S2). The \textit{T. brucei} SRP68 homolog (GenDB Tb04.29M18.150) shares 20\% identity and 37\% similarity with the mammalian protein, and the \textit{T. brucei} SRP72 (GenDB Tb10.61.0850) shares 20\% identity and 38\% similarity with the mammalian protein. The size of the \textit{T. brucei} SRP68 homolog is 64 kDa and that of SRP72 is 75 kDa. There is relatively little known about important motifs in these proteins (Lütcke et al., 1993). Recently, a domain near the N-terminus of SRP72 was identified that enables the protein to bind independently to the 7SL RNA. The sequence of this domain is located at positions 572-583 in the human protein (PDPRXRLPXXER) and is highly conserved in all species including trypanosomes (supplementary material Fig. S2) (Iakhiaeva et al., 2005). In highly conserved in all the trypanosome species (indicated by blue squares in Fig. 1A).

**RNAi silencing of SRP19, SRP68 and SRP72 affects growth and localization of the EP surface protein**

To examine whether the proteins identified by the bioinformatic searches are indeed bona fide SRP proteins, expression of these genes was knocked down by RNAi. To knock down the expression of these proteins, two strategies were taken. SRP19 and SRP72 were silenced by producing dsRNA from T7-opposing promoters (Wang et al., 2000). A 459 bp fragment
was amplified from the SRP19 gene, and a 545 bp one from the SRP72 gene (see supplementary material Table S1). The PCR products were cloned into the pZJM vector carrying the two T7-opposing promoters under the control of the tetracycline repressor (Wang et al., 2000). The SRP68 gene was also amplified from the genome, and a stem-loop construct was designed (see supplementary material Table S1). The construct is composed of a 473 nt stem and a 500 nt loop derived from the pex gene (Wang et al., 2000). This stem-loop construct was cloned downstream of the EP promoter and its expression was placed under the control of the tetracycline repressor. In both vectors, the antibiotic resistance genes are under the control of the T7 promoter, and the rRNA spacer region enabled the integration of the plasmid into the nontranscribed rRNA spacer (Wang et al., 2000). After linearizing the construct, the DNA was used to transfect T. brucei (29-13) cells that express the tetracycline repressor as well as T7 polymerase (Wirtz et al., 1999). After transfection, the cells were cultured for 24 hours, and then diluted to obtain a clonal population. After 3 weeks, cloned cells were used to establish pure cultures that were examined for inhibition of growth upon addition of tetracycline. The clones whose growth was clearly arrested upon addition of tetracycline were chosen for further analysis. The growth of the cells carrying the silencing constructs for SRP19, 68 and 72 was monitored following tetracycline induction and compared to uninduced cells. The results presented in Fig. 2A-C demonstrate that the growth of all cell lines was inhibited upon induction of silencing, as we previously described for SRP54-silenced cells (Liu et al., 2002), suggesting that the SRP pathway is essential in these parasites. Sudden death and lysis were observed in cells silenced for SRP68 or SRP72 proteins; we suspect that this cell death may result from the massive accumulation of the 7SL RNA in the nucleolus (see below). However, we cannot exclude the possibility that during SRP depletion the inability to synthesize sufficient new membrane proteins (see below) that are essential to maintain proper intracellular protein sorting as well as DNA segregation also contributed to the death phenotype in these cells. However, cells silenced for SRP54 died, but the death was not sudden (Liu et al., 2002), suggesting that indeed the sudden death observed in this study may stem from the secondary effect due to 7SL RNA accumulation in the nucleolus.

**Fig. 2.** Growth curves of cells silenced for SRP protein. (A) SRP72. (B) SRP68. (C) SRP19. The arrow indicates the time at which tetracycline was added. The growth of uninduced cells (−Tet) was compared with the induced cells (+Tet). The number of uninduced cells is designated by squares and of induced cells by circles.

**Fig. 3.** (A) Northern analysis of SRP72, SRP68 and SRP19 mRNA upon silencing. RNA was prepared from cells carrying the RNAi construct, uninduced (−Tet) and after 2-3 days of induction (+Tet). Total RNA (20 μg) was subjected to northern analysis with random-labeled probes. (1) SRP72-depleted cells. (2) SRP68-depleted cells. (3) SRP19-depleted cells. The transcripts of SRP72, SRP68, SRP19, tubulin mRNAs, rRNA and dsRNA are indicated by arrows. (B) Western analysis of SRP19 protein. Whole cell extract (10^6 per lane) was prepared from cells carrying the RNAi construct, uninduced (−Tet) and after 4 days of induction (+Tet). The extract was fractioned on a 12% SDS-polycrylamide gel and subjected to western analysis with the anti-SRP19 antibodies. SRP19 and a non-specific protein band used as a control for the amount of protein loaded are indicated by arrows.
To ensure that the silencing of SRP proteins is responsible for the growth arrest, the quality of the silencing was monitored by northern analysis. The results (Fig. 3A) suggest that the mRNAs for all SRP genes were almost completely eliminated 2-3 days following induction of silencing. In each cell line, the dsRNA production was induced upon tetracycline addition. The level of tubulin or rRNA was used to ensure equal loading of RNA in the different lanes.

Antibodies raised against the mammalian SRP68, SRP72 and SRP19 proteins (kindly provided by Katherina Strub, University of Geneva, Switzerland) were examined for their crossreactivity with the *T. brucei* proteins. Significant crossreactivity was detected only with anti-SRP19 and, indeed, the level of this protein showed 85% reduction upon silencing (Fig. 3B).

To further correlate the deleterious effects of SRP protein silencing with specific defects in protein translocation, the processing of the major parasite surface protein carrying a signal peptide, the EP procyclin, was examined (Mowatt and Clayton, 1987). We recently demonstrated that all the signal-peptide-containing proteins examined transverse the ER during SRP54 depletion, but are mislocalized (Liu et al., 2002). To examine the localization of the EP during silencing of the three SRP proteins, cells were fixed and then reacted with anti-EP antibodies. This type of staining detects only the protein present on the surface of the parasite. The results presented in Fig. 4 indicate that whereas in the wild-type cells the EP was uniformly distributed on the surface, forming a tight and heavy coat, upon silencing, the protein layer became much thinner. This is true for cells silenced for each of the SRP proteins (SRP19, SRP68 and SRP72), suggesting that all the cell lines were properly silenced, and resulting in the same phenotype that was previously observed for SRP54-silenced cells (Liu et al., 2002). Note, that the silenced cells also changed their normal shape as was observed during SRP54 silencing (Liu et al., 2002).

Pre-SRP is formed during the depletion of either SRP68 or SRP72

In yeast (*S. cerevisiae*), the depletion of individual SRP proteins each affected the SRP complex differently. For instance, no change in the sedimentation of the particle nor in

![Fig. 4. The effect of SRP silencing on EP translocation. Immunofluorescence assay (IFA) with anti-EP (surface staining). Cells were fixed with 4% formaldehyde for 25 minutes and incubated with anti-EP antibodies. Uninduced (−Tet) cells, induced cells on the third day after induction (+Tet) and the type of cell lines are indicated. Bar, 5 μm.](image)

![Fig. 5. The effect of SRP silencing on the size of the complex. Whole cell extracts (5 × 10^6 cells) were prepared from uninduced (−Tet) cells and from cells carrying SRP72, SRP68 and SRP19 RNAi constructs after 3 days of induction (+Tet). The extracts were layered on continuous 10-30% (w/v) sucrose gradients. Gradients were centrifuged at 4°C for 22 hours at 35,000 rpm using a Beckman SW41 rotor. RNA was prepared from the sucrose gradient fractions and subjected to northern analysis with random-labeled 7SL RNA and with oligonucleotides complementary to SL RNA probes. Fraction numbers are indicated (from top to bottom). S values were determined relative to 28S rRNA, 4S RNA and catalase (11S). The transcripts of 7SL RNA and SL RNA are indicated by arrows.](image)
The level of SRP RNA was observed upon depletion of SRP54 or SEC65 (SRP19 homolog), but the level of 7SL RNA was reduced in cells lacking SRP72, SRP68, SRP14 and SRP21 (Brown et al., 1994). We therefore examined the integrity of the SRP particle in cells depleted of each of the SRP proteins. Whole cell extracts were prepared from uninduced cells and from cells depleted of the individual SRP proteins, after 2 days of silencing. The results in Fig. 5 indicate only a slight change in the size of the particle (Michaeli et al., 1992) upon SRP19 depletion, but major changes in the size of the particles upon SRP68 or SRP72 depletion. The specificity of the change can be appreciated when it is compared to the sedimentation of the spliced leader RNA complex SL RNP (serving as an internal control), which was unaffected during SRP deletion. The results suggest that, in the absence of SRP68 and SRP72, only sub-particles were formed. However, the sedimentation of the particles did not change much in SRP19-depleted cells, as was previously observed for the yeast particle (Brown et al., 1994).

The T. brucei SRP assembles in the nucleolus
Studies in mammalian cells indicate that microinjected 7SL RNA rapidly becomes localized in the nucleoli, and only subsequently departs to the cytoplasm (Jacobson and Pederson, 1998). In mammals, SRP19, SRP68 and SRP72 displayed nucleolar localization upon transient transfection, suggesting that SRP assembly takes place in the nucleolus (Politz et al., 2000). In yeast, deletion of SRP72, SRP68, SRP21 or SRP14 caused 7SL RNA to accumulate in the nucleolus, suggesting that the yeast particle assembles in the nucleolus as well, and that these proteins are essential for the exit of the particle from

![Fig. 6. The effect of SRP72 and SRP68 silencing on 7SL RNA localization. (A) Localization of the 7SL RNA by biochemical fractionation. Cytoplasmic and nucleolar fractions were as described in Materials and Methods. RNA from these samples was subjected to northern analysis with random-labeled 7SL and U3 RNA probes from uninduced cells (–Tet) and cells after 3 days of induction (+Tet). (1) SRP72-depleted cells. (2) SRP68-depleted cells. (B) Localization of 7SL RNA during SRP72 and SRP68 silencing by in situ hybridization. Cells uninduced (–Tet) and after 3 days of induction (+Tet) were fixed and hybridized with DIG-labeled PCR probes to U3 and 7SL RNA. (1) Cells carrying the SRP72 RNAi construct hybridized with DIG-labeled PCR probe to U3 RNA. (2) Cells carrying the SRP72 RNAi construct hybridized with DIG-labeled PCR probe to 7SL RNA. (3) Cells carrying the SRP68 RNAi construct hybridized with DIG-labeled PCR probe to 7SL RNA. The nuclei (N) and nucleolus (Ne) are indicated. Bar, 5 μm.]
the nucleolus to the cytoplasm (Ciufio and Brown, 2000; Grosshans et al., 2001). It was therefore of interest to examine whether the assembly of the trypanosome SRP also takes place in the nucleolus. The nucleolar localization was examined by biochemical fractionation and by in situ hybridization with 7SL RNA and U3 snoRNA probes. The results presented in Fig. 6A (1 and 2) demonstrate that, during SRP68 and SRP72 silencing (+Tet), the level of 7SL RNA decreased by 70% in the cytoplasm and increased by 50% in the nucleolus. The in situ hybridization data presented in Fig. 6B also clearly demonstrate that in uninduced cells the 7SL RNA was found in the cytoplasm and only a minor amount was detected in the nucleolus. However, during silencing of both SRP68 and 72 (Fig. 6B, 1 and 3), the RNA accumulated mainly in a single dot, the nucleolus, which was detected by the U3 probe. These results demonstrate that, during SRP72 or SRP68 silencing, the 7SL RNA is trapped in the nucleolus and that SRP assembly most probably takes place in this compartment.

Purification of the SRP complex reveals homologs of SRP19, SRP68, SRP72 and SRP54

The bioinformatic studies described above failed to identify the Alu-binding proteins. It was therefore essential to purify the trypanosome complex to homogeneity and determine its protein composition. For purification we used the TAP-tag approach (Rigaut et al., 1999). The coding sequence of SRP19 was cloned into the pLew79TAP vector (kindly provided to us by Achim Schnaüfer, Seattle Biomedical Research Institute, WA) carrying the TAP sequence to create a C-terminally fused protein that is under the regulation of the Tet repressor (Schnaüfer et al., 2003). The TAP is a 21 kDa tag that consists of a calmodulin-binding peptide (CBP), a TEV protease cleavage site, and two protein-A-binding peptides (Rigaut et al., 1999). The vector was linearized with NotI, which directs its integration into the rRNA intergenic region. To confirm the expression of the tagged protein and its proper incorporation into the SRP complex, cells were induced for the production of the fused protein. Extracts were fractionated on a sucrose gradient for 22 hours to enable the separation of free-proteins from the SRP complex. The fractions were subjected to both biochemical fractionation and by in situ hybridization with the appropriate probes. The results (Fig. 7A) indicate that all the tagged protein was associated with the SRP particles (15S). To examine whether the complex can be affinity selected using IgG-coated beads, whole cell extracts were incubated with the beads as described in Materials and Methods, and the levels of the tagged protein were determined following selection. The results (Fig. 7B) indicate that 96% of the protein was selected using the IgG-coated beads. To purify the particle, cells were induced for the production of the tagged protein. The tagged SRP complex was first purified by selection on the IgG beads. The pattern of the selected proteins was compared with the proteins selected from cells in which the production of the tagged protein was not induced. The proteins were separated on a 10% SDS-polyacrylamide gel and stained with silver. The results (Fig. 7C, lanes 1 and 2) demonstrate specific selection of only four proteins [SRP75, SRP64, SRP54 and the tagged SRP19 (~40 kDa protein)]. Interestingly, no proteins of the size of the Alu-domain-binding proteins were observed. The complex was released from the IgG beads by TEV digestion and the released particles were further purified by chromatography using a DEAE Sepharose column. The pattern of the purified proteins (Fig. 7C, lane 3) demonstrates the selection of only four proteins [the 40 kDa tagged SRP19 was cleaved to generate a 21 kDa protein still carrying the CBP (~4 kDa)]. To demonstrate that the purified SRP complex is intact and includes the tRNA-like molecule, RNA was extracted from the purified particles and silver stained. Two RNA species were revealed (Fig. 7D) and their identity was confirmed by hybridization with the appropriate probes (results not shown). These results indicate that, as opposed to all other eukaryotic SRPs described in nature, the trypanosome complex is composed of only four SRP proteins, and lacks the Alu-domain-binding proteins.

The cell line expressing the tagged protein was further used to investigate the assembly of the SRP complex. The silencing experiments of SRP72 and SRP68 suggested that the trypanosome SRP complex assembles in the nucleolus. To follow the localization of the tagged SRP19 protein, the cells were subjected to immunofluorescence upon induction of the tagged SRP19. The results (Fig. 7E, 1 and 2) suggest that the protein first accumulates in the nucleus and only later enters the cytoplasm. These results are consistent with the observation that mammalian GFP-tagged SRP19 protein first accumulates in the nucleolus/nucleus before migrating to the cytoplasm (Sommerville et al., 2005).

Discussion

In this study we describe the unique protein composition of T. brucei SRP, which is the first eukaryotic complex described so far that lacks the Alu-domain-binding proteins. However, the SRP carries a tRNA-like molecule that may functionally replace the Alu-domain. Despite the deviation in SRP composition, trypanosome SRP assembles in the nucleolus similarly to the yeast and human homologues. The deletion of any of the SRP particle proteins is lethal, suggesting the necessity of this pathway. Moreover, the same defects in formation of the EP surface coat were observed during the depletion of each of the SRP proteins, SRP19, SRP72, SRP68 and SRP54 (Liu et al., 2002), suggesting that indeed the proteins identified in this study are true SRP protein homologs.

Unique and conserved structural features of the trypanosome SRP19 and SRP72 proteins

The overall relatedness of the trypanosome SRP proteins to their homologs in other eukaryotes is relatively low (20-30% identity and 40-50% similarity). However, the most conserved structural domains are also conserved in the trypanosome proteins. These include SRP19 regions that interact with tetraloop 6, and the TPR of SRP72. Of interest are trypanosome-specific changes involving the addition of short domains that are absent from the other homologs, for instance, a longer N-terminus domain in L. major SRP19 as well as six extra amino acids at the C-terminal part of the Leishmania protein. Most striking is the conservation in positions (90-104) of the T. brucei SRP19 which is well conserved in all trypanosome species but lacking in the other homologs.
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Silencing of SRP proteins elicits the same defects on EP surface coat

Studies in yeast were focused mostly on defects observed during SRP54 and SRP19 (SEC65) depletions. Although, these depletions are not lethal, the cells grow very slowly and exhibit 'petite' phenotype (Brown et al., 1994). Interestingly, however, the yeast SRP54-null mutant adapts to the loss of the SRP by eliciting the synthesis of chaperones and reducing the production of ribosomes (Mutka and Walter, 2001). In trypanosomes, however, silencing of each of the SRP proteins is lethal. No adaptive response was observed, and major morphological changes take place that are accompanied by

Fig. 7. TAP purification of SRP. (A) Fractionation of RNP particles on a sucrose gradient. Whole cell extract (150 ml culture, 10⁷ cells/ml) was prepared from induced cells carrying the SRP19TAP construct. The extract was layered on a continuous 10-30% (w/v) sucrose gradient. The gradient was fractionated as described in Fig. 5. RNA extracted from an aliquot of odd-numbered fractions was separated on a 10% polyacrylamide-7M urea gel and subjected to northern analysis with random-labeled 7SL RNA. Fraction numbers are indicated (from top to bottom). S values were determined relative to 28S rRNA, 4S RNA and catalase (11S). The transcripts of 7SL RNA and the SRP19TAP protein are indicated by arrows. (B) Immunoprecipitation of the SRP by IgG. Whole cell extract was prepared from induced cells (50 ml, 10⁷ cells/ml) carrying the SRP19TAP construct and subjected to affinity selection using IgG Sepharose. The immunoprecipitated products and 2% of total cell extract and supernatant were separated on a 12% SDS-polyacrylamide gel and subjected to western analysis with IgG antibodies. SRP19TAP is indicated by an arrow. (C) Purification of SRP proteins. Whole cell extracts (1 litre, 10⁷ cells/ml) were prepared from induced cells carrying the SRP19TAP construct and from uninduced cells (control) and the extracts were subjected to affinity selection using IgG Sepharose. Proteins from uninduced cells are shown in lane 1, and induced cells in lane 2. Complexes selected with IgG beads were treated with TEV protease and were further purified by DEAE column (lane 3). Proteins extracted from the eluate were fractioned on a 10% SDS-polyacrylamide gel and stained with silver. SRP21 (SRP19 homolog fused to CBP), SRP19TAP (~40 kDa), SRP54, SRP64 (SRP68 homolog) and SRP75 (SRP72 homolog) are indicated by arrows. (D) Purification of SRP RNAs. As for C except that RNA was prepared from the affinity selection fraction and separsted on a 10% polyacrylamide-7M urea gel and stained with silver. 7SL RNA and sRNA-76 are indicated by arrows. (E) Immunofluorescence assay (IFA) of the SRP19TAP protein. Cells carrying the SRP19TAP construct were induced for 4 hours (1) and 60 hours (2) and the tagged protein was visualized. Cells were fixed with 4% formaldehyde for 25 minutes, incubated with IgG and visualized as described in Materials and Methods. Bar, 5 μm.
protein sorting defects (Liu et al., 2002). Interestingly and as described in detail in our previous study, the defects in the EP surface coat appear to be secondary, since EP translocates to the ER but is mislocalized and accumulates in mega vesicles (Liu et al., 2002). Our recent studies suggest that during SRP depletion the EP as well as other signal-peptide containing proteins transverse the ER via the chaperone pathway. However, because the SRP-depleted cells fail to synthesize the polytopic membrane proteins, the composition of both intracellular membranes such as the ER and the Golgi apparatus as well as the plasma membrane are most probably defective and affect intracellular trafficking. The punctuated staining that looks like megavesicular structures of EP (Liu et al., 2002) may result from augmented production of ER transport vesicles due to a secondary sorting defect. The data presented in this study suggest that depletion of each of the SRP proteins affected EP translocation to the surface in the same manner, indicating that the sequences identified represent bona fide homologs of SRP proteins.

Trypanosome SRP assemblies in the nucleolus; SRP68 and SRP72 are essential for exit of the particle from the nucleolus

Not much is known about the effect of SRP68 and SRP72 depletion on SRP function. Depletion of SRP72 and SRP68 in *S. cerevisiae* results in the accumulation of SRP RNA in the nucleus/nucleolus (Grosshans et al., 2001). Early studies also suggest that the level of SRP RNA is reduced in the absence of SRP72 and SRP68 but not when SRP19 (SEC65) or SRP54 are depleted (Brown et al., 1994). Whereas depletion of SRP54 and SRP19 in yeast does not affect the sedimentation properties of the SRP, no data exist on the effect of SRP68 or SRP72 depletion on the SRP complex. Interestingly, depletion of SRP54 and SRP72 by RNAi in cultured mammalian cells is not lethal (Ren et al., 2004). However, it is not clear at this point whether these experiments suggest that the SRP pathway is not essential in mammals, as in *S. cerevisiae*, or that the knockdown was not complete and the residual levels of SRP proteins that remained in these cell lines could support the growth of these cells without overt cellular phenotypes. The results presented in this study highlight the role of both SRP68 and SRP72 for SRP stability and exit of the particle from the nucleolus. The reduction in the level of 7SL RNA in the cytoplasm as well as the accumulation of the 7SL RNA in the nucleolus suggest that the trypanosome SRP assembly, as in yeast and mammals, takes place in the nucleolus. Furthermore, this study demonstrates that SRP68 and SRP72 are essential for the stability of the RNA and its proper export from the nucleolus to the cytoplasm. The presence of the 7SL RNA in pre-SRP particles in cells depleted of either SRP68 or SRP72 suggest that each of these proteins alone can form a stable complex with the 7SL RNA. These results are in agreement with a recent study demonstrating the specific binding of different recombinant SRP72 proteins to the 7SL RNA (Jakhiaeva et al., 2005), which suggests that SRP72 can independently bind to the 7SL RNA. These recent results, as well as the data presented in this study, challenge the previous notion that SRP68 binds to the RNA first, leading to a conformational change, and allowing SRP72 to associate through protein-protein interactions (Lütcke et al., 1993). Our results (Fig. 5) clearly demonstrate that SRP68 can stably interact with the 7SL RNA even in the absence of SRP72, and indicate that SRP72 can stably interact with the 7SL RNA in the absence of SRP68. However, one cannot exclude the possibility that SRP68 enhances the affinity of SRP72 to the RNA. In yeast, SRP68 was not detected in a strain lacking SRP72 (Brown et al., 1994). The interaction of trypanosome SRP68 with SRP72 seems therefore to be different. The ability to detect a sub-particle in the absence of each of these proteins suggests that formation of the sub-particle does not require the presence of both proteins, again supporting the notion that each of these proteins can bind separately to the 7SL RNA.

The assembly of the SRP in the nucleolus seem to be conserved from trypanosomes to man. The biological significance of this phenomenon is currently unknown. It was suggested that since the SRP needs to bind the ribosome in the cytoplasm, and since ribosome assembly takes place in the nucleolus, this compartment can serve as a checkpoint to verify that SRP particles were properly assembled and can correctly interact with the ribosomes (Pederson and Politz, 2000). However, elegant microscopic studies suggest that the SRP localizes within the nucleolus at sites that differ from the classical sites of ribosome assembly, making the checkpoint hypothesis unlikely (Politz et al., 2002). The accumulation of the pre-SRP particles in the nucleolus during SRP68 or SRP72 silencing may, however, occur at sites that are normally populated with other factors (for instance ribosomes). The sudden cell death observed upon SRP68 or SRP72 silencing but not during SRP19 and SRP54 knockdown suggest that the accumulation of the SRP in the nucleolus may be related to this very dramatic cell death induced during depletion of these two proteins.

The trypanosome SRP is the first eukaryotic particle to lack the Alu-domain-binding proteins

The most intriguing finding of this study is that the trypanosome SRP particle lacks the Alu-binding proteins. The strongest support for absence of Alu-binding proteins in trypanosomes is the protein composition of the purified particle presented in this study (Fig. 7). A very thorough analysis was performed recently on SRP RNA and its binding proteins in protozoa and fungi. The detailed analysis demonstrated that the Alu domain region of SRP RNA is highly variable in both sequence and secondary structure. All Alu domain sequences appear to carry the conserved UGUNR motif but, besides this similarity, all the other parts of this domain show a large degree of variation. Several protozoans, including *Trypanosoma, Eimeria, Tetrahymena* and *Theileria*, possess a very truncated Alu domain. Truncation in the Alu domain was also observed in fungi and microsporidia although, in *Saccharomyces*, an additional sequence fragment is inserted near the 5′ end (Rosenblad et al., 2004). Interestingly, a correlation was found between the truncated Alu domain and the absence of Alu-binding proteins. In some cases, however, as in yeast and fungi, Alu-binding proteins exist. All yeast and fungi carry SRP14 and many also carry SRP21, which seems to be homologous to SRP9 (Rosenblad et al., 2004).

Of special interest are those organisms that seem to completely lack the Alu-domain-binding proteins. Such organisms are the trypanosome species (this study) and
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Thelerta annulata, Giradia lamblia, Entamoeba histolytica and Encephalitozoon cuniculi (Rosenblad et al., 2004). Since many of these parasite genomes are incomplete, Alu-binding proteins may be found in the future. However, our analysis of the complete trypanosome genome was performed recently and no Alu-binding proteins were detected. SRP purification data (Fig. 7) also strongly support the lack of Alu-binding proteins in trypanosomes. The absence of Alu-domain-binding proteins in trypanosomes and in eubacteria such as Bacillus that nevertheless carry an Alu-like domain, as well as in archaea, allows us to speculate that the ancestral Alu domain was comprised solely from RNA.

Does the tRNA-like molecule present in trypanosomes replace the Alu-domain-binding proteins?

Our previous studies (Béjà et al., 1993; Liu et al., 2003) suggest the existence of a tRNA-like molecule that co-migrates with the T. brucei and L. collosoma SRP complexes. Indeed, the purified SRP contains this novel RNA molecule. The absence of the Alu-domain-binding proteins strengthens our hypothesis that this tRNA-like molecule may functionally replace the Alu domain and may mediate the arrest function of the trypanosome particle. The recent cryo-EM structure of the SRP in complex with the ribosome suggests that all the contact sites of the Alu domain with rRNA are also used by the eukaryotic elongation factor 2 (eEF2), suggesting that Alu-ribosome interaction exhibits elongation factor mimicry (Halic et al., 2004). The RNA-RNA interaction between L2 of the 7SL RNA 3′ end and helix 43 of rRNA is strongly reminiscent of the interaction between the tRNA-T loop and the same ribosomal helix in the context of the EF-Tu-tRNA-GTP ternary complex bound to the ribosome. Binding of the Alu domain in this position directly competes with the elongation factors entering their binding site. It will therefore be of interest to identify the site at which the trypanosome tRNA-like molecule binds to the ribosome and to determine whether it occupies the same site as the Alu domain.

The Alu domain and its binding proteins were shown to be essential for transport of the SRP (He et al., 1994; Jacobson and Pederson, 1998). The truncated Alu domain and the lack of the Alu-binding proteins in trypanosomes suggest that this domain and especially the Alu-binding proteins are not involved in transport of the trypanosome complex to the cytoplasm. The finding that only SRPs lacking SRP68 or SRP72 accumulate in the nucleolus suggests that these proteins are essential for exit of the particle from the nucleolus. Interestingly, silencing of SRP19 did not affect the level of the 7SL RNA nor its localization (our unpublished results), suggesting that SRP19 does not have a role in transport of the particle from the nucleus/nucleolus to the cytoplasm. We have recently silenced the XPO1 by RNAi and observed the accumulation of the SRP complex in the nucleolus, suggesting that this export factor mediates the transport of the trypanosome SRP as in mammals and yeast (our unpublished results).

This study is the first to describe a eukaryotic SRP that lacks the Alu-domain-binding proteins. It still remains to be seen whether the tRNA-like molecule functionally replaces the missing Alu-domain proteins. It will be of great interest to search in SRPs of other lower eukaryotes, eubacteria and archaea for the presence of an additional RNA molecule, like the one present in trypanosomes.
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