Analysis of a nuclear localization signal in the p14 splicing factor in Trypanosoma cruzi

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A B S T R A C T

There are only a few reported nuclear localization signals (NLS) in trypanosomes despite intensive research on nuclear metabolic processes such as mRNA processing and transcription during the recent past. Moreover, there are only two reports for a monopartite (La protein) and bipartite (H2B histone, ESAG8) NLS in Trypanosoma brucei. We decided to investigate a NLS in Trypanosoma cruzi by selecting p14, a small RNA recognition motif (RRM) containing protein involved in the splicing process in the nucleus. Its small size (117 amino acids), and an optimized streamlined workflow for analysis in T. cruzi, allowed us to define a region of basic amino acids (RRKRRR) located at the C-terminus that is necessary for nuclear localization. However, the NLS for p14 appeared to be more complex since the signature RRKRRR alone is necessary but not sufficient to direct heterologous proteins, such as GFP, to the nucleus. Since p14 interacts strongly with splicing factor SF3b155, a much larger protein, we designed a p14 variant unable to interact with it. The results allowed us to discard the notion that p14 is entering the nucleus, or is retained within, as the sole consequence of being part of a larger complex. Extensive mapping showed that all of the information of nuclear import resides within the small p14 protein in a bipartite NLS composed of the signature RRKRRR and a region of the RRM domain. Thus, NLS definition in T. cruzi is more complex than previously described.

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1. Introduction

Trypanosomes are intriguing and amazing organisms in many aspects of their molecular biology. For example, RNA transcription in these parasites is polycistronic, encompassing several dozen kilobases and they lack conventional promoters (Clayton, 2002). Polycistronic pre-mRNAs are processed by two coupled reactions, trans-splicing and polyadenylation, and exported to the cytoplasm as monocistronic mRNAs (Liang et al., 2003). Thus, mRNA maturation in trypanosomes differs from most eukaryotes. Trans-splicing involves the joining of two different molecules, the polycistronic pre-RNA and a capped 39 nucleotide sequence named Spliced Leader RNA (SL-RNA) (Liang et al., 2003).

However, with so many different mechanisms (i.e., transcription, RNA processing) between these parasites and their mammalian hosts there is still a lack of available effective anti-parasitic drugs or disease treatments (Nwaka and Hudson, 2006).

For these reasons, during recent years a lot of effort has been focused on the understanding of metabolic processes in the nucleus in order to propose viable drug targets.

We have previously described several splicing and polyadenylation factors in the genomes of Trypanosoma cruzi and Trypanosoma brucei, including those homologous to the splicing E complex (U2AF65/U2AF35/SF1) (Vazquez et al., 2009), A complex (SAP155/p14) (Avila et al., 2007), polyadenylation factors (Fip1/Cpsf30) (Bercovich et al., 2009b) and Exon Junction Complex EJC (Mago/Y14/NTF2 related) (Bercovich et al., 2009a). Other extensive work has been published dissecting nuclear processes such as SL-RNA metabolism (Liang et al., 2003), polycistronic transcription (Clayton, 2002) and more recently epigenetic control (Figueiredo et al., 2008; Villanueva et al., 2009).

Despite this intense work, few nuclear localization signals (NLS) were described for trypanosomes: La and H2B (Marchetti et al., 2000), and ESAG8 (Hoek et al., 2000). These signals are classical NLS of the monopartite and bipartite-type.

In mammals, the physical barrier imposed by the nuclear envelope requires active transport to translocate proteins to the nucleus across the nuclear pore complexes for molecules above 40 kDa (Görlich, 1997). This transport requires NLS recognition by importins α and β in the presence of Ran-GDP, nuclear transport factor 2...
protein p14 has a minimalistic domain architecture composed of involved in the splicing process in the nucleus, named p14. The trypanosomes, we selected a small 117 amino acid RNA recognition by distinct receptors. The import of U small nuclear ribonucleoproteins (snRNPs) is also achieved by another pathway into the nucleus (Görlich, 1997).

With the aim of in-depth analysis of NLS classes present in trypanosomes, we selected a small 117 amino acid RNA recognition motif (RRM)-containing protein of T. cruzi suggested to be involved in the splicing process in the nucleus, named p14. The protein p14 has a minimalistic domain architecture composed of the RRM (80 amino acids) and a C-terminal α-helix extension (37 amino acids), leaving a confined space to look for a NLS (Avila et al., 2007).

The only known interaction of mammalian p14 during spliceosome assembly is with splicing factor SF3b155 in the SF3b complex (Golas et al., 2003). The molecular architecture of human SF3b was determined by single-particle electron cryomicroscopy at a resolution of less than 10 angstroms, allowing identification of p14 in the central cavity of the complex (Golas et al., 2003). The p14 also cross-links with the branch-point adenosine in the pre-mRNA. Thus, p14 is completely hidden within SF3b155 and the pre-mRNA. Interestingly, its RRM domain is involved in protein–protein interaction with SF3b155 and not in protein–RNA interaction as a classical RRM (Spadaccini et al., 2006).

In this work, we demonstrate that T. cruzi p14 is a nuclear protein that harbors a complex bipartite NLS composed of a run of basic amino acids in the C-terminus extension and other motifs that require the RRM domain but not the interaction with SF3b155. Thus, this novel NLS expands the small group of different NLS found in trypanosomes and indicates that NLS definition in these parasites is more complex than previously described.

2. Materials and methods

2.1. pTREX-GW construction and GFP fusion proteins generation

The vector pTREX (Vazquez and Levin, 1999) were digested (5 μg of DNA) with EcoRV. The 5' phosphates were removed with calf intestinal alkaline phosphatase (CIAP) and ligated to the Gateway™ cassette C1 to create the destination vector pTREX-GW (see Fig. 1A). The vector is maintained in the ccdB Survival™ 2 Ti E. coli strain. The p14 and SF1 open reading frames (ORFs) with GFP and GFP alone were transferred by performing the left–right (LR) recombination reaction from the entry vector pENTR-DTOPO (Invitrogen).

Fusion GFP proteins were generated by splice overlap extension (SOEing) PCR in both the C- and N-termini. All of the ORFs contain the signature CACC in the 5' end necessary for the 5 min TOPO cloning reaction as described by the manufacturer (Invitrogen).

2.2. Construction of mutant proteins

N-terminal and C-terminal deletion mutants were generated by PCR with specific primers (see kDa). Internal deletion mutants and alanine scan mutants were generated by SOEing PCR (Ben-Dov et al., 2005). All of the mutant products were fused to GFP as described above and cloned into a pENTR-DTOPO vector (Invitrogen) and then transferred, using the LR recombination reaction, to destination vector pTREX-GW.

All PCRs were performed using proofreading iProof™ high fidelity DNA polymerase (Bio-Rad) and all products were sequenced on a MegABACE 500 (Amersham Biosciences) capillary sequencer to verify the mutations introduced.

2.3. Yeast two-hybrid analysis

The ProQuest™ Yeast Two-Hybrid Gateway™ compatible System (Invitrogen) was used for protein interaction analysis. T. cruzi p14 (TcP14) variants were analyzed in both DNA binding (DB) and activation domain (AD) configurations against SF3b155 variants. Since the DB-SF3b155 configuration produced low levels of auto-activation, we present only the results for DB-p14 configuration for a clear interpretation of the results. Activation of the His3 reporter gene induced by interaction pairs was studied, analyzing the ability of yeast to grow on plates lacking His (SC L-W-H-leu-trp-his lacking medium) in the presence of 25 mM of 3AT (3-amino-1,2,4-triazole) and incubated at 30 °C for up to 48 h. Activation of the LacZ reporter gene was monitored by a β-galactosidase activity liquid assay. Reactions were performed in duplicate and measured at OD 420 nm. The data were normalized with the amount of yeast in the initial culture (OD 600 nm).

Simultaneously, we processed the ProQuest's negative Control A (empty DB and AD) and positive Control E (DB – full length GAL4 protein) as standard controls.

2.4. Transfection of T. cruzi and confocal microscopy analysis

T. cruzi epimastigote cultures and transfections were performed exactly as previously described (Vazquez and Levin, 1999). Transient transfections were observed for analysis 3–5 days after addition of G418, while stable transfections were analyzed 2 months later.

For GFP analyses, cells expressing GFP were collected by low speed centrifugation, washed in PBS, fixed for 30 min in 4% paraformaldehyde and allowed to settle on poly-l-lysine coated slides. Nucleic acid staining with propidium iodide (PI) was performed after membrane permeabilization with 0.2% Triton X-100 and a RNase treatment as described by the manufacturer (Invitrogen). Due to the high over-expression of pTREX-GW, RNase treatment of the samples was mandatory to obtain a specific staining of the DNA. The samples were then mounted in a medium with an anti-fade reagent. Confocal images were obtained in an Olympus FV300 microscope.

3. Results and discussion

3.1. Generation of a Gateway™ expression system for rapid localization of proteins in T. cruzi

We generated a Gateway™ compatible version of the successful T. cruzi expression vector pTREX (Vazquez and Levin, 1999) (Fig. 1A). When used in conjunction with pENTR-DTOPO, the cloning process of PCR amplicons was greatly accelerated. For instance, transient transfection experiments were ready for rapid analysis in less than 1 week after the PCR amplifications.

Since this is, to our knowledge, the first Gateway™ expression vector described for T. cruzi, we first demonstrated that the attB sequences generated after LR recombination did not affect expression of protein products in this parasite. This is important because the attB sequences are now transcribed as part of the 5' and 3' untranslated regions (UTRs) of the mRNA. To do so, we used the T. cruzi splicing factor SF1 as a proof of concept. We previously demonstrated that TsSF1 is located in the nucleus (Vazquez et al., 2009). Transfection of a fusion GFP-TsSF1 protein using pTREX-GW allowed us to demonstrate that the protein was accumulated in the nucleus as expected (see Supplementary Fig. S1A).
Moreover, a Western blot comparison between transfected parasite extracts of pTREX-GFP and pTREX-GW-GFP determined that the expression levels were comparable (see Supplementary Fig. S1B).

Interestingly, the most important conclusion is that high over-expression of the protein, approximately 10-fold with respect to endogenous levels, did not hinder its localization, demonstrating that our system is robust.

3.2. The p14 protein is localized in the nucleus

We generated two different constructs: p14 fused to the C-terminus of GFP (GFP-p14) and p14 fused to the N-terminus of GFP (p14-GFP) (Fig. 1A). After transfection, both fusion proteins were located in the nucleus of T. cruzi, demonstrating that p14 is a nuclear protein while GFP alone was located all over the cell (with the exception of the kinetoplast) (Fig. 1B). The size of GFP (27 kDa)
allowed the protein to enter the nucleus by passive diffusion through the nuclear pore (Marchetti et al., 2000). Interestingly, p14 directed selective accumulation of GFP in the nucleus independently of being fused to the N or C-terminus (Fig. 1B). Moreover, GFP-p14 size is about 40 kDa, which is below the limit allowed to enter by passive diffusion (Marchetti et al., 2000), suggesting that the protein is actively transported and/or retained in the nucleus.

It was previously suggested that T. cruzi p14 was a nuclear protein based on its interaction with the splicing factor SF3b155, although it was not demonstrated (Avila et al., 2007).

Thus, p14 is an attractive model for nuclear localization signal analysis since (i) it is a small protein (117 amino acids) with a minimalist architecture, (ii) it harbors only one known domain (RRM) occupying 70% of the protein length with a 37 amino acid helical C-terminal extension (Fig. 2A), and (iii) it is localized and retained in the nucleus despite high over-expression as a fusion protein.

3.3. p14 nuclear localization is independent of the complex formation with SF3b155

The only known interaction of Tcp14 is with SF3b155, a much larger protein of 155 kDa (Avila et al., 2007). This observation is in agreement with other organisms (Spadaccini et al., 2006; Thickman et al., 2006). The interaction occurs via the RRM domain of Tcp14 and a specific region of SF3b155 mapped to amino acids 182–243 (Avila et al., 2007). In mammals, p14 is hidden in a central cavity of SF3b155 in the fully assembled spliceosome while in contact with RNA (Golas et al., 2003; Schellenberg et al., 2006).

Three different hypotheses could arise to explain the import of p14 to the nucleus. One, the protein is transported in complex with SF3b155 instead of having its own NLS. Two, the size of p14 could allow its passive diffusion to the nucleus, and it is actively retained once in complex with SF3b155 in the spliceosome without the need for a specific NLS. Three, p14 could have its own NLS and is independent of SF3b155 complex formation.

To test these hypotheses, we generated a p14 mutant protein unable to interact with SF3b155. All of the evidence points to the RRM as the interacting domain with SF3b155 (Schellenberg et al., 2006; Spadaccini et al., 2006; Avila et al., 2007), probably by exposing the hydrophobic amino acids in β1 (RNP2) and β3 (RNP1) to establish contact with its protein partner.

As seen in Fig. 2, every mutation that disrupts the correct folding of the p14 RRM (Fig. 2A) abolished the interaction with SF3b155 as indicated by the yeast-two hybrid assays (Fig. 2B and C). This is the case for the deletion of the β1 (RNP2) region (p14_Δβ1 in Fig. 2A), for the deletion of the α-B helix (p14_ΔαB in Fig. 2A) and for the interruption of the β3 (RNP1) region with alanines (p14_β3_A in Fig. 2A). On the contrary, deletion of almost the entire α-helix C-terminal extension (p14_1–91 in Fig. 2A) did not impair interaction with SF3b155 (Fig. 2B and C). Interestingly, this C-terminal extension harbors a region of basic amino acids (RRKRRR) similar to the monopartite NLS in other organisms.

We selected p14_ΔαB as a proof of concept to distinguish among the proposed hypotheses. A fusion protein GFP-p14_ΔαB was found located in the nucleus after transfection (Fig. 3AI). The result shows that disruption of the interaction with the protein partner does not prevent the nuclear localization of p14 (Fig. 2), at least when p14 is over-expressed.

Fig. 2. Analysis of the p14 splicing factor mutations that disrupt protein–protein interaction with splicing factor SF3b155. (A) Schematic diagram of Trypanosoma cruzi p14 protein and the mutant proteins constructed. In p14_β3_A, 48–52 indicates the positions of five consecutive replacements by alanines. A brief summary of the interactions obtained is shown on the right. Tc155wt refers to SF3b155. AD, activation domain; RRM, RNA recognition motif. (B) Evaluation of the His3 reporter activation in L-W-H- (leu, trp, his lacking medium) plates containing 25 mM 3AT. (C) Evaluation of LacZ reporter activation by the ONPG (ortho-nitrophenyl-β-galactoside) assays. A, negative control; E, positive control. We performed the analysis for p14 in both DB and AD configurations against SF3b155; however, since SF3b155 produced low levels of auto-activation in DB the figure presents the results of p14 in DB for a clear interpretation of the results.
3.4. *p14* have a complex bipartite NLS

To identify the NLS within *p14*, we generated a series of mutant *p14* proteins fused to the C-terminus of GFP. Interestingly, the mutant protein GFP-*p14_1–91* lost its exclusive localization in the nucleus (Fig. 3AII). Since most of the C-terminal extension was deleted, this could point to the basic region RKRKR as a determinant for nuclear localization.

**Fig. 3.** Analysis of the nuclear localization signal (NLS) present in the *Trypanosoma cruzi* splicing factor *p14*. (A) Localization of GFP-*p14* mutant proteins. In each panel (I–VI), a schematic diagram of the *T. cruzi* *p14* mutant proteins is shown above and confocal microscopy images of transfected *Trypanosoma cruzi* epimastigotes is shown below. RRM, RNA recognition motif. (a) Differential interference contrast (DIC); (b) GFP detection; (c) merged images between DIC and GFP (for I and III, an additional merge with propidium iodide-stained DNA). 'All' indicates overall distribution, 'nuclear' indicates nuclear distribution. (B) Detailed amino acidic sequence of Tcp14. Secondary structure is indicated by B, β-sheet and H, α-helix. The basic amino acids present in the α-helix extension are shown in grey and underlined. Lines above the sequence indicate the precise start position of *p14_80–117* and *p14_91–117*.
To test this hypothesis, we generated a new construct to include the basic region where the last 17 amino acids from the C-terminus were deleted (p14_1–100). A fusion protein with GFP determined that this construct was localized exclusively in the nucleus (Fig. 3AIII). A comparison between p14_1–91 and p14_1–100 suggested that the inclusion of RRKRRR was necessary to re-locate the protein in the nucleus.

If this basic region composes a monopartite NLS, then its sole addition to GFP would re-locate it to the nucleus. Thus, we constructed the fusion GFP-p14_91–117 protein with the inclusion of the last 26 amino acids from the p14 C-terminus. However, the results in Fig. 3AIV clearly showed that the protein was distributed all over the cell. Integration of the analysis from I to IV in Fig. 3 suggested that RRKRRR was necessary but not sufficient to locate the protein in the nucleus.

Careful analysis of the p14 sequence showed that between the p4 of the RRM domain and the basic region RRKRRR, there are three other basic amino acids (Fig. 3B, between positions 80–90). We constructed a fusion GFP-p14_80–117 to analyze the possibility that the NLS was composed of a basic region that was larger than initially thought. However, results in Fig. 3AV, indicated that this was not the case since the protein was again distributed all over the cell.

To study more precisely the role of this basic region for p14 NLS, we generated a construct with alanine substitutions in the RRKRRR to RAAAAAR, namely p14_ANLS. This protein is exactly the same as p14_1–100, which is nuclearily localized, except for the alanine substitutions. A comparison between p14_1–100 and p14_ANLS showed that the motif RRKRRR was necessary for nuclear localization (Fig. 3AIII and VI), since p14_ANLS was distributed all over the cell.

Taken together, these results suggest that the motif RRKRRR was necessary but not sufficient to locate the protein in the nucleus and that the p14 NLS is a bipartite signal constituted by RRKRRR and another signature within the RRM domain.

Modeling of T. cruzi p14 by homology threading using mammalian p14 as a template (Schellenberg et al., 2006) clearly showed the two-domain structure of p14 present in other organisms: the RRM domain and the C-terminal α-helix C-D extension (data not shown). We suggest that the bipartite NLS of p14 could be conformational and composed of the linear motif RRKRRR and another conformational motif within the RRM, being that the two motifs are located very close in the space.

A recent article (Kuwasako et al., 2008) reports another nuclear magnetic resonance (NMR) solution structure of human p14 in complex with SF3b155 and RNA. They showed that p14 RRM, as previously known (Schellenberg et al., 2006), is in contact with SF3b155 and not with RNA. However, they discovered that p14 indeed contacts the RNA in an unusual way, using a charged groove within the RRM and a basic sequence of amino acids in the C-terminal α-helix extension (Kuwasako et al., 2008). They also suggested that this C-terminal extension acts as a NLS for human p14 although that was not demonstrated. Since the amino acids in the RRM charged groove are conserved in trypanosomes, it is tempting to speculate that the same signal involved in RNA binding could also be used as a bipartite NLS.

Interestingly, two of the three known NLS reported in trypanosomes are bipartite. One is present in the ESAG8 protein (Hoek et al., 2000) and the other in histone H2B (Marchetti et al., 2000). The ESAG8 protein contains the sequence RIGGRRKANPHLRREIADVTMELKRYRKCRGSG with the bipartite signal separated by 16 amino acids while histone H2B contains GKKSHKPRKRTWNVYNIRSLKS, a more scattered signal of basic residues. Typically, mammalian bipartite signals are composed of two sets of basic amino acids separated by approximately 10 residues. However, there are bipartite signals packed in less space, such as the one found in the LAN2A nuclear protein in herpes virus-infected B cells with the sequence RRHERPTTRRHRKRLRS, separated by only three amino acids (Muñoz-Fontela et al., 2003). Nevertheless, the construct GFP-p14_80–117 with three basic residues separated by three amino acids from the signature RRKRRR (Fig. 3B) did not accumulate in the nucleus, ruling out the possibility of such a bipartite signal.

Recently, it was reported that TcUBP1, a small RRM protein of T. cruzi, depends on the RRM domain for nuclear accumulation (Cassola and Frasch, 2009). The case of TcUBP1 is different from p14 since TcUBP1 is a cytoplasmic protein that is accumulated in the nucleus under particular circumstances, such as induced cellular stress, by arsenite (Cassola and Frasch, 2009), while p14 serves its function directly in the nucleus with no accumulation in the cytoplasm. It is interesting to note that both are small RRM containing proteins and that TcUBP1 needs the RRM in order to translocate to the nucleus. However, TcUBP1 binds RNA via its RRM domain and mutations affecting RNA binding (RNPI) also affected nuclear re-localization, suggesting that both functions are associated (Cassola and Frasch, 2009). In contrast, the p14 RRM is implicated in protein–protein interaction binding RNA in an unusual way and both functions (nuclear import and protein binding) could be dissociated (Figs. 2 and 3).

TcUBP1 lacks the basic residues signature and depends on the RRM as a non-classical NLS while we propose that p14 bipartite NLS is composed of the basic run motif and another motif within the RRM domain, constituting a novel class of NLS.

Interestingly, the RRM domain of the La nuclear protein from Saccharomyces cerevisiae was involved in nuclear import but not in its human counterpart (Rosenblum et al., 1998). This is not the case either in T. brucei where La protein presented a monopartite NLS (Marchetti et al., 2000).

There are only a handful of proteins with mapped NLS in trypanosomes (La, H2B, ESAG8, TcUBP1 and p14) from which to draw conclusions but it seems to emerge that NLS definition in trypanosomes is more complex than previously described. In higher eukaryotes, NLS is indeed complex, with a mixture of monopartite and bipartite basic residues, the M9 signal, the U snRNPs pathway and a novel NLS in the PPLase domain of Pin1 among others (Görlich, 1997; Lufei and Cao, 2009). It was postulated that a cell might need several distinct nuclear import signals to regulate import of distinct classes of substrates separately (Görlich, 1997). It would be interesting to find NLS signals/pathways which are trypanosome-specific in order to postulate those as molecular drug targets.

In fact, it would be useful to analyze whether the importin α (Tc00.1047053509057.20) and β (Tc00.1047053504105.150) homologs of trypanosomes bind any of the few NLS described to date.

We also noted that a basic signature of amino acids similar to that found in Tcp14 was found towards the C-terminal of other trypanosomes (La, H2B, ESAG8, TcUBP1 and p14) from which to draw conclusions but it seems to emerge that NLS definition in trypanosomes is more complex than previously described. In higher eukaryotes, NLS is indeed complex, with a mixture of monopartite and bipartite basic residues, the M9 signal, the U snRNPs pathway and a novel NLS in the PPLase domain of Pin1 among others (Görlich, 1997; Lufei and Cao, 2009). It was postulated that a cell might need several distinct nuclear import signals to regulate import of distinct classes of substrates separately (Görlich, 1997). It would be interesting to find NLS signals/pathways which are trypanosome-specific in order to postulate those as molecular drug targets.

In fact, it would be useful to analyze whether the importin α (Tc00.1047053509057.20) and β (Tc00.1047053504105.150) homologs of trypanosomes bind any of the few NLS described to date.

We also noted that a basic signature of amino acids similar to that found in Tcp14 was found towards the C-terminal of other T. cruzi proteins for which we have experimental evidence of nuclear accumulation such as U2AF35, U2AF65, SF1, FIP1, CPSF30, p14, Y14, SF3b155, U1–70 K (Vazquez et al., 2003, 2009; Avila et al., 2007; Bercovich et al., 2009a, b). It would be interesting to use mutational analysis in these proteins to determine whether this signature alone is sufficient for nuclear transport or if other sequences are necessary as in Tcp14.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2010.02.011.

References


