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Mapping of the protein-binding interface between splicing factors SF3b155 and p14 of *Trypanosoma cruzi*

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Abstract

SF3b155 and p14 are essential components of spliceosome core that recognize the branch point adenosine, a critical step in splicing in eukaryotes. Trypanosomes are unusual since every transcribed gene is processed by *trans*-splicing instead of *cis*-splicing. Thus, the *trans*-spliceosome emerges as an interesting anti-parasitic drug target since this process is not present in mammalian hosts. Here, we present the orthologues of these proteins in *Trypanosoma cruzi* that interact strongly with each other. To define similarities and differences with the human pair, we performed a detailed alanine scan analysis that allowed us to identify the regions and the critical amino acids of *T. cruzi* SF3b155 involved in interaction with p14. We demonstrate that the *T. cruzi* SF3b155 interface is larger and contains more complex elements than its human counterpart. Additionally, our results provide the first insights into the core of the putative mRNA processing complex of trypanosomes.

Keywords: *Trans*-splicing; Drug targets; Protein interaction

Trypanosomes are intriguing and amazing organisms in many aspects of its molecular biology [1]. The genome sequence of the three model trypanosomes (*Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania major*) were published in 2005 [2], thus, providing a major tool to the understanding of several of their unusual aspects. However, with so many different mechanisms between these parasites and its mammalian host there is still a lack of availability of effective anti-parasitic drugs or disease treatments [3].

RNA transcription in these parasites is polycistronic encompassing several dozen kilobases and they lack conventional promoters. Polycistronic pre-mRNAs are processed by two coupled reactions, *trans*-splicing and polyadenylation, and exported to the cytoplasm as monocistronic mRNAs [1,4] . 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 Splice Leader RNA (SL-RNA). Little is known about the complex and its components that carry-out the *trans*-splicing/polyadenylation reactions. However, it is important not only to dissect the basic aspects of the mechanism but also because it could provide several interesting potential anti-parasitic drug targets.

We have found several putative splicing factors in the genome of *T. cruzi* using bioinformatics analyses, including

Abbreviations: aa, amino acids; NMR, Nuclear Magnetic Resonance; RRM; RNA Recognition Motif; 3AT, 3-amino-1,2,4-triazole. Accession Nos. AY895171 for SF3b155; AY294609 for Tcp14.

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those homologous to the E (early) complex of mammalian systems (U2AF65/U2AF35/SF1). The E complex of trypanosomes presents several unusual features such as a divergent U2AF65 and the lost of interaction with its U2AF35 partner [5].

In this work, we focus our attention in the protein factors homologous to the A complex. Mammalian spliceosome assembly is dynamic and the initial recognition of the 3′ splice site by the E complex is followed by the A complex where splicing factor SF1 is replaced by a multiprotein component named SF3b [6]. This particle is coordinated by splicing factor SF3b155 that establishes protein interactions with SF3b145, SF3b130, SF3b49, SF3b14b, SF3b10 and p14 [7,9]. The function of SF3b is crucial in the recognition of the branch point adenosine in the splicing reaction. The molecular architecture of human SF3b was determined by single-particle electron cryomicroscopy at a resolution of less than 10 angstroms [8], allowing identification of p14 in the central cavity of the complex. Of particular interest was the strong interaction between SF3b155 and p14 that appeared in the A complex and persisted within the fully assembled spliceosome. Human p14 is a small RRM containing protein of 125 aa that cross-link with the branch-point adenosine [9]. Crystal structure and NMR analysis of the protein interaction interface between human SF3b155 and p14 were reported by independent groups [10,11].

Here, we found orthologues of these core spliceosomal proteins in the genome of *T. cruzi* that interact strongly with each other. To define similarities and differences with the human pair, we performed a detailed alanine scan analysis that allowed us to identify the region and the critical amino acids of *T. cruzi* SF3b155 involved in interaction with p14. Our results provide the first insights into the core of the putative mRNA processing complex of trypanosomes.

Materials and methods

*Data mining.*The protein sequences of human p14 and SF3b155 were used as probes in a TBLASTN to search of a locally licensed copy of the complete nucleotide sequence of the *T. cruzi* genome (http://www.tigr.org/tdb/e2k1/tca1/). Results were correlated with the annotations in GENEDB (http://www.genedb.org). ESTs coding for both proteins were found in the NCBI database dbEST (*T. cruzi* filtered) using the blastn algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

Cloning of TcSF3b155, Tcp14 genes in the Gateway™ system. The coding sequences of TcSF3b155 and p14 were PCR amplified from 100 ng of total genomic DNA of *T. cruzi* CL-B clone using the following primers: Tc155_up: 5′ GGATCCGATGACGGACGAAGAAAGAAGC 3′; Tc155_down: 5′ CAACTACAGAATCACCTCCAG 3′; Tcp14_up: 5′ GGATCCAATGCCGGATGAACGCATTC 3′; Tcp14_down: 5′ CTCGAGTTAGCTCTTCTCCTCAGTGA 3′. PCR products were cloned into pGEM-T Easy™ (Promega), subsequently digested with BamHI and EcoRI and subcloned in frame into the Gateway™ entry vector pENTR-2B (Invitrogen).

Construction of mutant proteins. N-terminal and C-terminal deletion mutants were generated by PCR with the specific primers (see Supplementary table S1). Internal deletion mutants and Alanine scan mutants were generated by SOEing PCR [12] using the primers listed in Supplementary tables S2 and S3. All the mutant products were Gateway™ cloned as described above.

All PCRs were performed using the proofreading AccuTaq™ enzyme (Sigma–Aldrich) and all the products were sequenced on a MegaBACE 500 (Amersham Biosciences) capillary sequencer to verify the mutations introduced.

Mapping of the interaction interface using yeast two-hybrid analysis. The ProQuest™ Yeast Two-Hybrid Gateway™ compatible System (Invitrogen) was used for the protein interaction analysis as previously described [13]. Tcp14 variants were always analyzed in DB configurations and SF3b155 variants in AD configurations.

Activation of *His3* reporter gene induced by interaction pairs was studied analyzing the ability of yeast to grow on plates lacking His (SC $L^-W^-H^-$) in the presence of 25 mM of 3AT and incubated at 30 °C for up to 48 h.

Activation of *LacZ* reporter gene was monitored by a β-galactosidase activity liquid assay. Reactions were performed in duplicates and measured at $OD_{420\ nm}$. The data was 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.

Results

Characterization of TcSF3b155 and Tcp14

Splicing factor Tcp14 is a small RRM containing protein of 117 amino acids. It shares 42% identical and 61% conserved residues compared to its human orthologue. This homology is restricted to the 77 aa long RRM domain. There is no significant sequence conservation in the C-terminal domain but both proteins present an extended alpha helix in this region (not shown).

Splicing factor SF3b155 is a large protein of 1112 aa and it is 192 aa shorter than its human counterpart. It is a modular protein with an unfolded N-terminal domain and a helical C-terminal region bearing several HEAT repeats [7,8]. TcSF3b155 shares 33% identical and 51% conserved residues compared to its human orthologue. However, the N-terminal domain of the *T. cruzi* protein is less conserved (27%) and 161 aa shorter than the same region in HsSF3b155.

Moreover, while HsSF3b155 N-terminal domain contains 22 TP dipeptide and 5 RWD repeats, TcSF3b155 contains 5 TP and 7 GGTTP repeats lacking RWD repeats (Fig. 1).

Using Proquest yeast two hybrid assays, we demonstrated that TcSF3b155 and Tcp14 interacted strongly with each other. In fact, they activated the *His3* reporter gene in plates lacking histidine with concentrations up to 75 mM 3AT (not shown) and the *LacZ* reporter gene with a strength of interaction equivalent to 80% of the strongest Proquest positive control (see E and Tc155 in Fig. 2C).

Mapping the essential domains in TcSF3b155 for interaction with Tcp14

In order to map the minimal region of TcSF3b155 involved in protein interaction with Tcp14, we generated

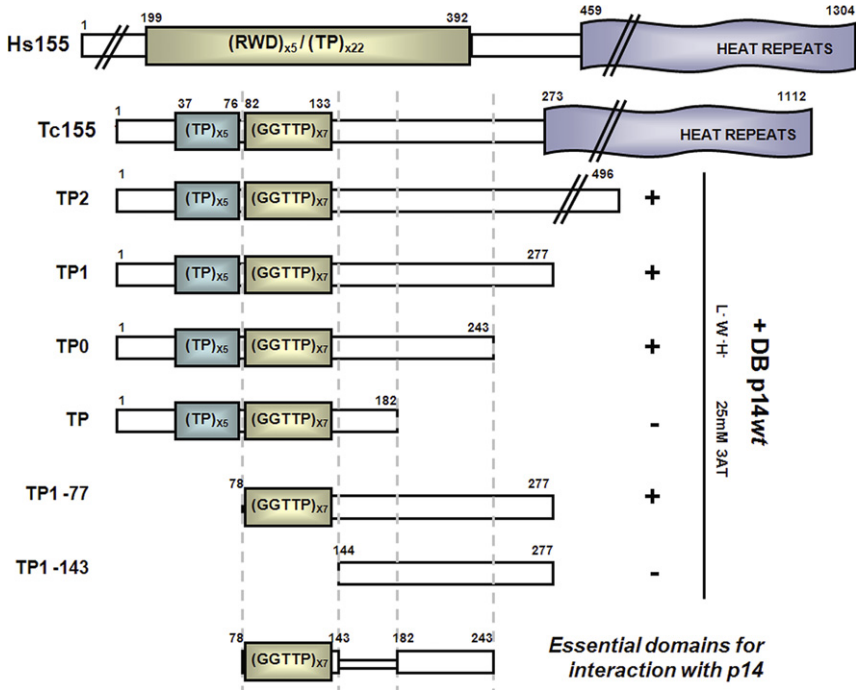

Fig. 1. Deletion mapping analysis of interacting domains in TcSF3b155. Hs155 and Tc155 refers to the wild type SF3b155 proteins of *H.sapiens* and of *T. cruzi*, respectively. TP2, TP1, TP0 and TP are the C-terminal deletion mutants generated; TP1-77 and TP1-143 are the N-terminal deletion mutants generated. A Summary of the TcSF3b155 regions essential for the interaction with Tcp14 is indicated below.

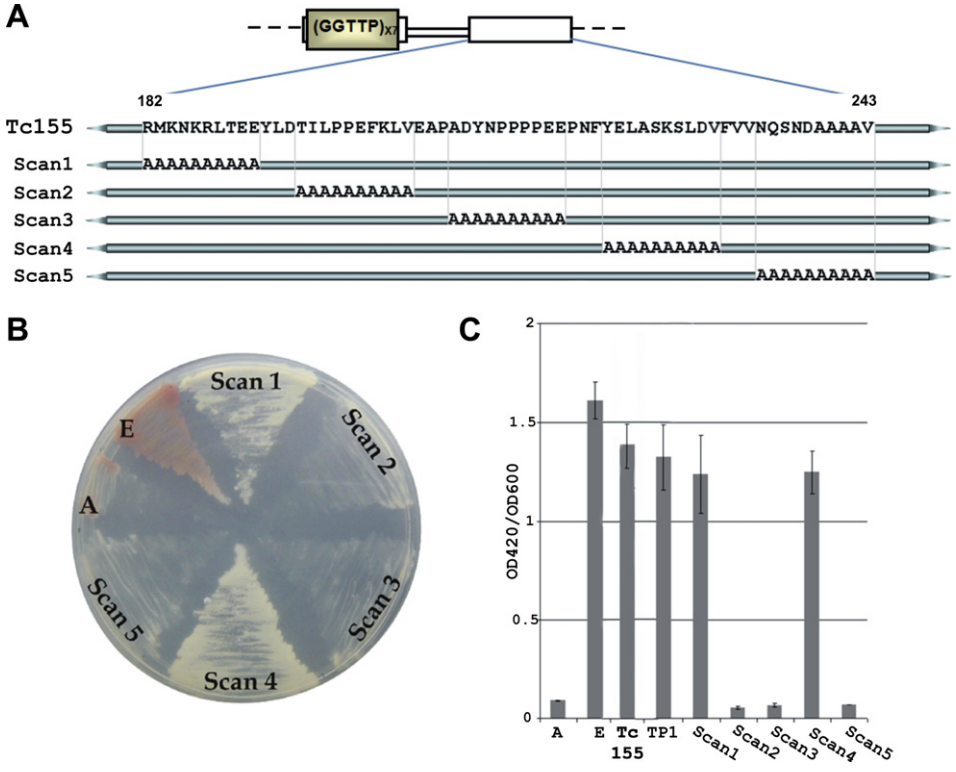

Fig. 2. Alanine Scan analysis in the 182–243 region of TcSF3b155. (A) Schematic representation of the mutants generated. Tc155, TcSF3b155 wild type sequence. The replacements in the Scans are indicated with an A. horizontal lines indicate sequence identical to the wild type. (B) Evaluation of the *His3* reporter activation. (C) Evaluation of *LacZ* reporter by the ONPG assay. Control A, negative control; control E, positive control.

several deletion mutants from the C-terminal domain named TP2, TP1, TP0 and TP (Fig. 1). This was based on the knowledge that the HEAT repeats are not involved in interaction with p14 in the human counterpart [8]. The constructs were evaluated by activation of the *His3* reporter gene in yeast two hybrid analyses. Our results showed that a region comprised between amino acids 182 and 243 was essential for interaction with Tcp14 (see constructs TP0 and TP in Fig. 1).

Then we generated deletion mutants from the N-terminal using the construct TP1 named TP1-77 and TP1-143. The results showed that another region located between amino acids 78 and 143 was also essential for interaction with Tcp14 (Fig. 1). Surprisingly, this region contained the GGTTP repeats. Our results also showed that the TP repeats were not involved in the interaction (Fig. 1).

In summary, deletion mapping indicated two regions necessary for interaction with Tcp14: 78–143 (GGTTP) and 182–243 (Fig. 1).

Scan analysis to determine critical amino acids in region 182–243

Since the region 182–243 showed no distinct hallmark in the primary sequence, we performed an alanine scan analysis to determine essential amino acids for the interaction. The scan was designed to replace ten consecutive residues separated by three residues gaps. Five scans were necessary to cover the complete region (Fig. 2A).

The scans were evaluated by the activation of two independent reporter genes, *His3* and *LacZ* (Fig. 2B and C, respectively). The results indicated that scans 1 and 4 were positive and scans 2, 3 and 5 were negative for interaction with Tcp14. Quantitative analysis of *LacZ* reporter activation showed that scans 1 and 4 did not affect the interaction strength respect to the wild type proteins while scans 2, 3 and 5 completely abolished interaction (Fig. 2C).

Interestingly, the human SF3b155 core peptide involved in binding to p14 overlapped with the end of our scan1 and the whole scan 2 (Fig. 3A). The human core peptide is a 15 aa long unfolded region that adopts alpha and beta secondary structures upon binding to p14 (Fig. 3A) [10,11]. When compared to the homologous region in the *T. cruzi* protein, five residues were identical, five were conserved and five were not conserved (Fig. 3A). In contrast, the core peptide was highly conserved across evolution from yeasts to vertebrates (Supplementary Fig. 1 online). The first two acidic residues homologous to the core peptide were covered by the end of scan 1 which did not affect interaction, thus, we focused our attention in the rest of the sequence (Fig. 3A). This 13 aa long peptide comprised the three residues gap between scans 1 and 2 and the complete scan 2. We decided to re-analyze the region in detail by a new alanine scan designed to replace two, or three, consecutive residues (Fig. 3A). Six scan constructs were generated to cover the peptide.

The scans were evaluated by activation of *His3* and *LacZ* reporter genes (Fig. 3B and C, respectively). Our results indicated that three scans were positive (DTI, PE and LV) and three were negative (YL, LP and FK) for interaction with Tcp14. Moreover, positive scans did not affect interaction strength while negative scans abolished it completely (Fig. 3C).

Interestingly, the three negative scans conserve at least one of the two residues with the human core peptide (Fig. 3A). To test if the conserved residues are relevant to establish binding to p14 in *T. cruzi*, we re-evaluate scan YL. We selected this scan in particular because the aromatic Y^{192} residue was a not conservative change. In fact, an acidic E residue was conserved at this position in human (Fig. 3A) and all organisms analyzed (Supplementary Fig. 1 online). In contrast, the L^{193} residue was highly conserved throughout evolution including *T. cruzi* (Fig. 3A and Supplementary Fig. 1 online). We generated new scans for each separate residue, named Scan2_Y192 and Scan2_L193 (Fig. 4A), which were evaluated as previously.

Surprisingly, our results showed that the not conserved Y^{192} residue was essential since its replacement by alanine abolished interaction (Fig. 4B). Instead, the scan of the highly conserved L^{193} residue was able to interact in plaques lacking histidine at 25 mM 3AT (Fig. 4B, upper panel). However, quantitative assays of *LacZ* activation indicated that the strength of interaction dropped below 30% of the wild type proteins (see TP1 and Scan2_L193 in Fig. 4B, lower panel).

These data demonstrated that the region homologous to the human SF3b155 core peptide presented interesting differences in *T. cruzi* to bind the p14 splicing factor.

Besides Scan 2, the Scan 3 was also negative for interaction with Tcp14 (Fig. 2). This region lays downstream of the region homologous to the human core peptide and is less conserved (Fig. 4A). In fact, there are only three identical and seven not conserved residues compared to the human protein. However, it was shown that a short peptide of four amino acids ($PAGY^{418–421}$) in human SF3b155 folds into alpha helix upon binding to p14 (Fig. 4A) [10]. A similar sequence was present in the *T. cruzi* protein in the region of Scan 3 ($PADY^{207–210}$) (Fig. 4A). To test if Scan 3 was negative due to interruption of this putative alpha helix, we generated a replacement of the sequence $DY^{209–210}$ to AA (Scan3_DY in Fig. 4A). The scan was evaluated by activation of *His3* and *LacZ* reporter genes (Fig. 4 C, upper and lower panels, respectively). Interestingly, the interaction with Tcp14 was completely abolished.

The Scan 5 was also negative and most important there are not conserved residues in this region compared to the human protein (Figs. 2 and 4A). Since four out of the ten scanned residues were alanines in the original sequence, we focused our attention in the charged/polar residues and we generated two new scans to analyze the dipeptides $NQ^{234–235}$ and $ND^{237–238}$ (Fig. 4A).

Surprisingly, neither scan NQ nor scan ND affected the interaction with Tcp14 (Fig. 4D). This result suggested that

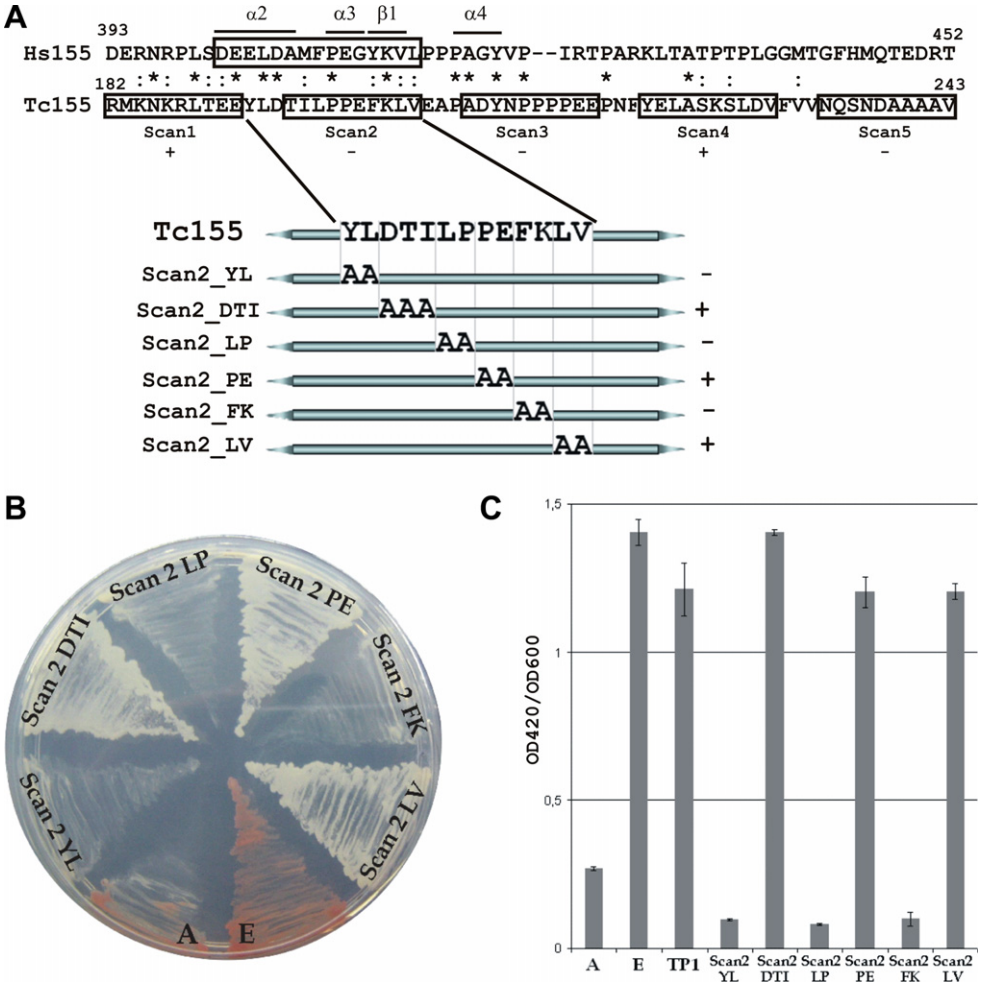

Fig. 3. Alanine scan re-analysis of the region 192–204 in TcSF3b155. (A) Alignment of the *H.sapiens* (Hs155) and *T. cruzi* (Tc155) SF3b155 between the positions indicated. Boxed sequences in Tc155 indicate scanned sequences and a summary of their interactions with Tcp14 (+/−). Boxed sequence in Hs155 indicates the core peptide for interaction with human p14. Above are indicated the secondary structure induced in the region upon binding. (*), identical amino acids; (:) conserved amino acids. Re-scan analysis schematic diagram is shown below the sequence. Replacements are indicated by A. Horizontal bars indicate identical sequence to the wild type. (B) Evaluation of the *His3* reporter activation; (C) evaluation of *LacZ* reporter by the ONPG assay. Control A, negative control; control E, positive control.

either S^{236} or V^{243} are the amino acids important for binding in this region or, alternatively, it is a structural and sequence independent related feature.

Discussion

The *trans*-spliceosome emerges as an interesting anti-parasitic drug target since this process is not present in mammalian hosts. However, it is necessary to demonstrate that the parasite *trans*- and the host *cis*-spliceosomes present substantial differences to be postulated as drug target. Little is known about the components of the *trans*-spliceosome, particularly those involved in the recognition of the 3′ splice site [4,14].

Here, we show that the genome of *T. cruzi* contains othologues for SF3b155 and p14 and that they interact strongly with each other.

The interaction interface of the human pair is comprised by a short N-terminal region of SF3b155 between amino acids 373–415 [11] which contained a core peptide that is absolutely necessary for interaction ^{401}DEELDAMF-PEGYKVL^{415} [10]. This region interacts with the RRM domain and part of the C-terminal extended alpha helix of p14, in particular with residues located in the β1 (RNP2) and the β3 (RNP1) sheets [11].

Interestingly, human SF3b155$^{373–415}$ is sufficient for full interaction with p14. In contrast, we demonstrated that a homologous region in *T. cruzi*, SF3b155$^{182–243}$, is necessary but not sufficient for interaction with Tcp14. In fact, another region located in 78–143 containing GGTTP repeats is also necessary but not sufficient for interaction. In summary, two distinct domains are involved in interaction in *T. cruzi* while a single small region is necessary in the human protein. Further work will reveal if the GGTTP repeats are a sequence or a structure dependant signature.

The TP repeats are conserved in both organisms although there are fewer in *T. cruzi* (Fig. 1). These repeats are not involved in binding to p14; instead they are

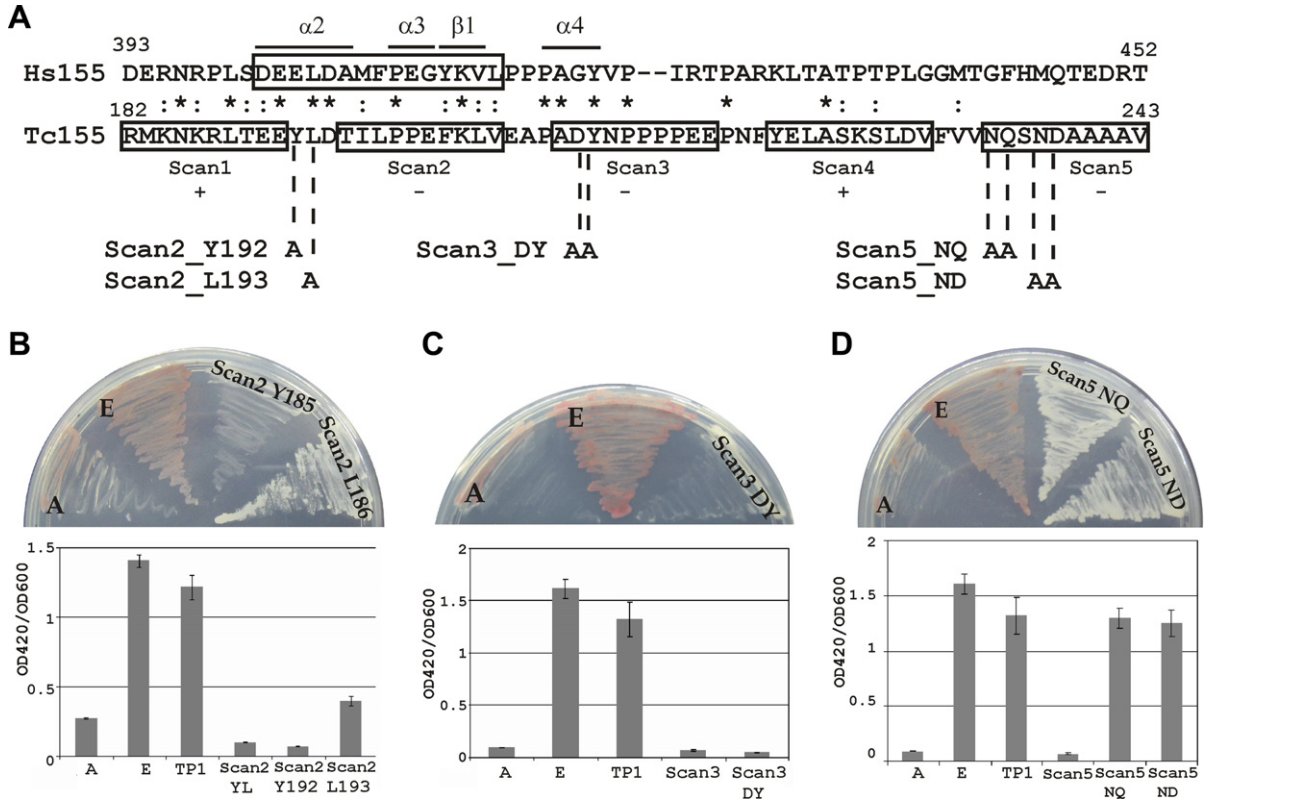

Fig. 4. Alanine scan re-analysis of the region 182–243 in TcSF3b155. (A) Sequence comparison description is the same as in Fig. 3. (B) Scan Y192 and Scan L193 analyses. (C) Scan DY analysis. (D) Scan NQ and Scan ND analyses. (Upper panels) evaluation of the *His3* reporter activation; (lower panels) evaluation of *LacZ* reporter by the ONPG assay. Control A, negative control; control E, positive control.

proposed to function as a localization signal in nuclear speckles [15]. In contrast, the human RWD repeats (Fig. 1) are absent in *T. cruzi*. The RWDs serve as binding sites for splicing factor U2AF65 [16] and we found that *T. cruzi* U2AF65 did not interact with TcSF3b155 in yeast two hybrid assays (data not shown), fact that could be explained by the absence of the RWDs.

Largest chemical shifts perturbations in NMR spectra for the human core peptide 401-415 upon binding to p14 are observed for D^{401}, A^{406}, F^{408}, E^{410}, G^{411} and L^{415} [10]. Interestingly, only two of these positions were partially conserved in *T. cruzi*, D^{401}/E^{190} and L^{415}/V^{204} (Fig. 3A). However, our alanine scan analysis showed that their replacements were not relevant for interaction (Fig. 3). In fact, our data indicated that only dipeptides $YL^{192–193}$, $LP^{197–198}$ and $FK^{201–202}$ within the *T. cruzi* homologous core peptide were important for interaction. Replacement of Y^{192} by alanine completely abolished binding to Tcp14, representing an example of a key difference in the interface of interaction between *T. cruzi* and human (Fig. 4A and Supplementary Fig. 1 online).

If the human core peptide is extended to amino acid position 421, a new alpha helix (α4) is induced upon binding to p14 and the affinity of interaction is increased two fold (Fig. 4A) [10]. Our results with Scan 3 and Scan DY point towards a similar situation in *T. cruzi* (Fig. 4). In contrast, our results with Scan 5 reveal another important difference between *T. cruzi* and human since this scan abolished interaction and there is no sequence conservation with the human protein in this region. In summary, our work reveals several important differences between the two SF3b155 interaction interfaces. In particular, the *T. cruzi* SF3b155 interface appeared to be larger and with more complex elements. On the other hand, we found no significant differences in the p14 interaction interface compared to its human counterpart. In fact, our results indicated that the Tcp14 folded RRM domain is necessary and sufficient to bind to TcSF3b155 (data not shown), probably by exposing the hydrophobic amino acids in β1 (RNP2) and β3 (RNP1) to establish contact with its protein partner [10,11].

In a recently published work, independent authors identified two different compounds, natural and semi-synthetic, inhibitors of the mammalian spliceosome that may be used as anticancer drugs. Interestingly, they demonstrated that the two compounds targeted the SF3b complex to inhibit splicing [17–19]. They also suggested that it may be possible to exploit species-specific distinctions in SF3b to adapt such compounds for treating parasitic and fungal infections [17].

We believe that our data combined with NMR spectra analysis could be a valuable tool to aid in a computer assisted drug design to exploit the differences between the *T. cruzi* and human interaction interfaces of SF3b155 and p14 [20].

Acknowledgments

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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.bbrc.2007.09.090.

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