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Biased cellular locations of tandem repeat antigens in African trypanosomes

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Abstract

Trypanosoma brucei subspecies cause African trypanosomiasis in humans and animals. These parasites possess genes encoding proteins with large tandem repeat (TR) domains as do the other trypanosomatid parasites. We have previously demonstrated that TR protein of *Leishmania infantum* and *T. cruzi* are often targets of B-cell responses. However, African trypanosomes are susceptible to antibody-mediated immunity, and it may be detrimental for the parasites to have such B-cell antigens on the cell surface. Here we show TR proteins of *T. brucei* subspecies are also antigenic: recombinant TR proteins of these parasites detect antibodies in sera from mice infected with the parasites by ELISA. Analysis of amino acid sequences revealed that, different from TR proteins of *Leishmania* species or *T. cruzi*, the presence of predicted signal peptides, trans-membrane domains and GPI anchor signals in *T. brucei* TR proteins are significantly lower than those of the whole proteome. Many of the *T. brucei* TR proteins are specific in the species or conserved only in the closely related species, as is the same case for *L. major* and *T. cruzi*. These results suggest that, despite their sharing some common characteristics, such abundance in large TR domains and immunological dominance, TR genes have evolved independently among the trypanosomatid parasites.

Keywords

trypanosomatid; tandem repeat; antigen

Introduction

The trypanosomatid is a group of flagellated protozoan parasites many of which cause diseases in humans and animals. The three major diseases caused by the trypanosomatid

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parasites are African trypanosomiasis, Chagas disease, and leishmaniasis, respectively. Although African trypanosomes (*Trypanosoma brucei*, *T. congolense* etc.), *T. cruzi* and *Leishmania* species belong to the same order and show structural similarity, the diseases caused by their infection are very different. Genome sequence projects of the trypanosomatid parasites are ongoing, and reports in 2005 regarding the genomes of three species (*T. brucei*, *T. cruzi* and *L. major*) have allowed the opportunity for systematic comparison of these parasites [1,2,3,4].

Genes encoding proteins with tandem repeat (TR) domains have been found in a wide range of organisms from prokaryotes to higher animals. Although very divergent among organisms, TR genes compose around 1–2% of the entire genes [5]. In some organisms, the TR protein family is dominated by molecules with common characteristic(s) besides the repetitiveness. For example, around 50% of TR genes in *Homo sapiens* are zinc finger proteins [5], one of the largest families of human proteins composing ~2% of the human proteome [6]. In fungi such as *Saccharomyces cerevisiae* and *Candida albicans*, most TR genes encode cell-surface proteins [5,7]. However, the functions of TR proteins could be disparate among different organisms because of diversity in their sequence patterns [5]. Interestingly, one feature appears to be shared in such divergent protein families: they are often potent B cell antigens. Many TR proteins have been identified as B-cell antigens from various organisms including protozoan parasites [8,9,10,11,12], and it is proposed that these antigens function for immune evasion [13,14,15]. In some organisms, having a variety of TR within a given protein may play an important role in generating variability in cell surface immunogens and adhesion molecules, thereby evading the immune system or enhancing pathogenicity [7,16,17]. Although the trypanosomatid parasites do not necessarily possess higher total numbers of TR proteins than other organisms, one common feature is their relative richness in proteins with TR domains that stretch over a larger number of repeats [5,18]. Through computational screening and immunological evaluations, we have recently demonstrated that proteins with large TR domains in *Leishmania infantum* and *Trypanosoma cruzi* are targets of B-cell responses [5,18,19].

Here we conducted a computational search for TR genes in *T. brucei brucei* and *T. brucei gambiense*. We then expressed selected TR proteins and examined if they are serologically relevant by analyzing the serum antibody response of trypanosome-infected mice. Finally, biochemical characterizations, including sequence analyses, were performed to identify any shared characteristics of these TR proteins, such as sequence similarity or biased cellular location predicted by the presence of signal sequence or trans-membrane domain(s). These analyses demonstrated uniqueness of TR proteins to individual species, and this may be explained by the distinct roles of antibodies in their infections.

Materials and Methods

Computational search for TR genes

DNA sequence data from *T. b. brucei* (TbruceiTreu927AnnotatedCDS_TriTrypDB-2.2.fasta1) [2] and *T. b. gambiense* (TbruceiGambienseAnnotatedCDS_TriTrypDB-2_2) [20] were obtained from TriTrypDB [21]. Tandem Repeats Finder, a program to locate and display TR in DNA sequences [22], was used to identify TR genes as previously described [5,18]. In this study, the genes were regarded as TR genes if the scores obtained from the Tandem Repeats Finder analysis were 500 or higher.

DNA/Protein sequence analyses on *T. b. brucei* TR genes

The properties of each *T. brucei* TR protein were analyzed for 1) a protein's molecular mass, isoelectric point, presence of a signal sequence, trans-membrane domain(s), or a GPI-anchor signal; 2) its known antigenicity and/or functions by Blast searches against the NCBI database using both DNA and deduced amino acid sequences; 3) evidence of protein expression reported in a previous study [23]. Characteristics such as isoelectric point and molecular mass, as well as the presence of predicted signal peptides and trans-membrane domains, were obtained from the TriTrypDB. The presence of a GPI-anchor signal was predicted using GPI-SOM [24].

Recombinant proteins preparations

Nucleotides coding a partial TR domain of Tbg972.10.12530, Tbg972.3.6010, Tbg972.10.13390, Tbg972.7.4290, Tbg972.7.3660, Tbg972.10.19140, Tbg972.7.2590, Tbg972.10.1660, Tbg972.7.4640, or Tbg972.11.17330, were synthesized by either Gene Dynamics LLC (Tigard, OR). The synthesized genes were inserted into the pET28 vector (EMD Biosciences, San Diego, CA). *E. coli* Rosetta was transfected with pET28 plasmids containing individual genes, and recombinant proteins were purified as soluble proteins using Ni-NTA agarose (Qiagen Inc., Valencia, CA) as previously described [19], or insoluble proteins from inclusion bodies under denaturing condition (8M urea) using the Ni-NTA agarose according to the manufacturer's instruction. Tc6 and TcCRA were produced in a previous study [5].

Lysate antigen preparations

The procyclic forms (PCF) of *T. b. gambiense* were cultured as previously described [25]. Crude trypanosome lysate antigens of the *in vitro* culture derived parasites were generated by sonication, using the same methods previously described for *Leishmania* parasites [26].

Antibody ELISA

Sera that had been previously collected from BALB/c mice at ~8 weeks post infection with *T. b. gambiense* IL3253 [27] were used for this study. For ELISA, 200 ng/well of individual recombinant antigens or 1 µg/well of lysate antigens were used to coat the MaxiSorp plates (Thermo Fisher Scientific Inc., Waltham, MA). Mouse sera were diluted at 1/100 and added to the plates (100 µl/well). HRP-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL) was used as to detect bound antigen-specific antibodies. The plates were developed with tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD), stopped with 1N H₃PO₄, and scanned by a microplate reader at 450 nm.

Analysis for conservation of TR genes in the trypanosomatid

Repeat domains of the top 10 TR genes from *T. b. brucei*, *T. cruzi* or *L. major* were analyzed for conservation among the trypanosomatid parasites (*T. b. brucei*, *T. b. gambiense*, *T. cruzi*, *L. infantum* and *L. major*) by conducting Blast searches using both DNA and deduced amino acid sequences against the TriTrypDB.

Results

Search of tandem repeat proteins in African trypanosomes

To identify proteins that have an over-representation of TR regions, protein coding genes (without pseudogenes) from 11 major chromosomes each of *T. b. brucei* and *T. b. gambiense* were surveyed. The 20 genes returning the highest TR scores obtained for *T. b. brucei* and *T. b. gambiense* are shown in Tables S1 and S2, respectively. When employing a

TR cutoff score of 500, 83 of the 8,898 analyzed *T. b. brucei* genes (0.93%) and 109 of the 9,824 *T. b. gambiense* genes (1.11%) were identified as containing TR regions. The identified TR genes often had large TR domains; 44 and 55 genes had the scores of 2,000 or higher in *T. b. brucei* and *T. b. gambiense*, respectively. Among the identified TR genes, at least eight genes, including GM6, MARP, 24-amino acid repeat, CRAM, I2, and I17, encoded previously characterized antigenic repeat motifs that have been evaluated for serum antibody binding [28,29,30,31]. Although the number of TR genes in each *T. brucei* subspecies was similar, differences in the copy numbers of the repeats could be observed. For example, Tbg4 was ranked by ninth in *T. b. gambiense* (Tbg972.7.2590), having a repeat domain of 15.5 copies of a 195bp motif, whereas the orthologue in *T. b. brucei* (Tb927.7.2330) had only 5.5 copies of this repeat and was therefore ranked only forty-third in the species.

Serological reactivity of African trypanosome TR proteins

To examine whether previously uncharacterized TR proteins of African trypanosomes are also antigenic, seven *T. b. gambiense* TR proteins listed in Table S1 or S2, as well as previously characterized ones, i.e., MARP, I2 and I17, were expressed as recombinant proteins. These recombinant proteins were then used to detect antibodies in sera from mice infected with *T. b. gambiense*. Crude lysates of *T. b. gambiense* parasites were used as positive controls for antibody binding, and *T. cruzi* TR proteins (Tc6 and TcCRA) were used as irrelevant, negative control antigens. The repeat motif of Tc6 or TcCRA was not predicted within any proteins of *T. brucei* subspecies. As expected, sera from mice infected with *T. b. gambiense* possessed strong antibody responses to the lysate antigen. Compared with sera from uninfected mice, sera from infected mice did demonstrate some, albeit weak, response to the irrelevant Tc6 and TcCRA antigens (Figure 1). Among the previously characterized antigens, TbgMARP detected high levels of antibodies in the infected mice, whereas TbgI2 or TbgI17 did not. All the 7 newly identified TR proteins detected statistically significantly higher levels of antibodies when compared to control protein (Tc6); results on selected antigens are shown in Figure 1.

Biased cellular location of *T. b. brucei* TR proteins

We then examined the characteristics of TR proteins in *T. b. brucei*. The TR proteome contained higher numbers of negatively charged proteins and large-sized proteins than the whole proteome. The TR domains of the 83 proteins were not dominated by certain repeat motifs; their TR motifs were variable in length of repeat unit, and no conserved sequence motifs were found to dominate the 83 proteins. Next, the presence of predicted N-terminal signal sequence, trans-membrane domain(s), or C-terminal GPI anchor signal was assessed for *T. b. brucei* proteins. As shown in Table 1, the prevalence of proteins with the indicated domains was lower in the TR proteome than in the whole proteome. There were only two TR proteins (Tb927.7.6590 and Tb927.10.7180) predicted to have both signal peptides and trans-membrane domain, and one (Tb11.02.2360) for both signal peptides and GPI-anchor signal. Tb927.10.7180 is cysteine-rich, acidic integral membrane protein precursor or CRAM, which is known to be expressed in the PCF forms [29], whereas Tb927.7.6590 and Tb11.02.2360 are hypothetical proteins..

It has previously been reported that the TR proteomes of *T. cruzi* and *L. infantum* did not under-represent proteins with N-terminal signal sequence or trans-membrane domain(s) in comparison with the corresponding whole proteomes [5,32]. Thus, the low ratios of N-terminal signal sequence, trans-membrane domain(s), or C-terminal GPI anchor signal are unique among African trypanosomes. Additional analyses of the TR proteomes of *T. b. gambiense*, *T. cruzi*, *L. infantum* and *L. major* revealed that the two *T. brucei* subspecies possess a lower prevalence of proteins with predicted N-terminal signal sequence, trans-

membrane domain(s), or C-terminal GPI anchor signal than the others trypanosomatids (Table 2).

Genus/Species specificity of TR proteins

The conservation of the top ten TR genes from *T. b. brucei*, *T. cruzi* and *L. major* was assessed among five trypanosomatid species (those listed, plus *T. b. gambiense* and *L. infantum*). Overall, many TR genes were genus- or species-specific based, and genes with similarity to a TR gene from a given species were often found only in the closely related species (Table S3). The two exceptions were genes for calpain-like cysteine peptidase and microtubule-associated protein; these genes were conserved as syntenic genes among all species evaluated while sequence similarity was still not very high. All genes for calpain-like cysteine peptidase, e.g., Tb11.57.0008 for *T. b. brucei* and LmjF27.0490 for *L. major*, had a 204 bp-long repeat motif whereas sequence identity between consensus repeat motifs of African trypanosomes and other trypanosomatid parasites was around 50% in amino acids. Also, all genes for microtubule-associated protein, e.g., Tb927.10.10360 for *T. b. brucei*, Tc00.1047053511633.79 for *T. cruzi* and LmjF27.0490 for *L. major*, had a 114 bp-long repeat motif whereas the encoded repeat motifs did not highly resemble.

Discussion

In this study, we have identified TR proteins of African trypanosomes as targets of B-cell responses. These results are consistent with our previous findings with other trypanosomatids (*L. infantum* and *T. cruzi*). We previously demonstrated that the copy number of repeats affects the antibody binding affinity [32]. Together, our data suggest that the strong antigenicity of a protein with large TR domain may be solely dependent on the repetitiveness and be less dependent on the source of the protein. Therefore, the reason why the trypanosomatid parasites are richer in proteins with large TR domains than the other organisms is of importance. A large amount of nucleotide repeats in genomic DNA sequence are found in divergent organisms, but cis-splicing can occur and thereby disrupt the translation of repeat sequences in the genome to repetitive proteins. In contrast, cis-splicing is rare in the trypanosomatids, and repeats in genome are therefore reflected in the corresponding proteins.

Our new studies also demonstrate that many of the TR proteins are genus- or species-specific, suggesting that the TR genes among the trypanosomatid parasites have evolved along independently. For *Leishmania* parasites it may be beneficial to have such strong B-cell antigens as TR proteins, because B-cells and immunoglobulins help the parasite grow efficiently in their mammalian hosts [33,34,35]. In fact, there is up-regulated expression of many *L. infantum* TR proteins in the amastigote stage, the developmental parasite stage that occurs in the mammalian host [32]. In *T. b. brucei*, TR genes may also somehow be beneficial to the parasite, because 1) the number of genes was comparable to those in the other trypanosomatid parasites and 2) the encoded TR proteins seemed to be expressed no less than the whole proteome. Interestingly, *T. b. brucei* appears to express the TR proteins inside the body as a very few possess cell surface exposure signals. In addition, even the only three TR proteins predicted for surface exposure showed minimal expression in the slender BSF parasites that are the proliferating form in the mammalian hosts. One of them, CRAM, has been characterized in a previous study as expressed specifically in PCF [29]. Taken together, no TR proteins are likely to be expressed on the cell surface of BSF. In contrast to *Leishmania*, African trypanosome BSF extracellularly parasitize in the blood and are vulnerable to antibody-mediated immunity. It is therefore understandable that African trypanosomes have evolved to limit exposure of such B-cell antigens on their cell surface at least while parasitizing in their mammalian hosts. Clonal elimination after an immune response is established to one VSG type ensures that the intracellular antigens of

lysing and dying trypanosomes are exposed richly to the immune system. However, antibodies to those intracellular antigens would not participate in clearance of live parasites. Therefore, keeping strong B-cell antigens inside the cell and exposing them to immune system only when the cell is dead may be beneficial to African trypanosomes as decoys to keep B-cells busy and away from critical antigens. Of course, it is possible that those proteins have other biochemical functions; further characterizations are required to understand the true requirement for such antigenic proteins within African trypanosome species. Together, it is intriguing to further study TR genes of the trypanosomatid as footprints of evolution required differently for individual parasites with varying conditions for parasitism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Serological reactivity of African trypanosome TR proteins

Newly identified and previously characterized TR proteins of African trypanosomes as well as lysate of *T. b. gambiense* were evaluated the reactivity by ELISA using sera from mice infected with *T. b. gambiense* (Tbg), or from naïve mice at 1:100 dilution. Mean and S.E.M. of OD values for each group are shown. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by unpaired *t*-test to Tc6.

Table 1Characteristics of *T. b. brucei* TR proteins

	All	TR	All	TR	TR/All	Chi-square
Total	8,898	83	(%)	(%)		P-value
Signal sequence (SP)	1,735	10	19.5	12.0	0.6	0.0852
Trans-membrane domain (TM)	1,862	6	20.9	7.2	0.3	0.0021
C-term GPI signal	1,161	4	13.0	4.8	0.4	0.0253
SP & TM	817	2	9.2	2.4	0.3	0.0318
SP & C-term GPI signal	374	1	4.2	1.2	0.3	0.1714

Table 2

Characteristics of TR proteins in the trypanosomatid parasites

	<i>T. b. brucei</i>	<i>T. b. gambiense</i>	<i>T. cruzi</i>	<i>L. infantum</i>	<i>L. major</i>
Total	83	109	203	57	59
Signal sequence (SP)	10 (12.0)	10 (9.2)	32 (15.8)	14 (24.6)	17 (28.8)
Trans-membrane domain (TM)	6 (7.2)	9 (8.3)	47 (23.2)	11 (19.3)	17 (28.8)
C-term GPI signal	4 (4.8)	8 (7.3)	31 (15.3)	8 (14.0)	8 (13.6)
SP & TM	2 (2.4)	4 (3.7)	25 (12.3)	8 (14.0)	15 (25.4)
SP & C-term GPI signal	1 (1.2)	0 (0.0)	15 (7.4)	5 (8.8)	7 (11.9)