RESEARCH ARTICLE

Preliminary evaluation of a *Trypanosoma brucei* FG-GAP repeat containing protein of mitochondrial localization [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract

Background: *Trypanosoma brucei*, a causative agent of African Trypanosomiasis, is known to cross the blood brain barrier during the second stage of the disease. It was previously suggested that this parasite crosses the blood brain barrier in a manner similar to that of lymphocytes. This would imply that trypanosomes possess integrins that are required to interact with adhesion molecules located on the blood brain barrier microvascular endothelial cells, as a first step in traversal. To date, no *T. brucei* integrin has been described. However, one *T. brucei* putative FG-GAP repeat containing protein (typical of integrins) encoded by the Tb927.11.720 gene, was predicted to be involved in cell-cell/cell-matrix adhesion. Therefore, this study sought to characterize a putative FG-GAP repeat containing protein (FG-GAP RCP) and to determine its cellular localization as a basis for further exploration of its potential role in cell-cell or cell-matrix adhesion.
Methods: In this study, we successfully cloned, characterized, expressed and localized this protein using antibodies we produced against its VCBS domain in *T. brucei*.
Results: Contrary to what we initially suspected, our data showed that this protein is localized to the mitochondria but not the plasma membrane. Our data showed that it contains putative calcium binding motifs within the FG-GAP repeats suggesting it could be involved in calcium signaling/binding in the mitochondrion of *T. brucei*.
Conclusion: Based on its localization we conclude that this protein is unlikely to be a trypanosomal integrin and thus that it may not be involved in traversal of the blood brain barrier. However, it could be

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involved in calcium signaling in the mitochondrion.

Keywords
Trypanosoma brucei, FG-GAP repeat, integrin, mitochondrion, calcium signaling

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Introduction

Trypanosoma brucei is a hemoflagellated extracellular protozoan parasite that causes a zoonotic disease known as African Trypanosomiasis. It comprises of three subspecies, namely, *T. b. brucei*, *T. b . rhodensiense and T. b. gambiense* with the former causing disease in animals and the latter two causing the disease in humans. In both humans and animals, the disease classically progresses in two stages; the early hemolymphatic stage (or acute phase in animals) where parasites are found in blood and lymphatic fluid, and the late stage (or chronic phase as it is referred to in animals) where trypanosomes and an elevated number of white blood cells (WBCs) are found in the brain parenchyma. *T. brucei* has been shown to invade brain parenchyma by crossing the blood brain barrier (BBB)[1]. Despite previous attempts to determine how the trypanosomes are able to cross the BBB[1–3], the underlying mechanisms still require further elucidation. In the last decade, the focus was mainly on host factors suspected to facilitate invasion of the brain parenchyma by lymphocytes and trypanosomes[4,5] leaving parasite factors largely unexplored.

In the GeneDB and TritrypDB genomic databases, *T. brucei* has six genes that encode putative FG-GAP repeat containing proteins (FG-GAP RCP). The FG-GAP (phenylalanyl-glycine and glycine-alanine-proline) is a consensus sequence usually found in adhesion molecules[6]. This repeat has been well characterized in integrins and is found within the seven blades of the beta propeller of alpha integrin subunits. In some integrins, it has been found to contain a Ca^{2+} binding motif and is involved in ligand binding[7,8]. This repeat is also found in the T-cell immunomodulatory protein that has been shown to be involved in cell-extracellular matrix adhesion in some parasites as well as in modulating the T-cell immune response[6,9]. A few intracellular proteins such as kaptin 2E4 and ITFG2 have been found to contain this repeat, whose function in these proteins is not clearly defined[8,10]. Among the six *T. brucei* FG-GAP repeat containing protein genes, only one has been found to contain more than one FG-GAP repeat and a well-defined N-terminus alpha integrin domain. This gene, annotated as Tb927.11.11720 in *T. b. brucei* or Tbg972.11.13150 in *T. b. gambiense*, is located on chromosome 11 and has a structure similar to the alpha subunit of integrins (a large predicted N-terminus extracellular domain, a short transmembrane domain and a short cytoplasmic tail). Based on the sequence and structural homology, this gene product is also referred to as a putative integrin alpha chain protein and predicted to be a membrane protein and to be involved in cell-matrix adhesion (https://www.genedb.org/#/gene/Tb927.11.11720 and https://tritrypdb.org/tritrypdb/app/record/gene/Tb927.11.11720#GOSlim). According to the GeneDB database, the expression of this protein has been mentioned in five mass spectrometry experiments but its localization is still unclear[11–15]. Three of these studies were focused on the mitochondrial or mitochondrial membrane proteomes but the Tb927.11.11720 gene product was found in either the mitochondrial or plasma membrane fraction. Thus its localization could not be established with certainty. Noteworthy is that the mitochondrial fraction can be easily contaminated with other organelle or organelle membrane proteins as well as with plasma membrane proteins[11,12]. Because this gene has properties similar to those of integrins, we hypothesized that this gene could encode a *T. brucei* integrin and aid in the traversal of the blood brain barrier. This hypothesis was also hinged on the fact that it was previously suggested by Masocha and colleagues that trypanosomes could traverse the blood brain barrier in a manner similar to lymphocytes[2] where the first step involves the use of lymphocyte integrins that interact with vascular adhesion molecules on the microvasculature[16]. In this study therefore, we sought to further characterize this putative FG-GAP repeat containing protein (FG-GAP RCP) and to determine its cellular localization as a basis for further exploration of its potential role in cell-cell or cell-matrix adhesion.

Methods

Trypanosome culture

T. b. brucei strains*: T. b. brucei* STIB920, *T. b. brucei* SmoxB4 and *T. b. brucei* Lister 427 were cultured and maintained in HMI-9[17] at 37ºC, in a 5% CO_2 humidified atmosphere unless otherwise stated. *T. b. brucei* SmoxB4 is a trypanosome cell line generated from *T. b. brucei* Lister 427[18] that expresses the T7 promoter and tet repressor was a gift from Professor Keith Gull (previously a Professor at Sir William Dunn School of Pathology, University of Oxford, England). This strain was thus cultured in the presence of 0.1µg/ml puromycin (Sigma, P8833). *T. b. brucei* STIB920 was originally isolated from a hartebeest in Tanzania in 1972[19] and was obtained from the Swiss Tropical Institute, Basel.

Cloning and Sequencing of the FG-GAP RCP gene

In order to verify the sequence of this FG-GAP RCP gene, the full length coding sequence of the Tb927.11.11720 gene as seen in the GeneDB database was cloned in pEGFP-C1 plasmid vector (NovoPro, V12024). To do this, genomic DNA was extracted from cultured *T.b.brucei* STIB920 using a genomic DNA extraction kit (DNeasy Blood and Tissue Kit, Qiagen, Cat.No/ID: 69506) and the gene amplified (using the BioRad S1000 Thermocycler) with primers designed in-house and synthesized by Sangon Biotech (Shanghai) Co.Ltd:

5’-CGTCTCGAGCTATGAATAGGTGCAATCCTATCTC-3’ (forward) and

5’-GACGAATTCCTACCTCAGCTTCAGCTCC-3’ (reverse). Amplification was done using a Pre-mix Ex-Taq HotStart Version kit (Takara, RR030A) under the cycling conditions: initial denaturation: 94°C 2min, followed by 35 cycles of 94°C for 30sec, 60°C for 30sec and 72°C for 1min, 72°C for 5min (final extension), 4°C hold at infinity and the fragment cloned between *XhoI* and *EcoRI* restriction sites so that it had an N-terminus GFP tag. The cloned sequence was confirmed by sequencing which was done by BioSune Platinum Biotechnology (Shanghai) Co. Ltd using the Sanger Sequencing method. The sequenced sample was analyzed using ClustalMAFFT, Clustal Omega, TMHMM Server v.2.0, Signal P-5.0 Server and Motif finder to demonstrate the presence of predicted

domains and structures. CAL-EF-AFi a server for predicting that predicts the presence of EF loops, was also used to determine the presence of predicted calcium binding motifs. In addition to this, the presence of glycosylations sites was determined using the NetNGlyc 1.0 Server.

Cloning and expression of two binding domains of the FG-GAP RCP gene in *E. coli*

In order to obtain antigens for the generation of antibodies against the FG-GAP RCP, the coding sequences of two different motifs/domains of the FG-GAP RCP gene, Tb927.11.11720 (i.e. FG-GAP repeat 1; 121-603bp from the start codon and the VCBS domain; 937-1671bp), were amplified from genomic DNA extracted from cultured *T.b.brucei* STIB920 using a genomic DNA extraction kit (DNeasy Blood and Tissue Kit, Qiagen, Cat.No/ID: 69506), cloned and expressed in *E. coli*. The first fragment, here in referred to as FG-GAP repeat 1, was amplified using the primers designed in- house and synthesized by Sangon Biotech (Shanghai) Co.Ltd:

5'CGTGAATTCGACTGCGTGTCACATCGCTTG 3' (forward) and

5'GACCTCGAGTCACGTCACAAACTCTTCCGGTTCGA 3' (reverse) and the second fragment, herein referred to as the VCBS region, was amplified with primers:

5'CGTGAATTCGAACTTCTTGCAGAGGCAGCTCTTGT 3' (forward) and

5'GACCTCGAGTCATGGCACGAGATTGGCTGACCA 3' (reverse). Amplification was done using a Pre-mix Ex-Taq HotStart Version kit (Takara, RR030A) under the cycling conditions: initial denaturation: 94°C 2min, followed by 35 cycles of 94°C for 30sec, 62°C for 30sec and 72°C for 1min, 72°C for 5min (final extension), 4°C hold at infinity. Each fragment was individually cloned in pGEX5X-3 plasmid vector (GE Healthcare, 28954555) downstream of the GST-tag and between *EcoRI* and *XhoI* restriction sites. Expression was done in *E.coli* BL21 cells (GE Healthcare, 27-1542-01) grown in Luria Bertnai Broth, induced using 0.2 mM IPTG (VWR Life Science Ameresco, 0487) and cells further grown in Luria Bertani Broth at 16°C for 12hrs post induction. The cultures were centrifuged and the cells lysed in Phosphate Buffered Saline (PBS) containing 1mg/ml lysozyme (Sigma, 62971), 1mM PMSF (Sigma P7626) at room temperature for 30min, followed by four freeze thaw cycles and sonication on ice (15sec, 30sec rest, 15 times, using the Scientz, JY92-IIN sonicator). To ensure proper solubility of the expressed protein, Triton X-100 (Farco Chemical Supplies, 30632-31) was added to the lysates to a final concentration of 1% and the lysates incubated at room temperature for 30min. They were then centrifuged at 10000g for 30min. The supernatants were mixed with glutathione sepharose beads (GE Healthcare, 17-5132-02) and incubated overnight at 4°C. The flow-through was removed, the beads washed with PBS and the protein eluted with Elution buffer (50mM Tris-Cl, 10mM reduced glutathione, pH 8.0). The purified protein was analyzed by SDS-PAGE followed by Western blotting using monoclonal mouse anti-GST antibodies (Upstate Biotechnology, 05-311) diluted 1:5000 in TBST and then with polyclonal goat anti-mouse IgG conjugated to horseradish peroxidase and diluted at 1:3000 (Biorad STAR207P). The blots were developed by chemiluminescence (Thermo Scientific ECL substrate, 32106) and images captured with a luminescent image analyzer (Image Quant LAS 4000 Mini, GE Healthcare). The purified and confirmed FG-GAP repeat 1 and VCBS domain expression products were stored at -80ºC till required for immunization of rabbits.

Production of anti-FG-GAP repeat 1 and anti-VCBS domain antibodies

In-order to produce antibodies against two domains/motifs of the FG-GAP RCP (i.e. the FG-GAP repeat 1 and the VCBS domain), three female, six months old New Zealand white rabbits were used for immunization. The rabbits were kept in the Shanghai Institute of Biochemistry and Cell Biology Laboratory Animal facility. Each rabbit was kept in its own stainless steel specific pathogen free (SPF) cage, at 25°C, with a twelve hour light/dark cycle, and fed with Rabbit pellets and sterile double distilled water *ad-libitum*. To minimize the use of many animals and since the purpose was to produce antibodies, three rabbits were used for this study. The three Rs were considered when determining the number of animals to use[20]. Prior to immunization, pre-immune serum was collected from each rabbit. Two rabbits were each immunized subcutaneously with 300µg of FG-GAP or VCBS expression products respectively contained in 500µl of PBS and emulsified in an equal volume of Freund's complete adjuvant (Sigma, F5881). The third rabbit was similarly immunized with 500µl PBS emulsified in an equal volume of Freund's complete adjuvant. All animals were boosted with half of the respective dose emulsified in Freund's incomplete adjuvant (Sigma, F5506) at day 21 and 35 respectively. Immune serum was collected on days 14, and 39 (taking the first immunization to be day zero) and analyzed by Western blotting as described in the next paragraph. All procedures were done in the afternoons and during all the procedures care was taken to ensure that the rabbits were calm and suffered minimum harm, this was done by gently patting the rabbits before immunization and bleeding. All procedures were done by trained personnel experienced in bleeding and immunizing rabbits.

For Western blotting, the expressed and purified FG-GAP repeat 1 and VCBS motif/domain were run on a 10% Separating SDS-PAGE gel and then electrophoretically transferred onto a piece of Nitrocellulose membrane (BioRad; 1620115) using the BioRad Transfer apparatus (BioRad, 1703930). After the transfer, the membrane was briefly stained with 0.1% (w/v) PonceauS (Sigma, P7170) in 1% (v/v) Acetic Acid to confirm that the proteins had been successfully transferred on to the nitrocellulose membrane and the membrane cut into four pieces so that two pieces each contained the FG-GAP repeat 1 and the other two each contained the VCBS domain. The membranes were washed with double distilled water to remove the PonceauS and then blocked with 5% skimmed milk in Tris-buffered Saline containing 0.05% Tween 20 (Sigma; P1379), (TBST) for 1hr.

After this, the membranes were incubated overnight at 4°C in either rabbit anti-FG-GAP serum, rabbit anti-VCBS or rabbit pre-immune serum each diluted at a dilution of 1:1000 in PBS. The rabbit anti-FG-GAP serum, rabbit anti-VCBS serum and pre-immune serum were poured off and the membranes washed with TBST for 5 minutes with shaking, three times. The membranes were then incubated in goat anti-rabbit IgG conjugated to horseradish peroxidase (BioRad, #1662408EDU), diluted at 1:3000 in PBS for 1hr at room temperature. The goat anti-rabbit IgG was poured off and the membranes washed with TBST, five times, 5min per wash with shaking. The membrane was then developed with chemiluminescence (Thermo Scientific ECL substrate, 32106) and images captured with a luminescent image analyzer (Image Quant LAS 4000 Mini, GE Healthcare). IgG antibodies were then purified from the serum collected after the second boost using Protein G Sepharose (GE Healthcare, 17-0618-05) according to the manufacturer's instructions. To obtain fragment specific antibodies, purified IgG was incubated with pure GST protein and glutathione beads overnight at 4°C to remove GST specific antibodies. To obtain the pure GST protein, *E.coli* BL21 cells were transformed with an empty pGEX5X-3 plasmid and expression induced using 1mM IPTG. The GST protein was purified from the lysed *E.coli* BL21 cells using Glutathione Sepharose beads following the same protocol used for purifying the FG-GAP repeat 1 and VCBS domain. The beads-GST-IgG complex was pelleted by centrifugation and the supernatant containing fragment specific IgG collected, quantified using the Bio-Rad Protein Assay using the Bio-Rad Dye (BioRad; 5000006) following the manufacturer's instructions and then stored at -20°C till needed for further experiments.

Over-expression of the FG-GAP RCP in *T. brucei*

Preparation of DNA constructs. The DNA construct was prepared using the pHD1700 (which was graciously provided by Dr. Julius Mulindwa, Department of Biochemistry, College of Natural Sciences, Makerere University) expression vector which allows tet-inducible ectopic expression of a protein with a C-terminal myc-tag. For expression, the coding region of the full length FG-GAP RCP gene (Tb927.11.11720) was amplified from genomic DNA using primers designed in-house and synthesized by Sangon Biotech (Shanghai) Co.Ltd:

5'-CCTAAGCTTCGATGAATAGGTGCAATCCTATCTC-3' (forward) and 5'-TATACGCGTCCTCAGCTTCAGCTCC-3' (reverse) and cloned in pHD1700 vector between HindIII and MluI restriction sites. The plasmid was amplified in DH5α E. coli cells (Invitrogen 18265017) and the cloned fragment confirmed by sequencing where sequencing was done by the BioSune Platinum Biotechnology (Shanghai) Co. Ltd using the Sanger Sequencing method.

Parasite transfection. Bloodstream form trypanosomes ($3x10^7$) were centrifuged at 800g for 10min. They were re-suspended in 100µl of Human T cell solution from the Lonza transfection kit (VPA-1002, Lonza), mixed with 10µg of recombinant vector pHD1700 DNA and electroporated once with the AmaxaNucleofectar using program X-001. Parasites were then re-suspended in 20ml of HMI-9 medium, two 10-fold serial dilutions made from this in HMI-9 medium and one millimeter aliquots of these dilutions dispensed in 48 well plates. They were incubated in a CO_2 incubator at 37ºC for about 6hrs and stable transformants selected by adding a final concentration of 5µg/ml hygromycin B (Invivogen, ant-hg-1). Expression was induced with 1µg/ml tetracycline (Sigma, 87128). The expressed protein was analyzed by Western blotting and Immunofluorescence as described in section below.

Immunolocalization of the putative FG-GAP RCP in the trypanosome

Immunolocalization was done by subcellular fractionation followed by Western blotting or with *in situ* immunofluorescence.

Subcellular fractionation. Different trypanosome protein fractions were prepared as previously described by 21. Briefly, parasites ($9x10^7$) from culture were centrifuged at 800g for 10min and the pellet washed twice in excess PBS. Cells were then re-suspended in PEME (10mM PIPES, 2mMEGTA, 0.1mM EDTA and 1mM MgSO4, pH 6.9) containing 1%NonIdet P40 (Sangon Biotech, CAS 09-0100) and 1x Complete protease inhibitor cocktail (Roche, C762Q78) and incubated on ice for 10min. An aliquot of this lysate (total lysate) was removed and mixed with SDS sample buffer and the rest centrifuged at 15000g for 15min at 4°C to obtain the pellet (cytoskeleton fraction) and supernatant (cytosolic fraction). Cytoskeleton fraction was further extracted by resuspending in PEME containing 1M NaCl, 200µg/ml DNaseI (Sigma; 5025) and 50µg/ml RNaseA (Sigma, R6513) and incubating on ice for 15min followed by centrifugation at 15000g, 15min at 4°C. An aliquot of the supernatant (cytoskeleton fraction) or pellet (flagella fraction) were mixed with an equal volume of SDS sample buffer and analyzed by Western blotting together with the rest of the aliquots. For Western blotting, trypanosome whole cell lysates and the different fractions of the protein were run on a 10% separating gel and then transferred onto nitrocellulose membranes. After determining that the transfer was successful, the blots were cut in a predefined manner. They were then blocked by incubating in 5% skimmed milk in PBS for 1hr. The membranes were then incubated in either pre-immune IgG, purified polyclonal rabbit anti-VCBS domain IgG (produced in house as described above) diluted 1:10,000 in PBS or monoclonal mouse anti-alpha tubulin diluted 1:1000 (Sigma; T9026) for 2hrs at room temperature. The primary antibodies were poured off and the membranes washed with TBST three times, with shaking, 5min per wash. The membranes were then incubated in with either polyclonal goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad, #1662408EDU) or polyclonal goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad, STAR207P) each diluted 1: 3000 in PBS. The blots were washed five times with shaking, 5min per wash and developed by chemiluminescence (Thermo Scientific ECL substrate, 32106) and images captured with a luminescent image analyzer (Image Quant LAS 4000 Mini, GE Healthcare).

Immunofluorescence. Using immunofluorescence, both the native and over-expressed proteins were immunolocalized. Trypanosomes ($2x10^6$) were centrifuged at 1000g for 10min.

To label the mitochondria, trypanosomes were re-suspended in HMI-9 medium containing mitotracker (Molecular probes, M7512 diluted at 1:10000) and incubated at 37°C, in a CO_2 incubator for 10min. They were centrifuged, washed once in PBS, the resulting pellet re-suspended in PBS and 40µl of this allowed to adhere to cover-slips each pre-coated with poly-l-lysine (Sigma, P8920) during a 20min incubation. The adhered trypanosomes were fixed with ice cold 4% Paraformaldehyde in PBS for 10min at 4°C. They were washed thrice in PBS and blocking done in 5% FBS in PBS for 1hr at room temperature. The slides were washed 3times in PBS-Tween20 (PBST) and incubated in for 1hr at room temperature in 1µg/ml of the purified polyclonal rabbit anti-VCBS domain IgG (produced in house as described) or monoclonal mouse anti-alpha tubulin diluted 1:1000 (Sigma; T9026) or monoclonal mouse anti-myc-tag (Merck Millipore, 05-724) diluted 1:100. They were washed and incubated in with Alexa Fluor 488 polyclonal donkey anti-mouse IgG (Invitrogen Molecular Probes, A21202), Alexa Fluor 647 polyclonal donkey anti-rabbit IgG (Invitrogen Molecular Probes, A31573), and DAPI (Invitrogen Molecular Probes, D1306) all diluted 1:1000 in PBS. They were washed as described and mounted with fluorescence mounting medium (DAKO, S3023) and analyzed with a Confocal microscope (Nikon A1).

Ethical considerations

All animal procedures were approved by the Institutional Animal Care and Use Committee of Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Approval number: SIBCB-S106-16-028). All experiments were performed in accordance with the relevant guidelines and regulations. Care was taken to ensure that the rabbits were calm during the bleeding and immunization processes. After the immunization study the animals were euthanized using a carbon dioxide chamber According to the Institutional Animal Care and Use Committee Guidebook (IACUC, 2nd edition, 2010) euthanasia using carbon dioxide is an acceptable method for humanly sacrificing rabbits. Carbon dioxide may cause some distress to the rabbits but because animals die fast when exposed to optimum concentrations of carbon dioxide it is argued that the animals experience minimum pain in the process of death making it an acceptable method for euthanasia.

Results and discussion

Sequencing and bioinformatics analysis of the *T. brucei* FG-GAP RCP gene (Tb927.11.11720) revealed predicted calcium binding motifs not previously reported

In this study, the coding sequence of the *T. brucei* FG-GAP RCP (Tb927.11.11720), located chromosome 11 as indicated in TritrypDB and GeneDB databases was identified. It was cloned into a mammalian expression vector, sequenced and detailed sequence and bioinformatics analysis carried out. Our sequencing data confirmed that this protein contains 622 amino acids (aa) (Figure 1). Using SignalP and TMHMM analysis we also confirmed that the FG-GAP RCP contains a long extracellular domain, a short trans-membrane domain and a short cytoplasmic tail. Using Motif finder this protein was predicted to contain two major FG-GAP repeats that are within the extracellular domain (one between aa residues 98–118 and the other between 267-280aa) and one major VCBS (i.e. *Vibrio*, *Colwellia*, *Bradyrhizhobium*, *Shewanella*) domain between 370-440aa (Figure 1). The VCBS domain is a type of domain found in many bacteria and known to be involved in cell adhesion[22]. Further sequence analysis using CAL-EF-AFi a server that predicts the presence of EF loops, revealed the presence of the DXXXD motif in the FG-GAP repeat 1 and FG-GAP repeat 2 as well as the DXDXDG motif in the VCBS domain. The DXXXD motif in addition to being found in EF-loops is known to coordinate Mg^{2+} binding[23] while the DXDXDG is a calcium binding motif present in the canonical EF loops and is found in a number of proteins involved in calcium binding[24,25]. In addition to containing metal ion binding sites, further analysis using the NetNGlyc software showed that the *T. brucei* FG-GAP RCP contains five N-Glycosylation sites located at positions 28, 125, 168, 295 and 397. The presence of predicted FG-GAP repeats, metal binding motifs as well as N-Glycosylation sites strongly suggests that this protein may have adhesion and ligand binding functions[7,8,26].

The generated anti-FG-GAP repeat 1 and anti-VCBS specifically recognized full length FG-GAP protein

The specificity of the antibodies generated against the FG-GAP repeat 1 and the VCBS domains of the FG-GAP RCP was determined by probing them against the full length FG-GAP RCP expressed in the mammalian expression system (i.e. HEK 293 T cells). The mammalian expression system, being a eukaryotic expression system, ensures proper folding and post-translational modifications of the protein. Thus, antibodies are expected to bind to conformational epitopes similar to the native protein. Analysis was done using an immunofluorescence assay. Both the anti-FG-GAP repeat 1 and anti-VCBS antibodies specifically recognized the target protein (Figure 2).

The *T. brucei* FG-GAP RCP is located in the trypanosome mitochondrion

According to GeneDB and TritrypDB genomic databases the *T.brucei* FG-GAP RCP; Tb927.11.11720 was predicted to be an integral membrane protein. However, it was not clear which membrane it was integral to. In this study, anti-VCBS specific antibodies were used to determine the localization the FG-GAP RCP in the trypanosome.

For localization using Western blotting, the whole cell lysate, detergent soluble, cytoskeleton and flagella fractions were prepared. A band of about 65kDa that was recognized by immune IgG, but not pre-immune IgG, was detected in both the whole cell lysate (Figure 3A) and the detergent soluble fraction (Figure 3B). Since this band was not recognized by the pre-immune IgG it was taken to be specific to the immune IgG, and therefore belonging to the FG-GAP RCP despite migrating at a slightly lower molecular weight than expected. However, membrane proteins can migrate not as expected on SDS-PAGE gel since they are capable of binding either more or less SDS, resulting in faster or slower

Figure 1. Deduced sequence of FG-GAP RCP (Tb927.11.11720) and its identifiable motifs. The FG-GAP RCP gene was amplified, sequenced and analyzed and the following motifs: FG-GAP repeats (red box), VCBS domain (brown box), calcium binding sights (dark green highlights) and N-Glycosylation sites (yellow highlights) were detected. The signal peptides, trans-membrane domains and cytoplasmic tails are shown in red, green and blue colored letters and the different amino acid in *T.b.brucei* TREU927 is shown by a black box. Motifs and domains were generated using Motif finder, CAL-EF-AFi, NetNGlyc 1.0 Server, Signal P-5.0 Server and TMHMM Server v.2.0 servers or prediction methods respectively.

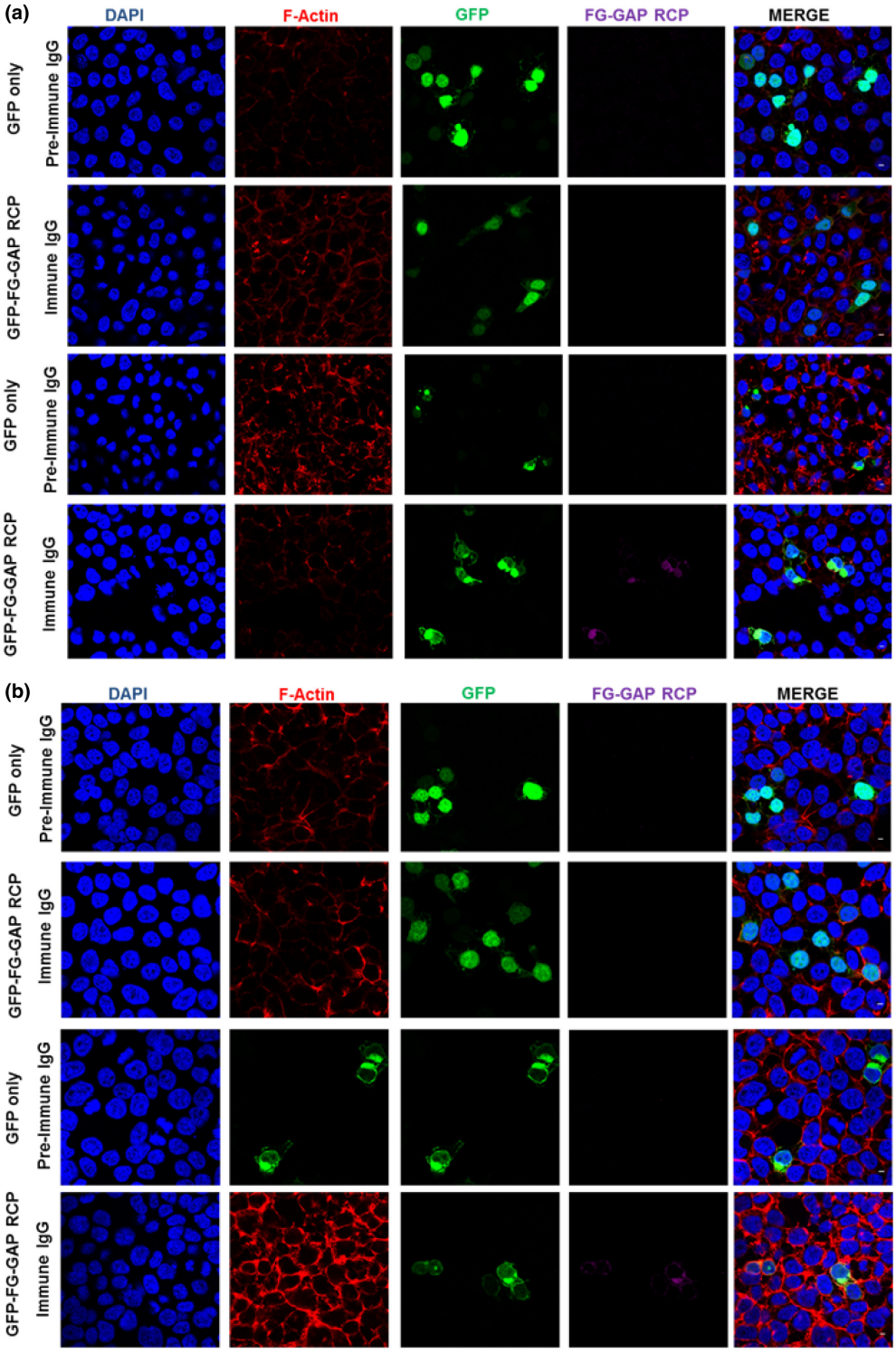

Figure 2. Immunofluorescence analysis of specificity of anti-FG-GAP repeat and anti-VCBS antibodies. The full length *T.b.brucei* FG-GAP RCP was expressed fused to GFP in HEK 293T cells. This was then used to analyze the IgG produced against the FG-GAP repeat 1 and the VCBS domains. After expression, the HEK 293T cells were probed with DAPI (lane DAPI), F-actin (lane F-ACTIN), and anti-FG-GAP repeat antibodies (**Panel A**) or anti- VCBS antibodies (**Panel B**) and the respective secondary antibodies. In both panels, the green fluorescence is GFP. Bar is 5μm. Please note in Figure 2 the brightness was increased so as to improve the visibility of the FG-GAP signal (magenta).

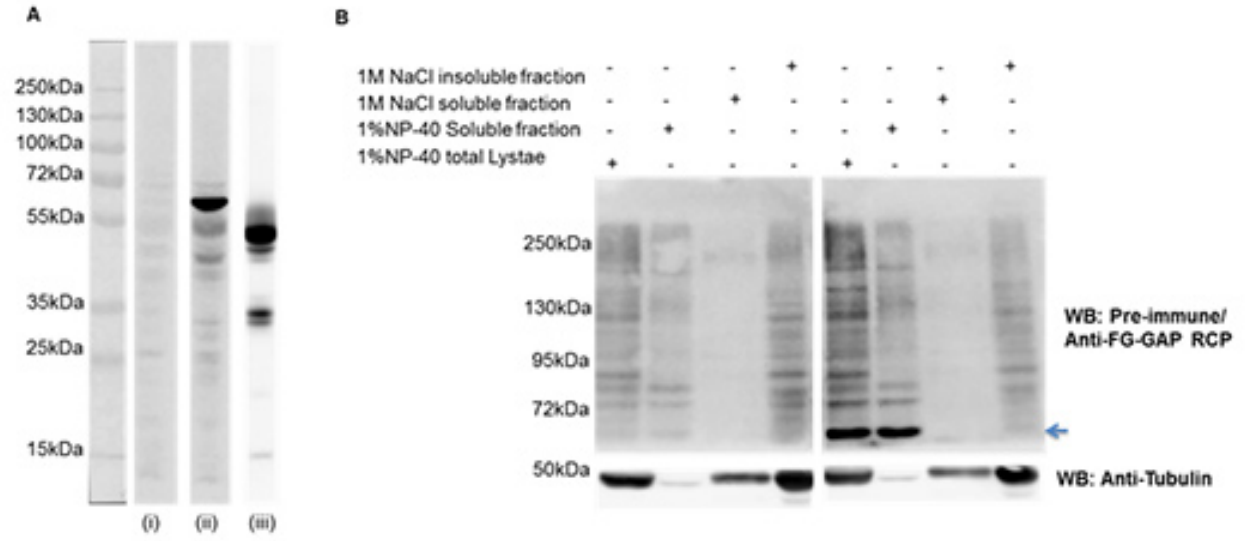

Figure 3. Western blot analysis of the subcellular fractions of the FG-GAP RCP. Whole cell lysates (**Panel A**) were separated on SDS-PAGE gel, transferred to nitrocellulose membrane and probed with Pre-immune IgG (lane i), anti- anti-VCBS IgG (lane ii) or mouse anti-α tubulin antibodies (lane iii). Then different trypanosome subcellular fractions were prepared using the different buffers indicated (**Panel B**), similarly separated, transferred to nitrocellulose membrane and probed with pre-immune IgG (sub-panel *Pre-immune*) or anti- VCBS IgG (sub-panel *Immune*) or with anti-α tubulin antibody (sub-panel, anti-α tubulin) which served as control for proper preparation and preservation subcellular fraction contents. Note that a band between 55 and 72 kDa was specifically recognized by the immune IgG in the whole cell lysates and in the 1% NP-40 fraction.

migration on an SDS-PAGE gel[27,28]. This could thus explain the anomaly in the exhibited molecular weight.

It is well known that when trypanosomes are lysed in the presence of a detergent and in the absence of PMSF, most plasma membrane proteins go into solution[29]. In our experiment, the protein was solubilized in the detergent fraction, suggesting that it could be located on the plasma membrane. The immunofluorescence data however, showed that this protein is instead located in the mitochondrion (Figure 4). Considering the fact that this protein has a predicted transmembrane domain, it is most likely located on the mitochondrial membranes. The presence of five N-glycosylation sites and the fact that N-glycosylation sites have been shown to target proteins to the apical membranes[30], further increases the likelihood of this protein's membrane localization within the mitochondria.

It was previously suggested that *T. brucei* may cross the blood brain barrier in a manner similar to lymphocytes[2]. The first barrier the lymphocytes encounter as they cross the blood brain barrier is the endothelium which they cross by using their integrins to interact with adhesion molecules such as ICAM-1 and VCAM-1 located on the endothelium[16,31]. It was demonstrated that although the cysteine protease (brucipain) may facilitate *T. brucei*'s crossing of the blood brain barrier, proximity and the ability of trypanosomes to attach to the endothelial cells is important[32]. Because of the presence of a predicted an N-terminal alpha chain and a transmembrane domain, we initially suspected this protein to be localized on the plasma membrane and hypothesized that it may have a role similar to that of cell surface integrins in other eukaryotic cells. Detailed sequence analysis showed that the *T. brucei* FG-GAP RCP shares only 20% identity with the extracellular domains of human alpha integrin subunits and only 33% identity with the cytoplasmic tail of one alpha integrin, ITGA6. The latter is known to bind to cell matrix components such as laminin, vascular adhesion molecule-1 and collagen to influence cell proliferation, differentiation, and migration[33]. Since the *T. brucei* FG-GAP RCP has been localized to the mitochondrion in this study, it is unlikely to have the same role as ITGA6. It could however be involved in other signaling roles initiated by the mitochondria.

Mitochondria play three vital roles in vertebrate cells. They are involved in bioenergetics, biosynthesis and signaling[34]. Though *T. b. brucei* bloodstream forms (BSFs) do not depend on oxidative phosphorylation, their mitochondria have been shown to be essential in other aspects. In fact one such mitochondrial protein, the mitochondrial calcium uniporter (MCU), previously thought to be essential only for oxidative phosphorylation, has recently been shown to be important for both BSFs and procyclic forms (PCFs) survival *in vitro* and *in vivo*[35] as well as for exponential growth of *Trypanosoma cruzi*and its growth in low glucose medium[36]. Another protein, the ATP synthase whose function is to generate ATP through oxidative phosphorylation in PCFs, is responsible for maintaining mitochondrial membrane potential in BSF[37,38] which is essential for transport of ions and nutrients. In this study, through sequencing and bioinformatics analysis, we have shown that the *T. brucei* FG-GAP RCP indeed contains FG-GAP repeats and in addition VCBS domains, both of which have a role in ligand binding. We also show that within the FG-GAP repeats are predicted calcium binding motifs. This seems to suggest that it could be involved in calcium signaling or a protein whose activity is regulated by

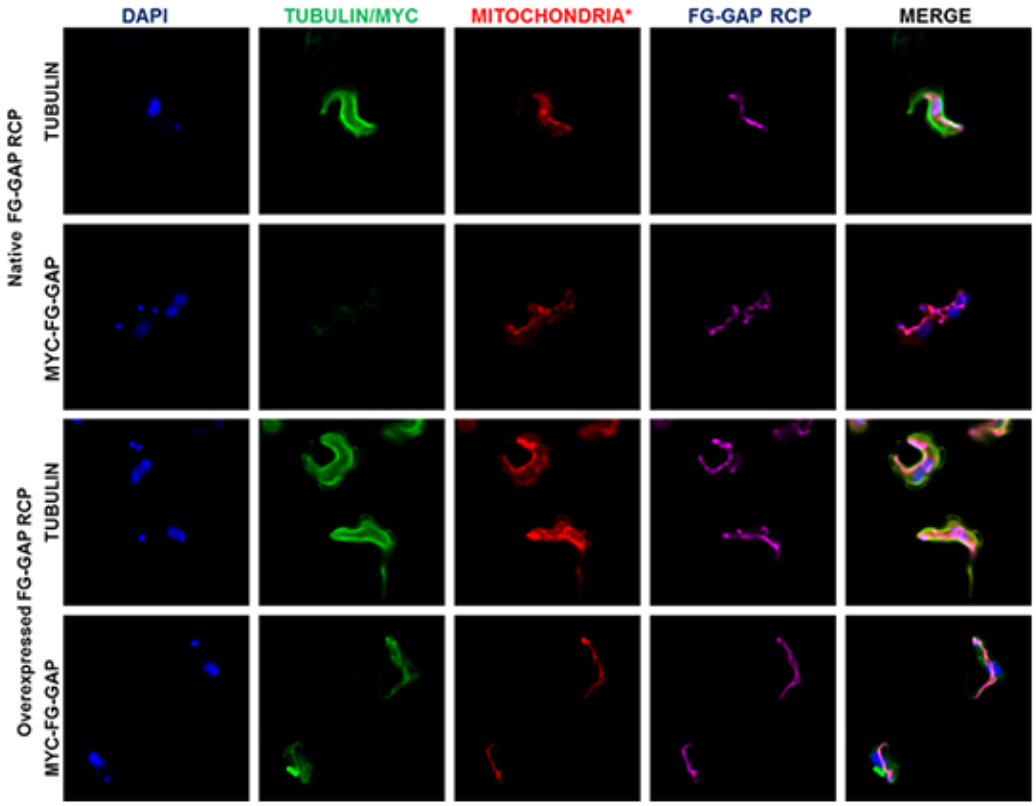

Figure 4. Immunofluorescence localization of the *T.b. brucei* FG-GAP RCP. The FG-GAP RCP, both native (sub panel tubulin) or over expressed (sub panel MYC-FG-GAP RCP) was localized by immunofluorescence using anti-VCBS IgG (lane **FG-GAPRCP**). The mitochondria were detected by pre-staining of the trypanosomes with mitotracker (lane **MITOCHONDRIA**) and probing with anti-alpha tubulin antibodies marked the morphology outline (lane **TUBULIN/MYC**) of the trypanosome, whereas the DAPI located the nucleus (lane **DAPI**). The over expressed protein was detected by probing with anti-myc tag antibodies (lane **TUBULIN/MYC**; sub panel **MYC-FG-GAP RCP**). Note that both the native and over-expressed staining co-localized with the mitotracker staining (mitochondria) but not the tubulin (outline) staining, thus suggesting that the FG-GAP RCP is located in the mitochondria. Bar 5μm.

binding to calcium, or even a calcium transporter. Some ligand binding proteins have been shown to be regulated by binding to Ca^{2+}[8,39,40]. Indeed the mitochondria are known to be involved in calcium signaling[41,42]; it therefore would not be surprising to find a protein with calcium binding motifs as here described.

Conclusion

Our data for the first time confirm the localization of this protein in mitochondria and not the plasma membrane. Using *in silico* analysis we show that the presence of predicted calcium binding motifs present within adhesion motifs/domain suggesting that this protein could be involved in some sort of adhesion and calcium binding or signaling. Based on our data it seems unlikely that this protein is a cell-cell or cell-matrix adhesion molecule and thus suggest that it may not act as a 'trypanosome integrin' that facilitates the crossing the blood brain barrier.

Data availability

Underlying data

T.b.brucei STIB920 FG-GAP RCP, GenBank Accession number: MN266897

Figshare: Preliminary evaluation of a Trypanosoma brucei FG-GAP repeat containing protein of mitochondrial localization; raw un-cropped immunofluorescence images for Figure 2A, https://doi.org/10.6084/m9.figshare.9248906.v1[43].

Figshare: Preliminary evaluation of a Trypanosoma brucei FG-GAP repeat containing protein of mitochondrial localization; raw un-cropped immunofluorescence images for Figure 2B, https://doi.org/10.6084/m9.figshare.9249014.v1[44].

Figshare: Preliminary evaluation of a Trypanosoma brucei FG-GAP repeat containing protein of mitochondrial localization; raw un-cropped immunofluorescence images for Figure 3 https://doi.org/10.6084/m9.figshare.9249764.v1[45].

Figshare: Preliminary evaluation of a Trypanosoma brucei FG-GAP repeat containing protein of mitochondrial localization; raw un-cropped immunofluorescence images for Figure 4 https://doi.org/10.6084/m9.figshare.9250037.v1[46].

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Open Peer Review

Current Peer Review Status: ✔ ?

Version 1

Reviewer Report 10 February 2020

https://doi.org/10.21956/aasopenres.14068.r27360

?

Torsten Ochsenreiter
Institute of Cell Biology, University of Bern, Bern, Switzerland

The authors report on the bioinformatics characterization of Tb927.11.720 that is annotated as a putative FG-GAP repeat protein. Furthermore, they tag the gene and raise antibodies against domains of the protein and finally show localization to the mitochondrion.

Overall the manuscript is well written. There is an excess of materials and methods some of which could be incorporated in the results section, which is very short.

Major concern - specificity of the antibodies:

- The authors could strongly improve the antibody results if they provide more evidence for the specificity, especially since they claim a novel localization that is different from the one reported by the TrypTag consortium. To be clear it is totally believable that the protein has a mitochondrial localization but the evidence could be much stronger.

- Since the authors have created a C-terminally tagged version of the protein in T. brucei, I suggest to verify their antibodies with this tagged version by western blotting and immunofluorescence microscopy.

- Alternatively, or in addition a depletion of the Tb927.11.720 by RNAi and the concomitant loss of a western blot signal would also be a very strong indication for specificity.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Parasitology, mitochondrial cell biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 20 January 2020

https://doi.org/10.21956/aasopenres.14068.r27338

Vyacheslav Yurchenko
Life Science Research Centre, Faculty of Science, University of Ostrava, Ostrava, Czech Republic

The work of Namyanja *et al.* is technically sound despite the fact that it presents findings which do not support the initial hypothesis.

Major concerns:

1. Please re-examine the genomic data/annotation of the T. brucei Tb927.11.11720 for putative mitochondrial localisation signal.

2. Please discuss available data on Tb927.11.11720 expression/RNA level (from the TriTrypDB).

3. Is it possible to visualise endogenous protein product of Tb927.11.11720 using the antibodies produced in this work?

4. Elaborate more on additional five FG-GAP-containing proteins.

Minor concerns:

1. Provide more general references on the biology of Trypanosomatide.

2. Unify style: sometimes enzyme names are in Italics, sometimes they are not.

3. Unify the style of the references: sometimes words are capitalised, but not in all references.

4. The Materials and Methods section is too long and can be shortened. Please delete trivial details.

5. Do not write catalogue numbers.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: molecular biology of trypanosomatids

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.