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Characterization of the *Trypanosoma brucei* homologue of a *Trypanosoma cruzi* flagellum-adhesion glycoprotein[1]

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Abstract

An immunodominant 72-kDa surface glycoprotein (Gp72) of *Trypanosoma cruzi* is involved in adhesion of the flagellum to the cell body (Cooper, R, Ribeiro de Jesus, A and Cross, G.A.M (1993) J. Cell Biol. 122, 149–156). We have characterized a gene, flagellum-adhesion glycoprotein gene1 (*fla1*), from *Trypanosoma brucei* that encodes a 546 amino-acid protein (Fla1) with high similarity to Gp72. Their sequence similarity and cellular localization suggest that Fla1 and Gp72 have similar functions. We could disrupt individual *fla1* alleles but not both, suggesting that *fla1* is essential in *T. brucei*, in contrast to the situation for *gp72* in *T. cruzi*. Using affinity-purified polyclonal antibody, raised against part of the amino-terminal domain of Fla1 expressed in *Escherichia coli*, we showed that Fla1 is concentrated along the flagellum and in the flagellar pocket in both bloodstream-form and procyclic trypanosomes. Fla1 from both life-cycle stages is *N*-glycosylated. Fla1 from bloodstream-form *T. brucei* contains additional glycans, which can be liberated by treatment with mild acid, suggestive of phosphodiester linkages.

Keywords: fla1.; Flagellum; Gene disruption; Glycosylation; Surface glycoprotein; *Trypanosoma brucei*

Abbreviations: bp, base pairs; *fla1*, flagellum-adhesion glycoprotein gene1; Fla1, protein encoded by *fla1*; PNGase, peptide *N*-glycosidase F; SDS, sodium dodecyl sulfate; TFA, trifluoracetic acid.

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[1] *Note:* The sequence data reported in this paper are available in the GenBank™, EMBL and DDBL databses under the accession number U43717.

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1. Introduction

Trypanosoma cruzi expresses an immunodominant 72-kDa glycoprotein (Gp72) [1,2], predominantly observed along the flagellum of epimastigote and metacyclic trypomastigote forms [3]. Deletion of both copies of *gp72* caused detachment of the flagellum from the cell body, after its emergence from the flagellar pocket, resulting in impaired mobility of the parasites [4]. The null mutant was severely impaired in its ability to survive in the insect host, *Triatoma infestans* [3]. Therefore, Gp72 appears to be essential for physical movement and natural transmission of *T. cruzi*. However, mechanistic details of structures of the glycoprotein and its conservation among the Kinetoplastidae is not known. Our previous attempts to identify a gene homologue in *T. brucei*, by DNA hybridization, were unsuccessful.

Plasmid T410, containing approximately 1.8 kbp of the 3′ portion of a *T. rhodesiense* cDNA, encoding a protein that showed significant similarity to *T. cruzi* Gp72, was identified while sequencing clones randomly selected from a cDNA library [5] and was kindly provided to us by N.M.A. El-Sayed and J.E. Donelson (University of Iowa). We have cloned and characterized the gene from which T410 was derived, which we name *fla1*, and its encoded protein, Fla1. Thus we confirmed that *T. brucei* expresses a homologue of Gp72. We show that the glycoprotein is concentrated along the flagellum and provide evidence that it may contain carbohydrates *O*-linked to the polypeptide via phosphodiester bonds and that, while the protein is expressed in both mammalian and insect infective stages, *O*-glycosylation is developmentally regulated.

2. Materials and methods

2.1. Parasites

Culture-adapted procyclic forms of *T. brucei* strain 427 were grown at 27°C, in SDM-79 medium [6] supplemented with 10% fetal-calf serum (FCS) and 7.5 μg/ml hemin, at a density of $5 \times 10^5 - 1 \times 10^7$ cells/ml. Bloodstream forms of the Molteno Institute Trypanozoon antigenic type 1.2 (MITat 1.2) clone 221a of *T. brucei* strain 427 were grown in rats or adapted to grow in liquid culture at 37°C, using HMI-9 medium as described [7]. Bloodstream-form cultures were maintained at a density of $5 \times 10^3 - 1 \times 10^6$ cells/ml. Epimastigote forms of *T. cruzi* Y_{NIH} strain [8] were grown in liver infusion tryptose medium [9] plus FCS at 26°C, as described previously [10].

2.2. DNA and RNA analyses

Trypanosome total DNA, total RNA and plasmid DNA, were isolated and purified as described previously [11–13]. Polyadenylated RNA was isolated with QuickPrep micro mRNA purification kit (Pharmacia Biotech, Piscataway, NJ). Agarose gel electrophoresis, nucleic acid transfer and hybridization, were performed by standard methods [4,11]. DNA and RNA hybridizations were quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Plasmid clone pBT100 [P. Hevezi and G. A. M. Cross, unpublished] containing a *Hin*dIII fragment of *T. brucei* α- and β-tubulin gene repeats, was used to produce a tubulin probe.

2.3. Cloning and characterization of the fla1 gene

On the basis of preliminary Southern hybridization experiments (see Section 3), 20 μg of total procyclic-form genomic DNA was digested with *Bam*HI and electrophoresed in a 0.7% agarose gel. Gel slices containing DNA fragments of 4–10 kbp DNA were excised and DNA was purified with a Geneclean kit (Bio 101, Vista, CA). The size-selected DNA was ligated to *Bam*HI-digested and dephosphorylated pBluescript II SK + (pBS, Stratagene, La Jolla, CA). The ligated products were transfected into the *E. coli* strain DH5α. Approximately 10 000 *E. coli* colonies were transferred to nylon membranes, hybridized with the 1.8-kbp insert of plasmid T410, and washed under high stringency (65°C, 0.1 × SSC, 0.1% sodium dodeycl sulfate (SDS)) [11]. A region containing the *fla1* open reading frame (ORF), in one (pfla) of several positive clones, was sequenced at least

twice on each strand, using subclones and synthetic oligonucleotides, on a DNA Stretch-Sequencer Model 373 (Perkin Elmer/Applied Biosystems, Norwalk, CT) by the Rockefeller University DNA/Protein Technology Center, using standard M13-reverse and T7 primers. The DNA sequence was assembled and analyzed using Lasergene software (DNASTAR, Madison, WI) on a Macintosh computer.

2.4. Replacement of *fla1* by drug-resistance genes

Upstream and downstream flanking regions of the *fla1* coding sequence and plasmid sequence were PCR-amplified as a single fragment from a plasmid (pflaBBg) containing a 4.8-kbp *Bam*H1–*Bgl*II fragment from pfla (including the 1.7-kbp *fla1* coding region, the 2.2-kbp upstream and and 0.9-kbp downstream flanking regions), using 'outward facing' 5′- and 3′-primers corresponding to nucleotides (nt) −1 to −15, upstream of the translation initiation codon and a 20 nt sequence approximately 260 bp downstream of translation-termination codon, using the LA PCR kit (Takara Shuzo) under the manufacturer's recommended conditions. The PCR fragment was end-trimmed and phosphatase-treated. The Tn5 neomycin phosphotransferase gene (*neo*) was obtained from p72neo72 [4] by digesting the plasmid with *Hin*dIII and *Pfl*MI. A hygromycin phosphotransferase gene (*hyg*) was obtained from p72hyg72 [4] by digesting the plasmid with *Hin*dIII and *Bam*HI. The fragments containing *neo* and *hyg* were end-filled and ligated to the PCR fragment to obtain knockout constructs, pKON and pKOH, respectively. Transfections were performed using a BTX ECM 600 electroporator (BTX, San Diego, CA) described previously [14,15]. Approximately 3×10^7 procyclic or bloodstream-form trypanosomes were suspended in ZFM or ZFMG medium, respectively, and electroporated with 10 μg of pKON or pKOH that had been digested with *Bam*HI plus *Xho*I or *Bss*HII, respectively. G418 or hygromycin were added to the bloodstream or procyclic form cultures at 1 or 10 mg/ml, respectively, 24 h after electroporation.

2.5. Production of Fla1 antiserum

A plasmid to produce histidine-tagged Fla1 fusion protein was created by cloning part of *fla1* into pET15b (Novagen, Madison, WI). A region of *fla1*, encoding amino acids 81–312, was amplified by polymerase chain reaction (PCR) using oligonucleotide primers, 5′-ct*ggatcc*atgtgatgttggaggtggt-3′ and 5′-ct*ggatcc*taaaccagtgagacaatcg-3′, where italicized letters underlined represent *Bam*HI sites used for cloning and underlining indicates an introduced termination codon. The PCR-amplified fragment was end-trimmed, cloned into *Hin*cII-digested pBS, and cut out with *Bam*HI. The 0.7-kb *Bam*HI fragment was cloned into *Bam*HI-digested pET15b to produce pHfla, which was transfected into *E. coli* strain BL21 ($F^- ompT r_{B-} m_{B-}$). The histidine-tagged protein was produced by incubating logarithmic cultures of the transformed *E. coli* in Luria-Bertani medium containing 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 3 h. Cells were disrupted by sonication in 50 mM Tris–HCl, pH 8.0, 0.2 mM ethylenediaminetetraacetate (EDTA) containing 100 μg/ml lysozyme and 0.1% Triton X-100. Inclusion bodies were collected by centrifuging the lysate at $10\,000 \times g$ for 10 min. The pellet was dissolved in 6 M urea and the histidine-tagged protein was purified with nickel resin columns under denatured condition, as suggested by the vendor (Novagen), then separated on an SDS-polyacrylamide gel. Two rats were immunized by intraperitoneal injection of the homogenized polyacrylamide gel slices containing approximately 100 μg of the purified protein. The same amount of the protein was injected 4, 6, and 10 weeks after the first immunization. Antiserum was prepared 1 week after the final booster. Approximately 50 μg of the histidine-tagged Fla1 was applied to a nitrocellulose membrane, air-dried and blocked in phosphate-buffered saline (PBS) pH 7.2 containing 0.05% Tween 20 (PBST), supplemented with 5% non-fat dry milk. The membrane was incubated with the antiserum diluted with PBST for 4 h, followed by washing

with PBST twice for 5 min. The anti-Fla1-specific antibody was eluted with 500 μl of 0.1M glycine pH 2.5, 0.15 mM NaCl, containing 0.05% Tween 20, and neutralized with 0.1 vol. of 1 M Tris–HCl pH 8.0.

2.6. Protein analysis

In different experiments, trypanosomes were lysed either by osmotic shock, or by three cycles of freezing and thawing, or by treating with 50 mM Tris–HCl, pH 7.4 containing 0.2 mM EDTA and 3% *n*-Octylglucoside, or by boiling in 0.5% (SDS) with 1% β mercaptoethanol in the presence of 40 μM leupeptin, 10 μM pepstatin, 0.2 mM phenylmethanesulfonyl fluoride and 1 mM EDTA. Lysates were centrifuged at $10000 \times g$ for 10 min to separate soluble and insoluble fractions. Portions of the parasite lysates were treated with peptide *N*-glycosidase F (PNGaseF) or neuraminidase, under conditions recommended by the vendor (New England Biolabs, Beverley, MA), or treated with 40 mM trifluoroacetic acid (TFA) at 100°C for 12 min [16]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis were performed as described [11]. After protein transfer, membranes were incubated sequentially in PBS containing 5% non-fat dry milk (PBSM) for 30 min, in PBSM containing the 100–1000-fold diluted anti-serum for 1 h, and in PBSM containing the 5000-fold diluted anti-rat IgG goat serum alkaline phosphatase-conjugate (Sigma) for 1 h. All incubations were carried out at room temperature.

2.7. Indirect immunofluorescence assay

Trypanosomes were washed with PBS and spread on polylysine-coated slides. The parasites were then fixed in 50 mM sodium borate pH 8 containing 3.7% paraformaldehyde for 20 min and permeabilized with PBS containing 0.1% Triton X-100 for 5 min. The slides were incubated with the 200–1000-fold diluted affinity-purified anti-Fla1 antibody in PBS containing 10% FCS for 1 h at room temperature and 20–30-fold diluted anti-rat IgG rhodamine-conjugate for 1 h. Finally, the slides were incubated with PBS containing 5 μg/ml Hoechst 33258 for 2 min, mounted and examined under Nikon Optiphot microscope equipped with appropriate dichromatic filters and photographed.

3. Results

3.1. Cloning of T. brucei fla1

When Southern blots of *Bam*H1-digested *T. brucei* DNA were hybridized with the 1.8-kbp insert from plasmid T410, encoding part of a *T. rhodesiense* protein showing significant similarity to *T. cruzi* Gp72 [5], bands of 4.8- and 8.0-kbp gave strong signals at high stringency. The insert was used to hybridize a size-selected *Bam*H1 genomic library from *T. brucei*, as described in Section 2. Approximately 10000 recombinant plasmid colonies were screened and seven positive clones were obtained after the second screening. Two and five plasmid clones contained 8.0- and 4.8-kbp *Bam*HI fragments, respectively. Further subcloning, restriction-site mapping, cross-hybridization and partial sequencing of these inserts revealed that the 8.0-kbp fragment contained an ORF showing highly significant similarity to *T. cruzi* Gp72. A representative clone (pfla) containing the 8.0-kbp insert was characterized. The signal from the 4.8 kbp-*Bam*H1 fragment was caused by strong similarity to 3′ untranslated region of the *T. rhodesiense* cDNA probe, and will not be further described in this report.

3.2. Genomic organization and RNA expression of fla1

The patterns of restriction-fragment hybridization, using several enzymes, were consistent with *fla1* being a single copy gene with a *Hin*dIII polymorphism in one allele (Fig. 1A, D). This premise was confirmed during replacement of

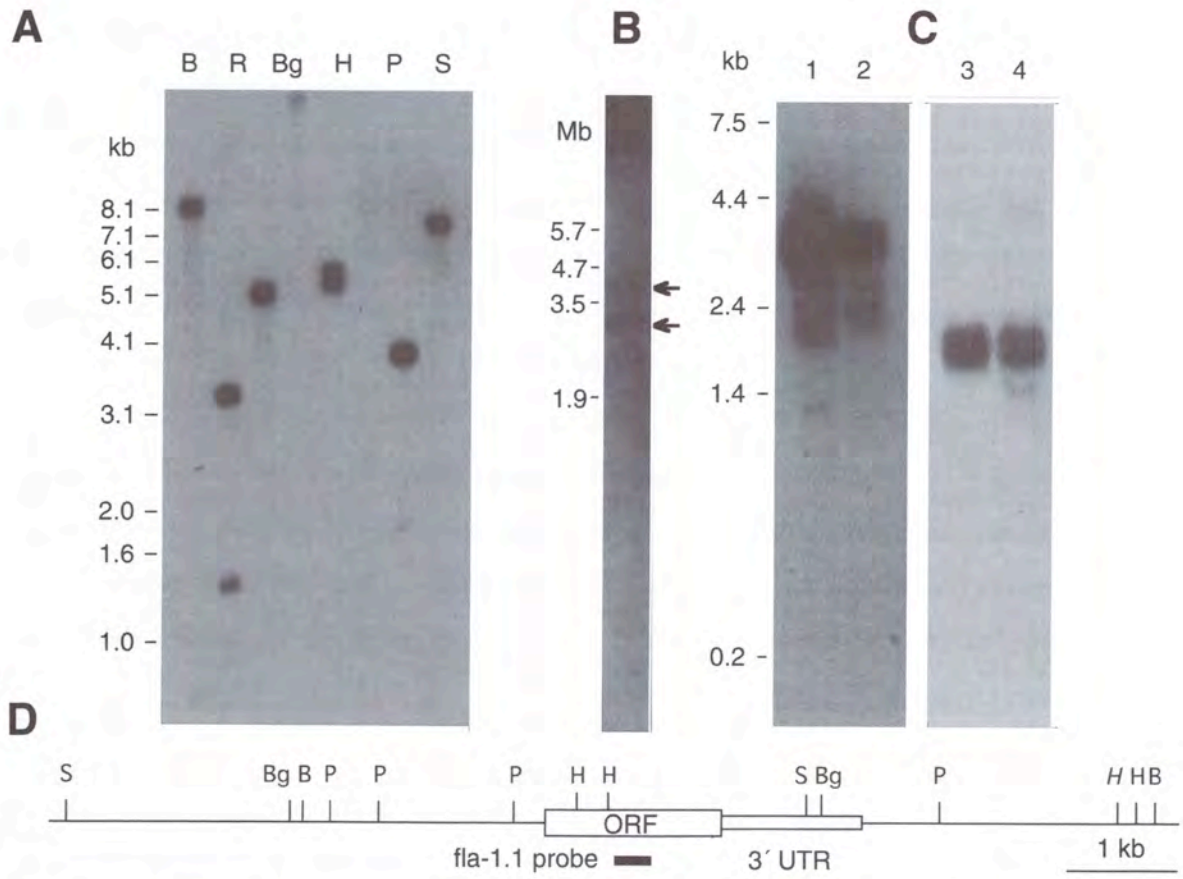

Fig. 1. Genomic organization and expression of *fla1*. (A) Southern blot hybridization of genomic DNA. Ten micrograms of endonuclease-digested procyclic-form *T. brucei* DNA was electrophoresed on 0.7% agarose gel, transferred to nylon membrane, and hybridized with the fla1.1 probe (panel D). (B) Pulsed-field gel electrophoresis of bloodstream-form chromosomal DNA. Arrows indicate the two chromosomes to which probe fla1.1 hybridized. (C) Hybridization of procyclic (lanes 1 and 3) and bloodstream-form RNA (lanes 2 and 4). 5 mg of polyadenylated RNA was electrophoresed on 1% gel, transferred to nylon membrane, and hybridized with fla1.1 (lanes 1 and 2) or tubulin probes (lanes 3 and 4). (D) Schematic representation of *fla1* organization. The polymorphic *Hin*dIII site is italicized. Restriction enzyme abbreviations: B, *Bam*HI; Bg, *Bgl*II; P, *Pst*I; H, *Hin*dIII; R, *Eco*RI; S, *Spe*I.

each allele with drug-resistance genes (see below). Hybridization of chromosomal-sized DNA, after pulsed-field gel electrophoresis, showed that the two alleles of *fla1* are located on chromosomes of approximately 3 and 4 Mbp (Fig. 1B). Polyadenylated *fla1* RNA was detected as a single 3.5-kb band in both procyclic and bloodstream forms (Fig. 1C). However, phosphorimager quantitation showed that procyclic forms contained approximately 7- fold more steady-state *fla1* mRNA than bloodstream forms.

3.3. Amino acid sequence of Fla1 and comparison to T. cruzi Gp72

The 8.0-kb insert of pfla was subcloned and a 2-kb region, containing the gene of interest, was sequenced. The 1638-bp coding sequence, flanked by stop codons in all frames, encodes a deduced 59.5-kDa protein of 546 amino acids, assuming that the first in-frame ATG is used for translation initiation (Fig. 2). The deduced Fla1 sequence showed 44% amino acid identity and 63% similar-

Fig. 2. Amino acid sequence comparison of Fla1 (top line) and Gp72 (bottom line). Dashes in the sequences are computer-inserted gaps. Identical amino acids are highlighted by vertical lines (|) and conserved cysteines by plus signs (+). Asparagines at possible *N*-linked glycosylation sites, the proline/threonine-rich putative *O*-linked glycosylation region, the RGD tripeptide and the putative transmembrane domain, are underlined. Asterisks indicate the predicted mature amino-termini.

ity to *T. cruzi* Gp72 (Fig. 2). The protein has a characteristic amino-terminal signal sequence, with a probable cleavage site [17] between amino acids 30 and 31, yielding a 57-kDa mature protein of 516 residues. The carboxy-terminal region has a putative membrane-spanning hydrophobic region, followed by a short putative cytoplasmic domain, in precisely the same location as Gp72, suggesting that Fla1, like Gp72, is membrane-associated. Neither protein has a carboxy-terminal sequence typical of a cleavable glycosylphosphatidylinositol-anchor signal sequence (reviewed in [18]). Eleven out of 12 cysteines in Gp72 are conserved in Fla1 and three out of five potential *N*-glycosylation sites in Gp72 are conserved in Fla1, which has four additional potential *N*-glycosylation sites compared to two alternative sites in Gp72. A proline/threonine-rich region of Fla1 (Ser_{336}–Thr_{353}; underlined in Fig. 2) is characteristic of *O*-glycosylated regions in other glycoproteins [19–21]. This putative *O*-glycosylated region in Fla1 is substantially shorter (18 residues) than in Gp72 (42 residues). The amino-terminus of Fla1 also contains an arginine-glycine-aspartate (RGD) tripeptide, which is found in fibronectin, collagens, fibrinogens, vitronectin, von Willebrand factor, snake disintegrins and slime mold discoidins, and is crucial for interaction of these proteins with cell surface integrins. Kyte-Doolittle hydrophilicity plots (Fig. 3) predict that Fla1 and Gp72 are structurally very similar, except for the length of the putative *O*-glycosylation region. The relative positions of the six carboxy-terminal cysteines in the hydrophilicity plots are almost identical, suggesting that the carboxy termini of these glycoproteins have a very

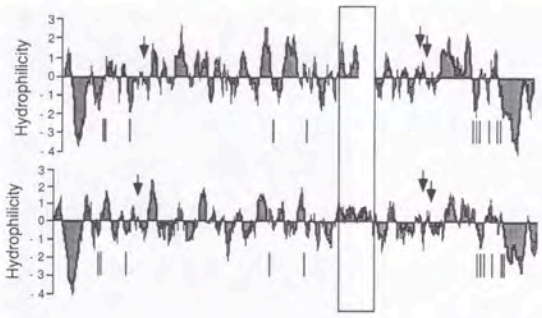

Fig. 3. Kyte-Doolittle hydrophobicity plot. The putative *O*-glycosylation region is boxed and a gap is inserted to compensate for the shorter length of this region in Fla1. Conserved cysteines and possible *N*-glycosylation sites are indicated by vertical lines and arrows, respectively.

conserved structure, which may be of functional importance.

3.4. Glycosylation of Fla1

An affinity-purified polyclonal antibody against the histidine-tagged amino-terminal region of Fla1, expressed in *E. coli*, was used to analyze Fla1 expression in parasites lysed with *n*-Octylglucoside or SDS. Bands corresponding to about 100 or 80 kDa, substantially larger than the predicted size (57 kDa) of the mature protein, were detected in lysates of bloodstream or procyclic forms, respectively (Fig. 4). Sub-cellular fractionation, by osmotic shock or freezing and thawing, followed by centrifugation, left Fla1 exclusively in the insoluble fraction in both developmental stages (data not shown). The anti-Fla1 antibody did not cross-react with a *T. cruzi* lysate and no cross-reaction was observed when *T. brucei* lysates were analyzed with the monoclonal antibody WIC29.26, which recognizes the unusual carbohydrates present on Gp72 [1,22,23], the *T. cruzi* Fla1 homolog (data not shown).

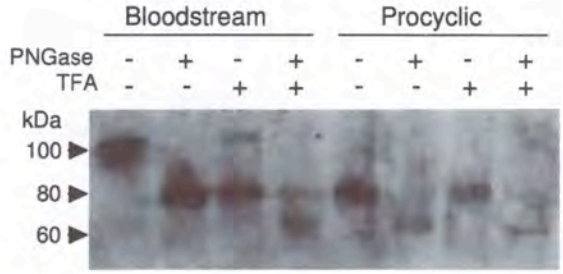

Fig. 4. Western blot analysis of Fla1 glycosylation. Lysates of approximately 10^7 bloodstream or procyclic forms were treated with PNGase F, TFA, or treated with both PNGaseF and TFA, and electrophoresed on a 7.5% SDS-polyacrylamide gel, transferred to nylon and reacted with affinity-purified anti-Fla1 antibody.

Treatment of lysates of bloodstream or procyclic forms with PNGaseF, to remove *N*-linked glycans, decreased the Fla1 bands to about 80 or 60 kDa, respectively (Fig. 4), but neuraminidase treatment had no effect (data not shown). Hydrolysis with 40 mM TFA at 100°C for 12 min, which selectively cleaves hexose-1-phosphate linkages [16], releases glycans phosphodiester-linked to threonine in *T. cruzi* Gp72 [23]. The same TFA treatment reduced the size of bloodstream-form Fla1 to approximately 80 kDa, but did not affect procyclic Fla1 (Fig. 4). TFA treatment following PNGaseF digestion reduced both bloodstream and procyclic-form Fla1 to approximately 60 kDa. These results suggested that procyclic Fla1 contains only conventional *N*-linked glycans but bloodstream-form Fla1 contains additional acid labile *O*-linked glycans.

3.5. Subcellular localization of Fla1

Indirect immunofluorescence, using paraformaldehyde-fixed and Triton X-100-permeabilized procyclic and bloodstream forms, showed that Fla1 was concentrated along the flagellum in both stages. The flagellum was evenly stained except at its proximal end, where a region corresponding to the flagellar pocket was stained more intensely (Fig. 5A, arrows). In those cells in which kinetoplast had already replicated, both daughter flagellae were equally stained (Fig. 5B).

3.6. Replacement of fla1 alleles

Two distinct fragments (5.4 and 5.2 kbp) were visualized when the *Hin*dIII-digested *T. brucei* genomic DNA was hybridized with a *fla1* probe (Fig. 6). In bloodstream forms transformed to G418 or hygromycin resistance, using *fla1*-targeting constructs pKON or pKOH, respectively,

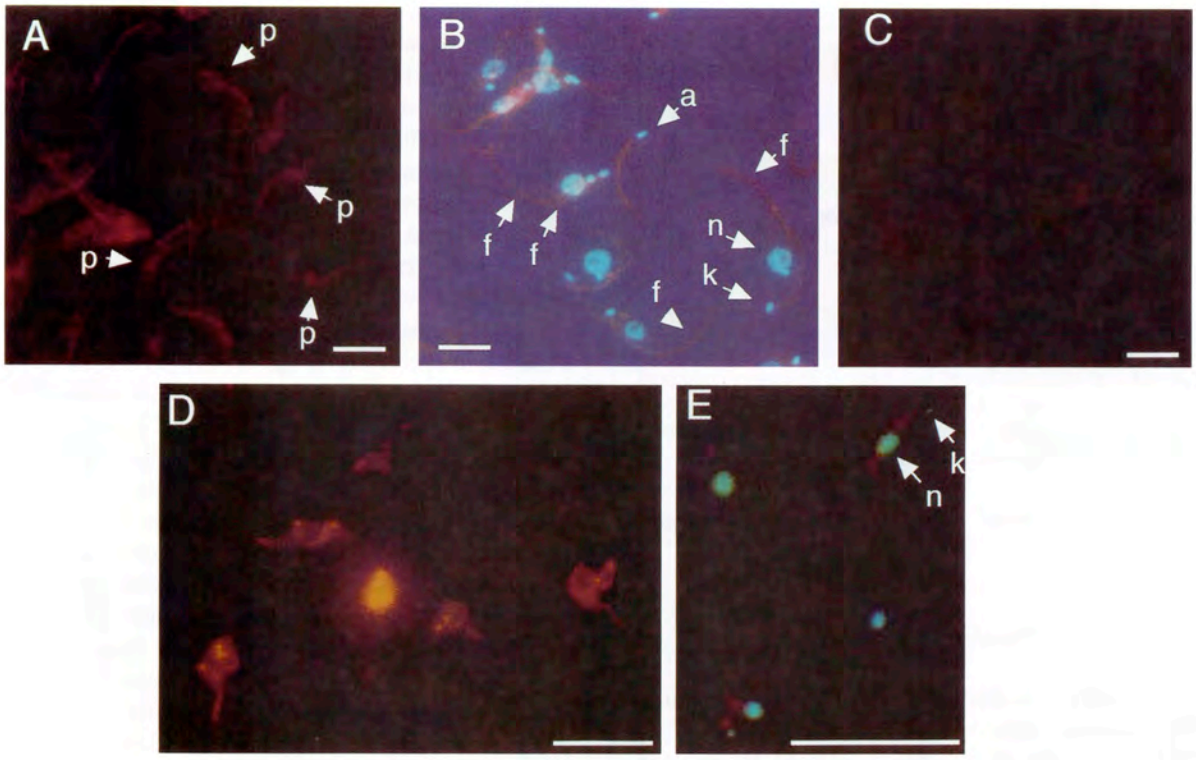

Fig. 5. Indirect immunofluorescence assay of Fla1. Procyclic (A–C) or bloodstream forms (D, E) of *T. brucei* were fixed and reacted with either the rhodamine-conjugated anti-Fla1 antibody (A, B, D, E), affinity-purified negative control antibody from pre-immune rat serum (C), or double-stained with anti-Fla1 antibody and Hoechst 33258 (B and E). Arrows marked p indicate flagellar pockets. Arrows marked f depict two daughter flagella in cells that have undergone kinetoplast division. Arrows marked k or n indicate kinetoplast and nucleus respectively. The cell indicated by the arrow marked a is an anucleate 'zoid' [42].

only the 5.2-kbp or the 5.4-kbp fragment was observed, confirming that *fla1* is a single-copy gene, and that individual alleles could be replaced by drug selectable markers. However, sequential replacement of both alleles, using either bloodstream-form or procyclic trypanosomes, was unsuccessful, despite repeated trials, suggesting that *fla1* is an essential gene.

4. Discussion

Gp72 was identified in *T. cruzi* epimastigotes because of its extreme immunogenicity, which is attributable to the presence of novel glycans, with which the Gp72-defining monoclonal antibody, WIC 29.26, reacts [1,22,23]. Polyclonal or monoclonal antibodies raised against native Gp72, which recognize the glycans, do not react with other life-cycle stages of *T. cruzi*, indicating that if Gp72 is expressed throughout the developmental cycle, as seems likely from recent experiments with an epitope-tagged Gp72 transgene [P.A. Haynes and G.A.M. Cross, manuscript in preparation], its glycosylation must be developmentally regulated.

Fla1 appears to be the *T. brucei* homologue of *T. cruzi* Gp72. The structural similarities are clear. Both Fla1 and Gp72 are membrane-associated glycoproteins of similar size and, as judged by the conserved cysteine residues and hydrophobicity profile, they have a similar structure. Fla1 and Gp72 contain seven and five *N*-glycosylation consensus tripeptides, respectively, and the posi-

tions of three of these potential *N*-glycosylation sites are conserved between them. However, Gp72 from *T. cruzi* epimastigotes appears to be minimally *N*-glycosylated (R. Cooper, P.A. Haynes and G.A.M. Cross, unpublished observations).

Fla1 from bloodstream-form *T. brucei* was sensitive to TFA treatment under conditions that selectively cleave hexose-1-phosphate linkages [16]. By analogy with the phosphodiester linked oligosaccharides of *T. cruzi* Gp72 [23], the most likely explanation for our current data is that the procyclic form of Fla1 contains only *N*-linked glycans but bloodstream-form Fla1 contains additional glycans that contain phosphodiester linkages. However, these glycans do not react with the monoclonal antibody WIC 29.26, so they are clearly distinct from the Gp72 phosphodiester-linked glycans. It is possible that the acid-labile material that causes the TFA-associated molecular weight shift could be attached to a subset of the *N*-linked oligosaccharides that are rendered resistant to PNGaseF by this modification, but we think this is unlikely. The proline/threonine-rich region is the most likely attachment site of the acid-labile glycan, as appears to be the case for the *O*-linked glycans of Gp72. Although *O*-glycosylation has been demonstrated in *T. cruzi* and *Leishmania* [22,24], it has not previously been reported in *T. brucei*. The secreted acid phosphatase of *Leishmania mexicana* contains both *N*-linked and acid-labile *O*-linked glycans [24–28]. The acid-labile glycans consist of polydisperse phosphoglycan chains, attached to the polypeptide backbone via Manα1-PO_4-Ser and composed of the unit sequence PO_4-6Galβ1-4Man [24,29], which is structurally related to *Leishmania* lipophosphoglycans. This type of *O*-glycosylation has also been reported in a lysosomal proteinase and a prespore-specific antigen (PsA) of *Dictyostelium discoideum* [30,31].

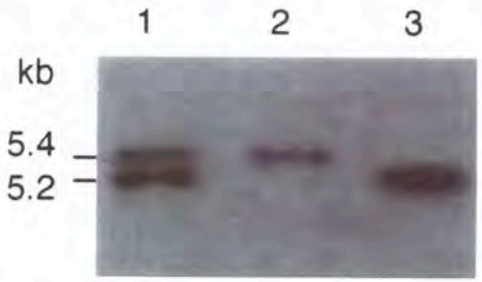

Fig. 6. Southern blot hybridization of genomic DNA from bloodstream forms of the wild-type (lane 1), hygromycin- and G418-resistant transformants that had been tranfected with pKOH and pKON respectively (lanes 2 and 3, respectively). Ten micrograms of *Hin*dIII-digested *T. brucei* genomic DNA was electrophoresed on 0.7% agarose gel, transferred to nylon membrane, and hybridized with the fla1.1 (Fig. 1D) probe.

The RGD tripeptide present in the amino-terminal domain of Fla1, at the same position as RGN in Gp72, is found in many extracellular-matrix proteins, including collagen I, fibronectin, laminins, tenascin, thrombospondins, and vitronectin [32–35]. The RGD tripeptide is involved in cell-adhesion interactions [35], self-assembly into multimeric structures, interactions with other extracellular-matrix components, interactions with small molecules and ions, and interactions with growth factors and other soluble factors [32]. The presence of the RGD tripeptide suggests that Fla1 may be involved in the interaction of the flagellum with either the cell body and/or with the cellular epithelium of its vertebrate and invertebrate hosts. It is noteworthy that the sequence of Fla1 showed some similarity to cellulase and endoglucanases (20% identity and 45% similarity). Therefore, Fla1 may also have enzymic functions, such as degrading extracellular-matrix in the hosts, although we have no evidence for such activities in either Fla1 or Gp72.

By immunofluorescence, Fla1 appeared to be enriched along the line of the flagellum. This distribution is similar to that of Gp72 in *T. cruzi* where, in the flagellated epimastigote form, anti-Gp72 staining does not appear to extend to the end of the flagellum (P.A. Haynes, D.G. Russell and G.A.M. Cross, submitted for publication). The anti-Fla1 flagellum-staining pattern is very similar to the distribution of an antigenically novel flagellum adhesion-zone-associated β-tubulin in *T. brucei* [36] and an 88 kDa flagellum-associated glycoprotein identified in procylic *T. brucei* [37]. Other investigators, who attached lectins to intact and trypsinized (to remove the variant surface glycoprotein) bloodstream-form *T. brucei*, found that ricin specifically associated with the anterior region of the flagellar pocket and the cell

body in the adhesion zone of the flagellum [38]. The glycoprotein responsible for ricin binding was not identified, but its location was similar to that of Fla1. Taken together with the intimate association between flagellum and kinetoplast, and the fact that basal body and flagellum are involved in the kinetoplast segregation [39], we propose that Fla1 might be necessary for the physical process of kinetoplast segregation and cell cleavage, in which the flagellum-attachment zone [40,41] has been proposed to be important [42]. Further ultrastructural studies on *T. brucei* and on the null mutants of *T. cruzi* may clarify these issues.

Both Fla1 and Gp72 are encoded by single-copy genes. However, whereas both alleles of *gp72* can be deleted, *fla1* is apparently essential, as we can delete individual alleles but not both. Deletion of *gp72* in *T. cruzi* leads to detachment of the flagellum from the cell body [4] and greatly impairs establishment of infection in the gut of the vector, *Triatoma infestans* [3]. Whether the defect in vector transmission is due to loss of adhesion to the midgut epithelium, or is a secondary consequence of impaired motility, is unknown.

Why should *fla1* be essential in *T. brucei* when its *T. cruzi* homologue is not? Assuming that the function of Gp72 and Fla1 is similar in both species, and is somehow necessary for flagellum adhesion, this implies that flagellum adhesion fulfills some essential but undetermined role in both life-cycle stages of *T. brucei* that is optional in *T. cruzi*. One of the major morphological differences between *T. cruzi* epimastigotes and *T. brucei* bloodstream or procyclic forms is that kinetoplast and basal body of the former are located in the anterior half of the cell body, whereas in the latter they are located in the posterior half of the cell body, and thus the extent of the interaction between the flagellum and cell body is much greater in *T. brucei*.

Identification and characterization of the flagellum components of kinetoplastids, and understanding their post-translational modifications, could identify targets for treatment and prophylaxis of diseases caused by this family of parasites.

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