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Subcellular localization of *Trypanosoma cruzi* glycoprotein Gp72

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SUMMARY

We have investigated the subcellular location of the *Trypanosoma cruzi* surface glycoprotein, Gp72, by introducing epitope-tagged copies of *gp72* into *gp72* null-mutant cells. A tagged Gp72, containing three tandemly repeated copies of a human influenza hemagglutinin nonapeptide (HA) adjacent to the mature Gp72 amino terminus, was able to complement the null mutant phenotype, as well as being recognized in western blots by both anti-HA antibody and the carbohydrate-specific monoclonal antibody WIC29.26. Integration of this epitope-tagged *gp72* into the chromosomal *gp72* locus produced a clonal cell line, 72HAN3.1G7, which was used for studies of the subcellular location of the epitope-tagged Gp72. Indirect immunofluorescence microscopy of fixed 72HAN3.1G7 epimastigotes showed that Gp72 was evenly distributed over the cell body and somewhat concentrated in the proximal region of the flagellum. No fluorescence could be detected in the distal tip of the flagellum. Immunoelectron microscopy of fixed 72HAN3.1G7 epimastigotes revealed that Gp72 was predominantly membrane-associated and located on the cell surface. Indirect immunofluorescence microscopy of live 72HAN3.1G7 epimastigote cells showed a similar pattern of fluorescence on the flagellum, but no fluorescence was detected on the cell body, which was attributed to masking by other cell-surface components. Indirect immunofluorescence microscopy of fixed 72HAN3.1G7 amastigotes revealed that Gp72, which has long been considered to be expressed only in epimastigotes and metacyclic trypomastigotes, can be expressed in amastigotes, but it no longer contains the WIC29.26 carbohydrate epitope.

Key words: *Trypanosoma cruzi*, Epitope tag, Surface glycoprotein 72, Glycosylation

INTRODUCTION

Trypanosoma cruzi is the etiological agent of Chagas disease, which is endemic in South and Central America. This protozoan parasite undergoes distinct biochemical and morphological changes during a complex life-cycle, involving reduviid bugs and mammals. The morphologically distinct life-cycle stages are named according to the point of emergence of the flagellum from the cell body. The parasite multiplies within the gut of its insect vector, the reduviid bug, as an epimastigote form. Differentiation occurs in the hind gut to produce metacyclic trypomastigotes, which are non-dividing invasive forms transmitted to the mammalian host by contaminative infection via insect faeces. The metacyclic trypomastigotes invade different cell types in the mammalian host, including macrophages and muscle cells, and undergo division as amastigotes before release from the cells as trypomastigotes. This cycle of cell invasion continues indefinitely (Brener, 1973). The biochemical changes that occur during differentiation between stages are important factors in establishing infection of insect vectors, invading mammalian cells, and evading the host immune sytem.

A number of stage-specific *T. cruzi* surface glycoproteins have been characterized, examples of which include Gp85 and other members of the *trans*-sialidase gene family from tissue culture trypomastigotes (Cross and Takle, 1993; Takle et al., 1989), GP 82 from metacyclic trypomastigotes (Araya et al., 1994) and amastin from amastigotes (Teixeira et al., 1994). In epimastigotes, a 72 kDa glycoprotein, Gp72, was first identified on the surface of epimastigote cells using a carbohydrate-specific monoclonal antibody, WIC29.26 (Snary et al., 1981). The WIC29.26 epitope was subsequently found on the surface of epimastigotes of all *T. cruzi* strains examined, despite variations in post-translational modification, which caused some masking of glycan epitopes (Kirchhoff et al., 1984; Schechter et al., 1986). Immunofluorescence microscopy showed that WIC29.26 reacted uniformly with the surface of epimastigotes and insect-derived metacyclic trypomastigotes, and less strongly with metacyclic trypomastigotes dervied from epimastigotes in culture (Kirchhoff and Sher, 1985). The WIC29.26 epitope is not found in the amastigote or tissue culture trypomastigote life-cycle stages (Snary et al, 1981). Although it has often been regarded as specific for, or synonymous with, Gp72, WIC29.26 in fact recognizes a novel glycan epitope (Haynes et al., 1996) that is present on a number of glycoproteins, including Gp72 (Cooper et al., 1993).

Previous work in our laboratory has involved cloning and sequencing the gene encoding Gp72 (Cooper et al., 1991) and preparing a *gp72* null mutant strain by targeted gene replacement (Cooper et al., 1993). The deletion of *gp72* caused a

striking morphological change, in which the flagellum was detached from the cell body after emerging from the flagellar pocket, and the overall shape of the parasite was dramatically altered. The null mutant was unable to survive in the insect vector (Ribeiro de Jesus et al., 1993). The null-mutant cells could be restored to normal morphology by complementation with episomal vectors expressing *gp72*, and glycosylation of the mature protein appeared to be the limiting step in the expression of functional Gp72 (Nozaki and Cross, 1994).

The exact function of Gp72 remains uncertain, although it clearly plays an important role in the maintenance of normal parasite morphology. It has been shown to be an acceptor for complement factor C3, which triggers the lysis of epimastigotes by mammalian sera via the alternative pathway (Joiner et al., 1985). It has also been suggested to play an important role in differentiation, as it has been shown that WIC29.26 antibody can inhibit metacyclogenesis in a model system (Sher and Snary, 1982). As Gp72 is directly involved in flagellar adhesion, it is highly likely Gp72 plays an important role in the mammalian stages of the life-cycle. Since the WIC29.26 antibody recognizes carbohydrates that are expressed only in the epimastigotes and metacyclic trypomastigotes found in the insect vector, it is not possible to use this antibody to study Gp72 in other life-cycle stages. Attempts to do this using antisera raised against the peptide backbone of Gp72 were unsuccessful in microscopy (Cooper et al., 1993), although weakly positive in western blots (Nozaki and Cross, 1995), suggesting that little of the polypeptide chain is exposed on the cell surface. We have overcome this problem by investigating the location of Gp72 using an epitope-tagging approach. An oligonucleotide encoding a peptide epitope tag was inserted towards the 5′ end of *gp72* and reintroduced into *gp72* null mutant cells, which were then examined by western blotting, immunofluorescence microscopy and immunoelectron microscopy, in both the epimastigote and amastigote life-cycle stages.

MATERIALS AND METHODS

Trypanosomes

Epimastigote stocks of Y-NIH strain *T. cruzi* were grown in liver infusion tryptose (LIT) medium at 26°C without agitation (Bone and Steinert, 1956). A *gp72* null mutant strain, in which the *gp72* alleles had been replaced by hygromycin and bleomycin resistance genes (Nozaki and Cross, 1994), was maintained under the same conditions. Amastigotes were produced by incubating metacyclic trypomastigotes in the presence of gamma-irradiated L6E9 rat skeletal muscle cell monolayers maintained in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum. Metacyclic trypomastigotes were purified from stationary-phase epimastigote cultures by lysing remaining epimastigotes with guinea pig complement.

Construction of epitope tagging plasmids

The efficacy of various epitope tags was investigated using the pTEX shuttle vector (Kelly et al., 1992). A summary of the different modifications used is presented in Fig. 1. Oligonucleotide sequences encoding epitope tags were inserted into the *gp72* coding sequence using the polymerase chain reaction (PCR) with a template plasmid (pGp72) containing *gp72* coding and flanking sequences in pBluescript. PCR products were subcloned using the pCR-script kit (Stratagene) before being ligated to pTEX. A 1.8 kb fragment encoding the influenza haemagglutinin (HA) nonapeptide tag sequence (-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-) inserted at the carboxyl terminus of the coding region was prepared using the oligonucleotides 5′-GACTAGTATGTTTTCAAAAAGGACG-3′, which contains the *gp72* translation-initiation codon immediately following an *Spe*I site, and 5′-CCGCTCGAGCTAGGCGTAATCTGGCACGTCTACGGGTACATGGGTGGA-3′, which encodes the HA tag immediately before the TAG translation-termination codon, followed by an *Xho*I site. The resulting 1.8 kb product was subcloned, and excised from pCR-script with *Spe*I/*Xho*I and ligated to *Spe*I/*Xho*I-digested pTEX to produce the plasmid p72HAC1. A similar strategy was used to generate p72FLAGC1, with the 8-residue FLAG (IBI/Kodak™) tag (-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-) inserted at the carboxyl terminus, using the oligonucleotide 5′-GCTCGAGCTATTTATCATCGTCATCTTTGTAATCCATGGGTGGA-3′.

The insertion of tag sequences into the amino-terminal domain required several steps. The 8-residue FLAG tag was inserted at position 41 of the deduced amino-acid sequence, 5 residues downstream of the predicted amino terminus at Gln-35, thus replacing the amino acids QQQLVIQD with DYKDDDDK, and changing the Leu at position 39 to a Ser in order to incorporate an *Xba*I site and facilitate cloning. A 1.9 kb fragment containing 0.3 kb of 3′ untranslated region, most of the coding region and the tag was amplified and subcloned using the oligonucleotides 5′-ATTCTAGAGATTACAAAGATGACGATGATAAATTCTTCATCAGTC-3′ and 5′-TTATATCGATACAGACACACATGCACCCTTCG-3′. A second fragment of 0.8 kb was amplified, spanning the region from the *Sac*I site at position 17 of the *gp72* 5′-untranslated region nucleotide sequence to the site of the start of the first fragment, corresponding to position 40 in the protein sequence. The oligonucleotides used were 5′-CGAGACTAGTCTGCAGGTCAACGGATCTTA-3′ and 5′-CTGTCTAGAATCAAACTGCGCTCCTGCAGG-3′. The second fragment was excised using *Sac*I and *Xba*I and ligated to a vector consisting of the first fragment in pCR-script, digested with *Sac*I and *Xba*I. The resulting product was used as the template in a PCR reaction, using primers to amplify the tagged *gp72* coding region, with *Spe*I and *Xho*I sites added to facilitate cloning into pTEX to produce p72FLAGN1.

A similar strategy was used to introduce the HA tag in the same position in the amino-terminal domain, but in this case the tag was inserted without replacing any of the original sequence. The oligonucleotides used were 5′-ATTCTAGATACCCGTATGATGTGCCAGACTACGCCCAGCAGCAGCTGG-3′ and 5′-TTATATCGATACAGACACACATGCACCCTTCG-3′. This was then ligated to the same second fragment described above. The resulting product was used as the template in a PCR reaction using primers to amplify the tagged *gp72*-coding region, with *Spe*I and *Xho*I sites added to facilitate cloning into pTEX to produce p72HAN1. The p72HAN1 plasmid was subsequently used as the starting template in the construction of a plasmid containing three tandem copies of the HA tag sequence, which also involved several steps. A 1.9 kb fragment was amplified by PCR using p72HAN1 as a template, and the oligonucleotides 5′-GACTAGTTACCCGTATGATGTGCCAGACTACGCCTCTAGATACCCGT-3′ and 5′-TTATATCGATACAGACACACATGCACCCTTCG-3′. The product, after subcloning, has two tandem copies of the HA tag, separated by an *Xba*I site, and also contains an *Spe*I site at the 5′ end to facilitate further cloning steps. A second fragment of 0.8 kb was amplified using pGp72 as a template and the oligonucleotides 5′-CGAGACTAGTCTGCAGGTCAACGGATCTTA-3′ and 5′-CACTAGTGGCGTAGTCTGGCACATCATACGGGTAATCAAACTGCGCT-3′. This was subcloned to give a product which contained the HA tag sequence at the 3′ end, and could be excised by *Sac*I and *Spe*I. This was ligated to a vector consisting of the first fragment in pCR-script digested with *Sac*I and *Spe*I. The resulting product was used as the template in a PCR reaction using primers to amplify the tagged *gp72*-coding region, with *Hin*dIII and *Xho*I sites added to facilitate cloning into pTEX to produce p72HAN3.

(A)

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                                        ↓
MFSKRTSPAP FRALLLPVVV VVVVVVASVA LPAGAQFDLR QQQLVIQDFF ISRSCAGCSQ GQTDGPSGAG   70
TLFTAAGGSL GKDASTLLLC DQGGGGSSVR LVNKSGIFTL AGSKTTRGNQ NGPAATALFN MPRAVVLEDG  140
ALYVADSANN LVREISNGIV TSFITEGLLG PSYIKPYSRP NGAHDLFVSD TGKSRIIFAP LQKQTFITVF  210
ITGFQPDVLQ ISEKSRLMFA ICNSTKILSI NMQGATTPKD YWQVGNADCM GYQSSLMLTT EEDKLLYYGI  280
LNGTPSIMSL PATKTKTEAP RICPDVLLRW PHGPIVSLVN INKHAFYVVT ASNVYIVHDG SYHPTVTPTP  350
PLTPTPTPEV TPTPTVTPTP TPEVTPTPPV TPSPTITIHR GFAVAAFPAQ SLPIEDPRLM HELLSWLMKD  420
VGIAFESTDF FAVFPPDREV LVPGYVNVST WNNLTVLFNF DRTIVITEYF TPEGMSSEEG QARLFASPWY  490
WTRNFLDSLK KTVAWKDLEA FCMVNCVEHC ETMTFHKSEC VGYVRPPVCN DVCVGAVVSS VVLGATGIAL  560
IALMVGSSAN LRSAVILVPPMZ
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(B)

72HAC1

```
VGYVRPPVCN DVCVGAVVSS VVLGATGIAL IALMVGSSAN LRSAVILVPP MYPYDVPDYA Z          591
```

72FLAGC1

```
VGYVRPPVCN DVCVGAVVSS VVLGATGIAL IALMVGSSAN LRSAVILVPP MDYKDDDDKZ            590
```

72FLAGN1

```
                                        ↓
MFSKRTSPAP FRALLLPVVV VVVVVVASVA LPAGAQFDSR DYKDDDDKFF ISRSCAGCSQ GQTDGPSGAG   70
```

72HAN1

```
                                        ↓
MFSKRTSPAP FRALLLPVVV VVVVVVASVA LPAGAQFDSR YPYDVPDYAQ QQLVIQDFFI SRSCAGCSQG   70
```

72HAN3

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                                        ↓
MFSKRTSPAP FRALLLPVVV VVVVVVASVA LPAGAQFDYP YDVPDYATSY PYDVPDYASR YPYDVPDYAQ   70
```

Fig. 1. (A) Deduced amino acid sequence of Gp72. The glutamine at position 36 indicated by an arrow is the predicted N-terminal amino acid after cleavage of the presumptive signal sequence. The thr- and pro-rich region of potential O-glycosylation is indicated in italics. (B) Amino acid sequence of epitope-tagged Gp72 constructs, with altered residues underlined and the predicted N-terminal glutamine at position 36 indicated by arrows. 72HAC1 and 72FLAGC1 contain the HA and FLAG tags, respectively, inserted at the carboxyl terminus of the protein. 72FLAGN1 contains the FLAG tag replacing residues 41-48, with leucine at position 39 changed to serine. 72HAN1 contains the HA tag inserted between residues 40 and 41, with leucine at position 39 changed to serine. 72HAN3 contains three copies of the HA tag inserted between residues Asp-38 and Gln-41.

Construction of a Gp72 integration vector

A plasmid that could be used to integrate tagged copies of *gp72* into the original *gp72* locus in null mutant cells was constructed by inserting the neomycin phosphotransferase gene into the *gp72* 3′-UTR (Fig. 3). A 2.4 kb fragment containing the *neo* gene flanked by the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) intergenic region (5′) and GAPDH-II downstream region (3′) was amplified from the pTEX plasmid (Kelly et al., 1992) by PCR, with *Eco*RI and *Hin*dIII sites added at the 5′ and 3′ ends, respectively, using the primers 5′-CGGAATCCCCATTTACGACTCCAAGG-3′ and 5′-GCGAAGCTTCACACGGCTAGCATACT-3′. A 1.7 kb fragment containing the remainder of the Gp72 downstream region, from the point 0.3 kb downstream of the stop codon, where the initial fragment used in the 72HAN1 construct terminated, to the end of the 3′-UTR, was PCR-amplified with *Sal*I and *Kpn*I sites added. The primers used were 5′-GCGTCGACATATGCCCCCGCATGTA-3′ and 5′-CCG-GTACCCTTTACTCCTGCCGCAC-3′. The Gp72HAN3 sequence fragment, with the upstream region and 0.3 kb of 3′-UTR, was then assembled in the correct sequence with the neomycin resistance cassette and the remainder of the Gp72 3′-UTR to produce p72HAN3.neoR.

Indirect immunofluorescence microscopy

For analysis of fixed cells, trypanosomes were suspended at 5×10^6/ml in phosphate buffered saline (pH 7.6) (PBS) and allowed to settle onto poly(lysine)-coated microscope slides for 10 minutes. Cells were fixed by immersion in 3.7% paraformaldehyde (Sigma) in PBS for 15 minutes followed by immersion in methanol at –20°C for 5 minutes. Slides were rehydrated by washing three times in PBS, incubated in 5% fetal bovine serum in PBS for 10 minutes, washed in PBS, and incubated with primary antibodies for 1 hour. After washing three times for 10 minutes in PBS, slides were incubated in secondary antibody solution plus 0.0025% Hoechst 33258 stain for 1 hour, washed three times in PBS for 10 minutes and air dried. For analysis of live cells, the initial washes and antibody incubations were all carried out in solution at 0°C. After the final washing the cells were resuspended in 0.5% paraformaldehyde in PBS for 5 minutes then washed again in PBS before being spread on slides prior to analysis. Slides were mounted in 25 mM Tris, pH 8.0, 50% glycerol, and examined under a Nikon optiphot microscope using a ×63 objective and ×5 teleconverter. For FITC staining, excitation was at 450-490 nm and a 520 nm emission filter was used. Exposures of 30-250 seconds were recorded on Kodak Ektachrome P1600 film. Monoclonal antibody 12CA5 (Boehringer), which recognizes an influenza hemaglutinin nonapeptide (HA), was dissolved at 1 mg/ml in water. WIC29.26 was purified from mouse ascites fluid using protein-A agarose (Pierce) and adjusted to 1 mg/ml. Antibody 2C2, which recognizes the amastigote specific antigen Ssp4 (Andrews et al., 1987), was also adjusted to 1 mg/ml. Monoclonal antibodies and FITC-conjugated goat anti-mouse antibodies (Boehringer) were used at dilutions of between 1:100 and 1:500 in PBS, 5% fetal bovine serum, and all incubations were carried out in a humid chamber.

Transfection of trypanosomes

Parasites were transfected at mid-log phase at 6×10^7/ml in Zimmerman fusion media (Bellofatto and Cross, 1989). Parasites (0.5 ml) were mixed with plasmid (50 μg) in a 2 mm cuvette and electroporated twice using a BTX electro cell manipulator set at 1.5 kV and

24 ohms resistance. Parasites were diluted into 10 ml LIT and incubated for 48 hours before adding 500 μg/ml of G418. Surviving transfected parasites reached mid-log phase after 24-28 days, and were cloned by limited dilution in 96-well plates for a further 28 days. For transfections using pTEX-based episomal plasmids, cloning and subsequent cell maintenance was performed in the presence of drug, while for chromosomal integration transfections, drug selection pressure was removed prior to cloning.

DNA and protein purification and analysis

T. cruzi genomic and plasmid DNA was purified as described previously (Medina-Acosta and Cross, 1993). Plasmids for transfection were purified using a maxi-prep plasmid kit (Qiagen). Southern blots were probed using a Fluorescein Gene Images kit (Amersham). Total cell lysates were prepared at 5×10^8 cells/ml in 50 mM Tris pH 7.4, 0.1 mM EDTA, 3% *n*-octyl glucoside (Boehringer). Western blots were performed with 1:5,000 dilutions of the anti-HA, anti-Flag M2 and WIC29.26 primary antibodies, and 1:1,000 dilution of the anti-Gp72-polypeptide rabbit polyclonal antibody (Nozaki and Cross, 1994), in PBS, 5% nonfat milk, and detected using appropriate alkaline phosphatase-conjugated secondary antibody (Schleicher and Schuell) and BCIP/NBT detection (Gibco BRL) or horseradish peroxidase-conjugated secondary antibody (Schleicher and Schuell) and ECL western blotting analysis system (Amersham).

Immunoelectron microscopy

Immunoelectron microscopy was performed on frozen sections from epimastigotes fixed in 4% paraformaldehyde (Sigma) and 0.5% glutaraldehyde (Sigma EM grade) in Hepes-buffered saline (pH 7.0, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$) and infiltrated in PVP/sucrose as described previously (Russell et al., 1992; Russell, 1994). Sections were probed with rabbit antibody raised against a synthetic peptide, corresponding to the HA tag, coupled to ovalbumin. The anti-HA antibody was affinity-purified on peptide sepharose and used at 1 mg/ml diluted in Hepes-buffered saline with 5% fetal bovine serum and 5% goat serum. In double-labelling experiments, WIC29.26 antibody was added to the primary antibody incubation at 1:1,000 dilution. Binding of primary antibodies was visualized by incubation with goat anti-rabbit IgG conjugated to 6 nm or 18 nm gold and goat anti-mouse IgG conjugated to 18 nm gold (Jackson Immunoresearch Laboratories). Controls were conducted with the *gp72* null mutant cell line, and by omission of the respective primary antibody. In initial experiments some nuclear labelling of the non-transfected controls was observed with the anti-HA antibody, but this was overcome by inclusion of 10 μg/ml of a heat-treated sonicate from control trypanosomes in the primary antibody incubation. In both instances, under the conditions used, background labelling remained below 7% of the specific label.

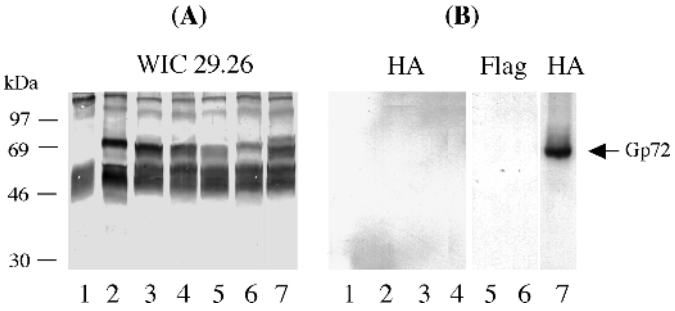

Fig. 2. Western blot analysis of cell lines containing epitope-tagged Gp72, using: (A) WIC29.26, (B) anti-HA or anti-FLAG. Lane 1, *gp72* null mutant; lane 2, Y-NIH wild type; lane 3, 72HAN1; lane 4, 72HAC1; lane 5, 72FLAGC1; lane 6, 72FLAGN1; lane 7, 72HAN3.

RESULTS

Epitope tagging of Gp72

The deduced protein sequence of Gp72 is shown in Fig. 1A. A series of epitope-tagged versions of *gp72* (Fig. 1B), were constructed and expressed in *T. cruzi gp72* null mutant cells using the pTEX episomal shuttle vector (Kelly et al., 1992). The use of *gp72* null mutant cell lines enabled us to assess the various constructs of epitope-tagged *gp72* by three criteria: restoration of a normal flagellar phenotype, indicating that the expressed glycoprotein was fully functional; restoration of a WIC29.26 reactive band on western blots, indicating that the expressed

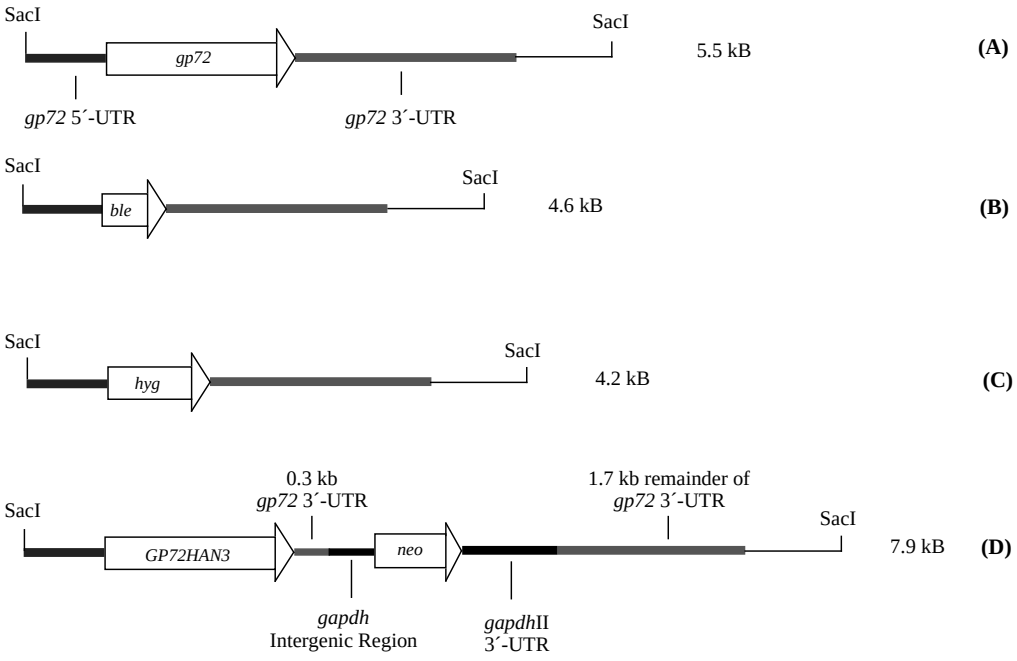

Fig. 3. Arrangement of the *gp72* locus. (A) *gp72* locus in Y-NIH wild-type cells; (B and C) *gp72* alleles in *gp72* null mutant cells, where the two copies of the *gp72* gene have been replaced by the hygromycin and bleomycin resistance genes; (D) *gp72* locus after integration of the insert contained in the p72HAN3.neoR vector. The fragment sizes indicated are those generated by digestion at the two endogenous *Sac*I sites as shown. The 0.7 kb 5′ upstream region and the 1.7 kb segment of the 3′ UTR downstream constituted homologous targeting sequences.

glycoprotein was authentically glycosylated; and appearance of a band on western blots probed with anti-tag antibodies, indicating that the inserted epitope tag was intact and accessible.

Insertion of a single copy of either the HA or FLAG epitopes at either the carboxyl terminus or in the amino-terminal region (Fig. 1B) produced cells of normal morphology, which gave a WIC29.26-reactive band for Gp72 in western blots (Fig. 2A, lanes 3-6). However, none of these produced a band in western blots when probed with anti-tag antibodies (Fig. 2B, lanes 3-6). A cell line in which a tagged Gp72 was detected by an anti-tag antibody, thus satisfying all three criteria outlined above for a functional epitope tag, was produced when three tandem copies of the HA tag were inserted in the amino-terminal region (Fig. 2A,B, lane 7). The use of multiple copies of the HA epitope tag in tandem arrays has been previously reported to give considerably higher sensitivity than single copies of the same sequence (Tyers et al., 1993).

Construction of a cell line expressing epitope-tagged *gp72* as an integral gene

In order to investigate the true subcellular location of Gp72, it was first necessary to produce a cell line in which the epitope-tagged *gp72* was integrated into the genomic *gp72* locus, to avoid artifacts that might result from the dramatic protein over-expression supported by the pTEX episomal vector (Kelly et al., 1992). Details of the integration vector used, p72HAN3.neoR, and the sequence regions used in the homologous integration, are shown in Fig. 3.

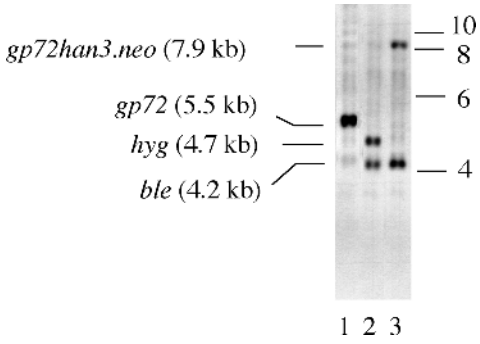

Fig. 4. Southern blot analysis of 72HAN3.1G7 clone. Genomic DNA from Y-NIH wild type, *gp72* null mutant and 72HAN3.1G7 cells was digested with *Sac*I, fractionated on an agarose gel, transferred to nitrocellulose, and probed with a fluorescein-labelled 0.7 kb Gp72 5′ fragment. Lane 1, Y-NIH; lane 2, *gp72* null mutant; lane 3, clone 72HAN3.1G7. Markers are indicated in kilobases.

When *gp72* null mutant cells were transfected with p72HAN3.neoR, the majority of the transformed population was of normal morphology. A single copy of the *gp72* gene is

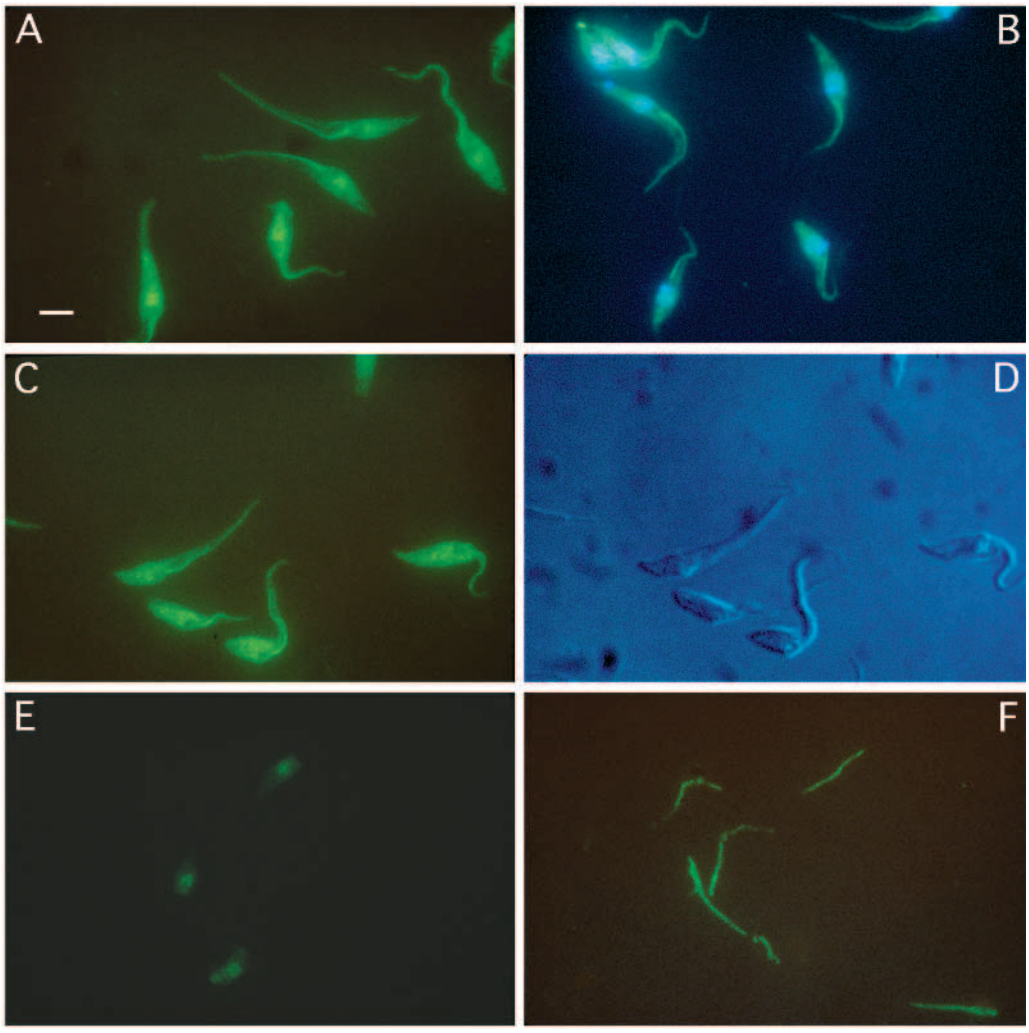

Fig. 5. Indirect immunofluorescence analysis of expression of Gp72HAN3 in epimastigotes using anti-HA antibody. (A and C) FITC staining of 72HAN3.1G7 epimastigotes; (B) FITC and Hoeschst staining of 72HAN3.1G7 epimastigotes; (D) the same field as C, under phase microscopy; (E) FITC staining of *gp72* null mutant epimastigotes; and (F) FITC staining of live 72HAN3.1G7 epimastigotes. Scale bar, 7 μm.

known to be sufficient for maintenance of normal function, as both alleles must be deleted before a null mutant phenotype becomes apparent (Cooper et al., 1993). A clone, designated 72HAN3.1G7, in which the hygromycin phosphotransferase gene of the *gp72* null mutant cells had been replaced by the *gp72han3.neo* gene, was identified by southern blotting (Fig. 4). Growth of this clonal cell line under different selection conditions confirmed that it was resistant to neomycin and bleomycin, but sensitive to hygromycin.

Western blots of the 72HAN3.1G7 clone revealed the same pattern of bands with both anti-HA and WIC29.26 antibody, as was seen for the previous 72HAN3 cell lines. Western blots with anti-Gp72-polypeptide antibody showed that the level of Gp72 protein expression was very similar in the wild-type and 72HAN3.1G7 cells, confirming that Gp72 was being expressed at normal levels in the transformed cells (data not shown).

Immunofluorescence microscopy of fixed and living 72HAN3.1G7 epimastigotes

The results of immunofluorescence microscopy of fixed 72HAN3.1G7 epimastigote cells, using the anti-HA antibody, are shown in Fig. 5. The anti-HA antibody reacted with the entire cell body, but showed a marked concentration in the region of the flagellum (Fig. 5A-C). The location of the nucleus and kinetoplast can be seen in Fig. 5B, which shows both the anti-HA staining and reaction with a nuclear stain. The reaction of the anti-HA antibody with the flagellum appeared to be restricted to the portion of the flagellum adjacent to the cell body (Fig. 5A-C). This can be seen more clearly when the anti-HA reactivity is compared with a phase microscopy image of the same cells (Fig. 5C,D). The flagellum of several cells can be seen by phase microscopy to extend beyond the end of the cell body (Fig. 5D). In contrast, the anti-HA reactivity of the same cells ends abruptly at the point where the flagellum reaches the end of the cell body (Fig. 5C). Thus, it appears that the anti-HA reactivity is limited to the proximal end of the flagellum, where it is attached to the cell body. The non-transformed *gp72* null mutant cells showed only faint reactivity with the anti-HA antibody (Fig. 5E), which was confined to the nuclear region and absent from the flagellum.

Living 72HAN3.1G7 epimastigote cells were also examined by immunofluorescence microscopy, with a light fixation applied after the antibody incubations to facilitate image capture. This produced a quite different pattern of staining. The anti-HA reactivity was seen exclusively on the flagellum (Fig. 5F), with no staining of the cell body, as was seen in analysis of the same cells with prior fixation (Fig. 5A-C).

Immunoelectron microscopy of 72HAN3.1G7 epimastigotes

Reaction of the anti-HA antibody with the cell body (Fig. 5A-C) may have been due to either a cell-surface or interior localization. To clarify this, immunoelectron microscopy was performed on fixed 72HAN3.1G7 cells using anti-HA antibody. The labelling of the cells with anti-HA antibody (Fig. 6A-C) indicated an abundance of protein on the cell surface, including the flagellar membrane, on the lumenal face of the flagellar pocket membrane, and in vesicles particularly abundant in the region between the flagellar pocket and the nucleus. Cells co-labelled with WIC29.26 exhibited an extensive overlap in the distribution of the two staining patterns (Fig. 6A,B).

Immunofluorescence of 72HAN3.1G7 amastigotes

Immunofluorescence microscopy was also performed on amastigotes of the 72HAN3.1G7 cell line, to examine the localization of epitope-tagged Gp72 in cells of the *T. cruzi* life-cycle stage that lack a flagellum. To facilitate comparison, amastigotes and epimastigotes were prepared separately and mixed prior to analysis. The amastigotes are the smaller, round cells, without flagella (Fig. 7A,B). The anti-HA staining of amastigotes is of a similar intensity to that seen in the cell body of the epimastigotes, and does not appear to show any preferential localization to specific organelles.

The reactivity of 72HAN3.1G7 epimastigotes and amastigotes with antibodies to stage-specific antigens was also examined in order to confirm their identity. An epimastigote that has reacted with WIC29.26 antibody shows staining principally on the flagellum and also on the cell body. (Fig. 7C). The nuclear stain of the same cells (Fig. 7D) shows that an amastigote cell is also present in the same field, but is only visible as a faint outline. Similarly, an amastigote cell reacts very strongly with the amastigote-specific 2C2 antibody (Andrews et al., 1987) (Fig. 7E), while visualization with nuclear stain (Fig. 7F) reveals the presence of an epimastigote which does not react with the antibody.

DISCUSSION

Epitope tagging, to study the expression, location and function of proteins, has become widely used in variety of biological systems. One of the major concerns with any such approach is the possibility that insertion of a foreign peptide sequence may alter the conformation or structure of the protein sufficiently to interfere with its function. In this study, we were able to use *gp72* null mutant cell lines to ensure that epitope-tagged Gp72 was, at the very least, able to complement the null mutant morphological phenotype. This functional assay assured us that insertion of foreign peptide sequences had not significantly altered the structure or conformation of the protein. In addition, we were able to use the WIC29.26 antibody to show that epitope-tagged Gp72 was glycosylated in a similar fashion to the native glycoprotein. These two criteria enabled us to examine transfected cell lines and be assured that functional, normally glycosylated epitope-tagged Gp72 was being expressed even when we could not detect the epitope tag in western blots, as was the situation when single HA or FLAG epitope tags were inserted at either the carboxyl terminus or in the amino-terminal region.

Although multiple copies of epitope tags have been reported to be advantageous in terms of sensitivity (Tyers et al., 1993), the reason for the lack of reactivity with anti-tag antibodies in western blots of cell lines transformed with the 72HAC1 and 72FLAGC1 constructs is unclear. The exact amino terminus of Gp72 is unknown (Cooper et al., 1993), but the glutamine at position 35 is the most likely mature amino terminus according to predictive algorithms (Von Heijne, 1986). Hence, the single-tag 72HAN1 and 72FLAGN1 constructs were designed with epitopes that start 5 amino acids downstream of this position, but the epitopes could have been destroyed if the cleavage position was not as predicted. The 72HAN3 construct, in contrast, could be trimmed within the first copy of the epitope, and still retain two complete copies of the epitope.

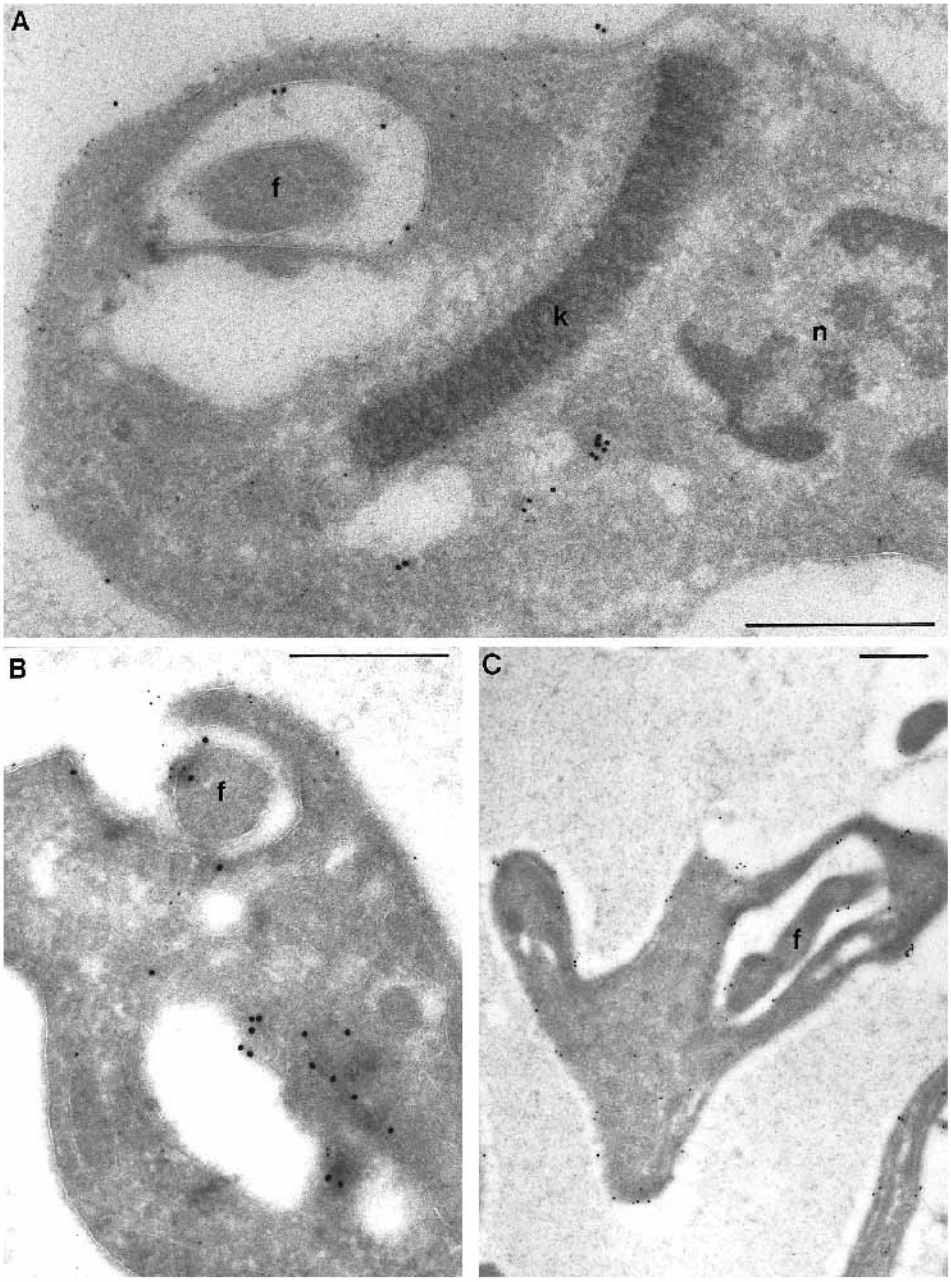

Fig. 6. Immunoelectron microscopy of 72HAN3.1G7 epimastigotes with anti-HA antibody, revealing the distribution of epitope-tagged Gp72 on the cell surface, within the flagellar pocket (f), and in vesicles located between the flagellar pocket and the cell nucleus (n), near the kinetoplast (k). (A and B) Cells were probed with rabbit anti-HA (6 nm gold) and mouse monoclonal WIC29.26 (18 nm gold); (C) this cell was probed with rabbit anti-HA (18 nm gold). Bars, 0.5 μm.

One of our concerns in studying the location of Gp72 in epimastigotes was to ensure that the translation of epitope-tagged *gp72* was controlled by authentic *gp72* regulatory sequences. As very little is known about the characteristics of promoters or transcription signals in *T. cruzi*, we sought to ensure that Gp72 was expressed at normal levels, and in the correct location, by using the p72HAN3.neoR integration vector rather than the pTEX episomal vector, which over-expresses proteins to high levels (Kelly et al., 1992). The immediate 5′ and 3′ flanking sequences of p72HAN3.neoR are identical to those of the native *gp72*. The insertion of the neomycin resistance cassette 0.3 kb downstream from the end of the *gp72* coding region, however, means that the level of expression of Gp72HAN3 in other life-cycle stages is not necessarily the same as that of authentic Gp72. The 3′ flanking regions of many genes in trypanosomes are known to play important roles in differential regulation between life-cycle stages (Nozaki and Cross, 1995). Further studies are underway in our laboratory to study the stage specificity, or otherwise, of Gp72 expression, using constructs with uninterrupted 3′ flanking regions.

Despite this caveat, the immunofluorescence microscopy of amastigotes of the 72HAN3.1G7 cells provides the first direct evidence that Gp72 can be expressed in life-cycle stages other than epimastigotes. The fact that Gp72HAN3 is clearly expressed in amastigotes, but is not recognized by the WIC29.26 antibody, indicates that there is developmentally regulated modification of the carbohydrate epitopes recognized by WIC29.26. As the amastigotes do not have a flagellum, there appears to be little need for expression of Gp72 if its only function is to participate in flagellar adhesion. This suggests that Gp72 may have another distinct function, which is as yet

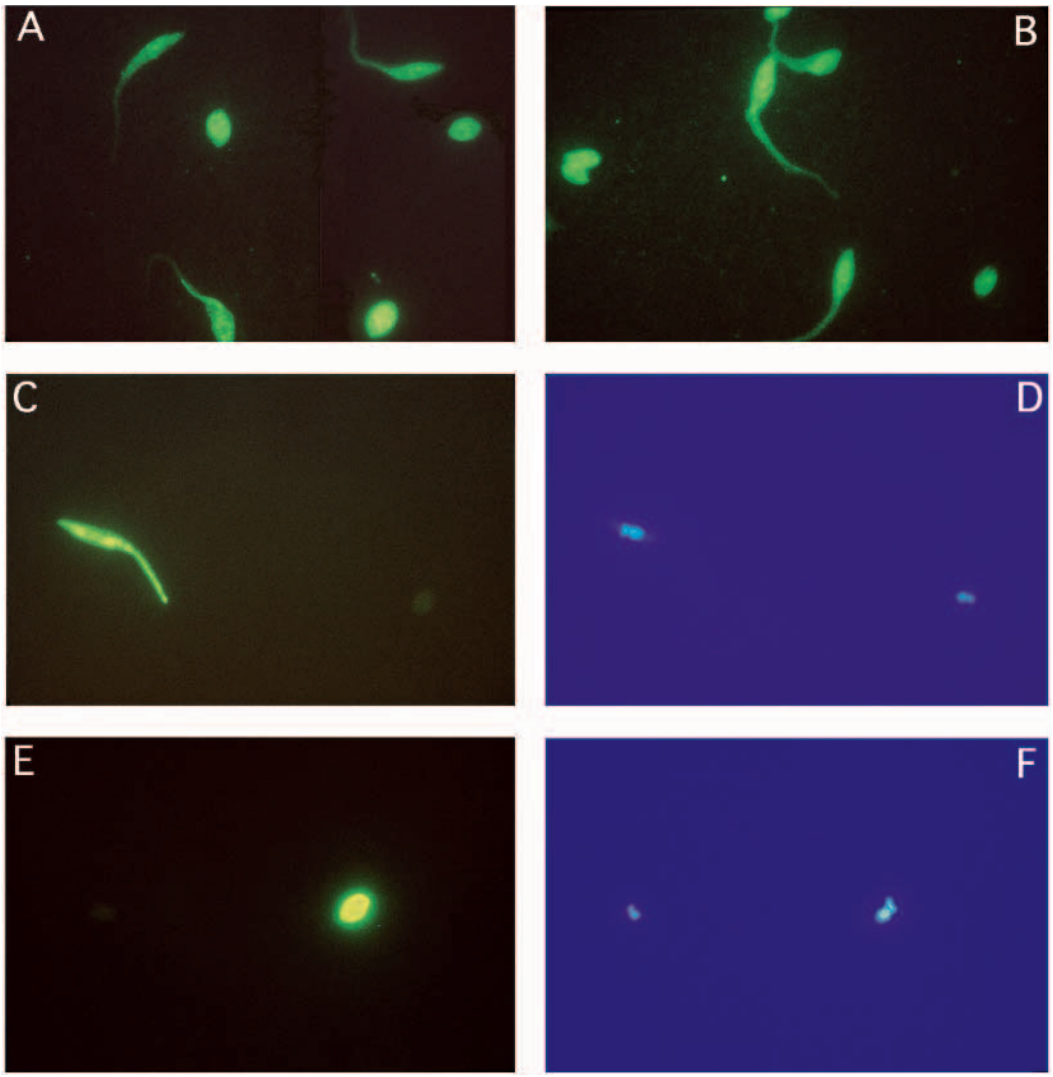

Fig. 7. Indirect immunofluorescence analysis of expression of Gp72HAN3 in amastigotes and epimastigotes of 72HAN3.1G7 cells. (A and B) FITC staining of a mixture of epimastigotes and amastigotes, with anti-HA antibody. (C) FITC and (D) Hoechst staining of an amastigote and an epimastigote with WIC29.26 antibody. (E) FITC and (F) Hoechst staining of an amastigote and an epimastigote with 2C2 antibody. Bar, 7 μm.

unknown, or alternatively, the developmental regulation of functional Gp72 may occur at the post-translational level.

The localization of Gp72 within epimastigotes is consistent with data previously reported. WIC29.26 epitopes were shown to be evenly distributed over the entire cell surface, including the flagellum (Kirchhoff et al., 1984). Using two additional antibodies (8B2 and 7C6), which recognize distinct epitopes of a purified 72 kDa protein, fluorescence was observed throughout the cell surface membrane including the flagellar pocket and cytosome (Harth et al., 1992). It is not certain, however, that the 72 kDa glycoprotein of Harth et al. is the same molecule as Gp72. In the current study, we can be certain that we are looking at a single glycoprotein, expressed as the product of a single gene, which also carries the WIC29.26 epitope. Immunofluorescence microscopy of *gp72* null mutant cells with WIC29.26 antibody showed the glycan epitopes were still distributed evenly over the cell surface in a similar fashion to that observed in wild-type cells. The major difference observed in the *gp72* null-mutant cells was a much weaker pattern of fluorescence in the cell body and proximal region of the flagellum, while the distal tip of the flagellum was still recognized very strongly (Ribeiro de Jesus et al., 1993). We have observed that epitope-tagged Gp72 is distributed over the cell body, but shows a concentration in the proximal region of the flagellum and is noticeably absent from the distal tip of the flagellum. The enhanced fluorescence we observed in the nucleus of some cells was also present in non-transfected controls and is therefore most likely to be a non-specific artifact. Thus, the localization of epitope-tagged Gp72 to the cell body and the proximal region of the flagellum agrees with previous findings obtained using less specific reagents. Immunoelectron microscopy confirmed that an abundance of epitope-tagged Gp72 was present on the cell surface, including the flagellar pocket and the flagellar membrane.

We observed a very clear difference in the reactivity of epitope-tagged Gp72 with anti-HA antibody in live cells as opposed to fixed cells. In the live cells, fluorescence was seen only on the flagellum, while in the fixed cells fluorescence was observed to be distributed over the whole cell body, including the proximal region of the flagellum. We think it unlikely that this apparent distribution is an artifact caused by the use of bivalent primary antibodies. In studies of *T. brucei*, antigen redistribution did not occur at 0-4°C and, under 'capping conditions', antibodies did not localize to the flagellum but moved to the posterior end and thence to the flagellar pocket (Barry, 1979). The present results suggest that there may be significant

differences in antigen accessibility between Gp72 on the flagellum and Gp72 on the remainder of the cell surface. A similar observation has been made in *Leishmania major*, where antibody recognition of the major surface protease (GP63) was strongly masked on promastigote cells by the presence of a lipophosphoglycan surface coat (Pimenta et al., 1991). A similar situation may occur in *T. cruzi*, with, for example, the lipopeptidophosphoglycan (De Lederkremer et al., 1990) or the more recently described mucin-like glycoproteins (Schenkman et al., 1993; Previato et al., 1995) forming a surface coat that masks antibody recognition of Gp72 on live cells.

An 88 kDa glycoprotein displaying a transmembrane attachment to a unique cytoskeletal structure, consisting of a narrow region parallel to the flagellar attachment zone, has been reported in *T. brucei* (Woods et al., 1989). This glycoprotein was identified by binding to the lectin ConA, which was also observed to bind to the distal tip of the flagellum. Glycoproteins of similar molecular mass that bind con-A have also been reported in *T. b. gambiense* and *T. b. rhodesiense* (Frommel and Balber, 1987). Several antigens have been localized to the flagellar attachment region of *T. cruzi*, but they are of high molecular mass and do not appear to be related to Gp72 (Cotrim et al., 1990; Souto-Padron et al., 1989). The localization of Gp72 to the proximal region of the flagellum, where it is adjacent to the flagellar adhesion zone, suggests that flagellar Gp72 may be interacting with another molecule that is found in this region. It has been suggested that two distinct mechanisms are responsible for flagellar attachment in trypanosomes. The first is a weak attraction that is distributed over the whole flagellar attachment region, while the second is a strong interaction that is localized to discrete maculae (Vickerman, 1969). Our results suggest that the putative Gp72 interaction with the flagellar adhesion zone may be involved in, or solely responsible for, the weak attraction, as is also evidenced by the striking flagellar non-adhesion phenotype observed in Gp72 null mutants (Cooper et al., 1993).

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