Abstract. Null mutants of the Trypanosoma cruzi insect stage-specific glycoprotein GP72 were created by targeted gene replacement. Targeting plasmids were constructed in which the neomycin phosphotransferase and hygromycin phosphotransferase genes were flanked by GP72 sequences. These plasmids were sequentially transfected into T. cruzi epimastigotes by electroporation. Southern blot analyzes indicated that precise replacement of the two genes had occurred. No aberrant rearrangements occurred at the GP72 locus and no GP72 gene sequences had been translocated elsewhere in the genome. Western blots confirmed that GP72 is not expressed in these null mutants. The morphology of the mutants is dramatically different from wild-type. In both mutant and wild-type parasites, the flagellum emerges from the flagellar pocket. In the null mutant the normal attachment of the flagellum to the cell membrane of the parasite is lost.

We previously characterized the gene that encodes GP72 (Cooper et al., 1991). The glycoprotein is encoded by a single pair of non-telomeric allelic genes with no obvious homology to any sequence in DNA and protein data banks. The derived protein sequence is consistent with the biochemical properties of GP72 (Ferguson et al., 1983).

Although there is some evidence that GP72 may play an important role in the control of cellular differentiation within the insect vector through interaction with gut lectins (Sher and Snary, 1982), the function of GP72 remains unclear. Functional studies of GP72, and of other genes in T. cruzi and related Kinetoplastids, have been hampered by the lack of genetic manipulation techniques. Recently, several breakthroughs in DNA transfection technology in Leptomonas seymouri (Bellofatto and Cross, 1989), Leishmania enrietti (Laban and Wirth, 1989), and T. brucei (Clayton et al., 1990; Rudenko et al., 1990; Zomerdijk et al., 1990) (for review see Bellofatto, 1990) have made these parasites more amenable to genetic analyses. In particular, it has become increasingly evident that stable integrative transformation by homologous recombination is a rapid and practical method to obtain mutants in these parasites. Strains of parasites with single and double gene replacements have been engineered in T. brucei and Leishmania (Cruz and Beverley, 1990; Lee et al., 1990; ten Asbroek et al., 1990; Cruz et al., 1991; Eid and Sollner-Webb, 1991). Transient (Lu and Buck, 1991) and, very recently, stable transfection (Otsu et al., 1993; Harrihan et al., 1993) has been reported in T. cruzi.

In this paper we describe the creation of a GP72 null mutant by targeted gene replacement. The null mutant has an unexpected morphology: the flagellum, instead of adhering to the cell body after emerging from the flagellar pocket, is completely free and the overall shape of the parasite is altered.
Materials and Methods

Parasites

*T. cruzi* Y strain epimastigotes were grown in liver infusion tryptose (LIT) medium (Nogueira et al., 1981) at 26-28°C without agitation. Two Y strains were used in these studies: YNIH has been maintained for several years in our laboratory, YLH was obtained from B.E. Hall at Yale University (New Haven, CT) and was derived from our YNIH strain several years ago.

Construction of Plasmids Used for Transfection

A previously described (Cooper et al., 1991) 4.6-kb genomic DNA clone (pGP72), which contains the entire protein coding region of GP72 plus 0.8 and 1.9 kb of 5' and 3' flanking sequence, was used as a source of fragments to construct neomycin phosphotransferase (Neo) (p72neo72) and hygromycin phosphotransferase (Hyg) (p72hyg72) hybrid plasmid DNAs. The PCR was used to amplify the entire 5' and 3' noncoding regions of GP72 from pGP72, and the coding sequences of Neo from pNeO (Pharmacia LKB, Piscataway, NJ) and Hyg from pSV2hygAI3 (generously provided by David Strehlow, Stanford University, Stanford, CA). All fragments were amplified using primers with restriction sites at their 5' ends, obtained from Oligos Etc. (Wilsonville, OR). Each PCR product was blunt-end cloned into EcoRV-digested and -dephosphorylated pBluescript SKII+ (Stratagene, La Jolla, CA) and then excised using the specific restriction enzymes sites within the PCR primers. The fragments were then cloned sequentially into pBluescript SKII+ so as to flank the Neo and Hyg coding sequences with the 5' and 3' noncoding sequences from GP72 in the correct orientation (see Fig. 1, a and b). The use of PCR to generate all fragments allowed us to make exact replacements of the GP72 coding region and retain the integrity of the 5' and 3' regions. However, to facilitate cloning, in p72neo72 a HindIII site was inserted between the 3' end of the upstream GP72 noncoding sequence and the start codon of the Neo gene. In addition, at the 3' end of the Neo coding sequence three base pairs were inserted (the remnants of a BclI/BamH1 compatible-end ligation). Similarly, in p72hyg72, a HindIII site and a BamH1 site were inserted between the 5' and 3' noncoding GP72 sequences, respectively, and the Hyg start and stop codons. PCR reactions were carried out with AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) according to the manufacturer's recommendations in a Techne (Princeton, NJ) or GeneAmp PCR System 9600 (Perkin Elmer Cetus) thermal cycler. All subsequent DNA manipulations were carried out according to well-established procedures (Ausubel et al., 1990). The final constructs were checked by sequencing across the junction of each ligation. The plasmid DNA used for the transfections was purified by CsCl gradient ultracentrifugation, digested with either SauI or EcoR I (p72neo72) or SauI (p72hyg72), extracted once with phenol/chloroform, precipitated with ethanol, and washed several times with 70% ethanol. The DNA was then dissolved in sterile water to a concentration of 2 mg/ml and used in the electroporations.

Transfection of *T. cruzi*

Transfections were carried out using mid-log phase parasites that were washed and resuspended to 6 × 10⁶/ml in Zimmermann Fusion Media (Bel-lafatto and Cross, 1989). 70 µg of linearized plasmid was mixed with 0.5 ml of parasites in a 2 mm BTX cuvette and pulsed twice at room temperature using a BTX Electro Cell Manipulator 600 (BTX Corp., San Diego, CA) set at 1.5 K, and 24-ohms resistance timing. The parasites were immediately diluted into 5 ml LIT and incubated for 60 h before adding either 500 µg/ml G418 (Geneticin, Gibco, Grand Island, NY) or 400 µg/ml Hygromycin B (Calbiochem-Behring Corp., La Jolla, CA). 3 to 4 wk after transfection the surviving parasites were cloned by limiting dilution in 96-well plates containing LIT media (Novy and MacNeal, 1904; Nicolle, 1908) overlaid with LIT plus G418, Hygromycin B, or both. After cloning, the parasites were grown without added drug.

Purification and Analysis of Genomic DNA from Transfected Parasites

10 ml cultures of stably transformed parasites were washed once in PBS and then lysed in 1 ml of 10 mM Tris-HCl, pH 8.0, 0.25 M NaCl, 0.5% NP-40 (Calbiochem-Behring Corp.). After centrifugation at 15,000 g in a micro-

centrifuge, the pellets were resuspended gently using a loose fitting Dounce homogenizer in 1 ml of 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10 mM NaCl, 0.5% SDS. Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to 50 µg/ml and the lysate incubated at 37°C for 1 h followed by four extractions with phenol/chloroform (50:50) and three extractions with water saturated ether. Southern blots using these genomic DNAs and pulsed field gel electrophoresis were carried out as previously described (Cooper et al., 1991).

Electron Microscopy

For transmission EM the parasites were processed essentially as described (Lewengrub et al., 1988). Thin sections were cut using an Ultra Microtome (Sorvall Instruments, Newton, CT), stained with uranyl acetate and lead citrate and observed using a transmission microscope (EM 300; Phillips Electronic Instruments, Inc., Mahwah, NJ).

Parasites for scanning EM were adjusted to 10⁷ parasites/ml and processed as described (Andrews et al., 1987), except for additional incubation of the dehydrated samples for 1 h in hexamethyldisilazane (Polysciences, Warrington, PA), before air drying. The samples were then coated with ~100 Å of gold/paladium (60:40) using a Denton Desk II sputter coater (Denville, NJ) and viewed using a scanning electron microscope (S450; JEOL USA, Peabody, MA).

Results

Creation of a GP72 Null Mutant

The plasmids that transformed *T. cruzi* to drug resistance were essentially identical, in the arrangement of coding and noncoding sequence, to a 4.6-kb genomic clone, pGP72, we had previously isolated (Cooper et al., 1991). In effect, in p72neo72 and p72hyg72 the coding sequence of GP72 was replaced by the coding sequences of the drug-resistance genes (Fig. 1, a and b). Both recombinant plasmids were linearized so that one end was homologous to the 5' or 3' noncoding regions of GP72 in the genome. The other end was vector sequence (Fig. 1 b). Southern blot analysis of genomic DNAs from G418-resistant cloned parasites from two separate transfections showed that nine out of the 10 clones analyzed had the Neo gene integrated at the expected position in the GP72 locus (data not shown). No vector DNA had been integrated. In the tenth clone, integration of vector DNA had occurred. This clone was not analyzed further.

One of the clones with the correct integration, YNIH D5, was then transfected with p72hyg72. We analyzed 10 clones resistant to both G418 and Hygromycin B. In contrast to the results observed with the initial transfections using p72neo72, only one clone (YNH D5C3) had the Hyg gene integrated into the expected location. In the other nine clones, preliminary data indicated that a single intact GP72 gene was still present and that the Hyg gene had been amplified (data not shown).

After obtaining the first null mutant clone, we generated three others either by retransfecting G418-resistant YNIH D5 (clones YNIH D5D2 and YNIH 13), or by carrying out the same sequential transfections using a different Y strain (clone YLH 4). In addition, starting with wild-type YNIH, we carried out single gene replacements using p72hyg72 (clone YNIH D6).

Diagnostic Southern analysis of the wild-type, single deletion, and null mutant clones was carried out. The probes and anticipated fragment sizes are shown in Figure 1 c. Analysis of YNIH D5C3 confirmed that this clone was a true double deletion of GP72 and that the coding regions of both GP72 genes had been replaced by the Neo and Hyg genes. After
Figure 1. Construction of plasmids used for transfection and partial restriction maps of the GP72 locus in normal, single mutant, and double mutant clones. (A) Detail of the 5' and 3' junction between coding and noncoding sequences of pGP72. (B) Detail of the 5' and 3' junction between coding and noncoding sequences of p72neo72 indicating the insertion of 6 bp at the 5' end and 3 bp at the 3' end of the coding sequence of the neo gene. Open boxes and the boxed sequences indicate 5' and 3' noncoding fragments from the GP72 locus that remain unchanged in the new clones. The coding sequences are italicized with the encoded amino acid sequence beneath. p72hyg72 was constructed in a similar way. (C) A partial restriction map of the normal GP72 locus (i), the Neo transformed GP72 locus (ii), and the Hyg transformed GP72 locus (iii) is shown with the probes used and SacI digestion products diagnostic of the correct integration event. Open boxes indicate 5' and 3' noncoding sequences. The solid box, stippled box, and hatched box indicate the GP72, Neo, and Hyg coding sequences, respectively.

SacI cleavage, the two parental GP72 genes appeared as a 5.5-kb band when probed with GP72† (Fig. 2 a, lane 1). In clone YNH D5, the remaining GP72 gene appeared as a 5.5-kb band and the new band of 4.4 kb corresponded to the Neo gene replacement of the other GP72 gene (Fig. 2 a, lane 2). In the single gene deletion clone generated by using p72hyg72, YNH D6, the size of the new band was 4.7 kb, corresponding to the Hyg gene replacement (Fig. 2 a, lane 3). In the double-mutant clone, YNH D5C3, the 5.5-kb band was replaced by the two new bands of 4.7 and 4.4 kb (Fig. 2 a, lanes 4 and 5). In a similar blot using a GP72 coding region probe, a 5.5-kb band was visible in either the parental, or both single deletion clones (Fig. 2 b, lanes 1–3) but not in the double deletion (Fig. 2 b, lanes 4 and 5). Conversely, Neo and Hyg coding sequence probes detected either a 4.4 or a 4.7-kb band with the Neo and Hyg probes, respectively, only in the single or double mutant clones (Fig. 2, c and d, lanes 1–5). The genotype of two independently obtained null mutants, YNH D5D2 and YNH 13, is also shown. YNH D5D2 is identical to YNH D5C3 (Fig. 2, a–d, lane 6). YNH 13 is a true null mutant, as no bands are evident when hybridized with the GP72 coding region probe (Fig. 2 b, lane 7), but additional bands of smaller than 4.4 and 4.7 kb are visible when probed with GP72†, Neo and

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Figure 2. Southern blot analysis of GP72 null mutant clones. Genomic DNA from normal, single, and double mutant clones were digested with SacI, fractionated on four identical gels and blotted to nitrocellulose. The 32P-labeled probes used were (A) GP72t, a 5' flanking probe, (B) the GP72 coding sequence, (C) the Neo coding sequence, and (D) the Hyg coding sequence. Lane 1, YNIH; lane 2, YNIH D5, a Neo single mutant; lane 3, YNIH D6, a Hyg single mutant; lane 4, YNIH D5C3, a Neo/Hyg double mutant; lane 5, YNIH D5C3F4, a clone derived from YNIH D5C3; lane 6, YNIH D5D2, and lane 7, YNIH 13, two other independently derived Neo/Hyg double mutants. The smaller SacI fragment which should hybridize to the GP72t probe in all the clones (See Fig. 1 c) is not visible in this figure.

Figure 3. Pulsed field gel electrophoresis of GP72 mutant clones. Chromosomes from normal (lane 1), Neo single mutant (lane 2), and Neo/Hyg double mutant (lane 3) clones were resolved using a rotating agarose gel electrophoresis apparatus. (A) Ethidium bromide; (B) GP72 coding; (C) Neo coding; (D) Hyg coding. Markers (M) are in kilobases.

Figure 4. Western blot analysis with mAb WIC 29.26 of GP72 mutants. Equal numbers of parasites were analyzed (5 x 10^6). Lane 1, YNIH, normal parasites; lane 2, YNIH D5, Neo single mutant; lane 3, YNIH D6, Hyg single mutant; lane 4, YNIH D5C3F4, Neo/Hyg double mutant; lane 5, YNIH D5D2, Neo/Hyg double mutant; lane 6, YNIH 13, Neo/Hyg double mutant; lane 7, YLH, normal parasites; lane 8, YLH G5, Neo mutant; lane 9, YLH 4, Neo/Hyg double mutant derived from YLH G5. GP72 is indicated by the arrow.
Figure 5. Scanning electron micrographs of normal (A) and GP72 null mutant (B) T. cruzi epimastigotes. Bars, 10 μm.
Figure 6. Transmission electron micrographs of normal (A) and GP72 null mutant (B) T. cruzi epimastigotes. Higher magnification cross-sectional views of the flagellae are shown in the insets. Bars: (A and B) 1 μm; (insets) 0.1 μm.

kD, a broad area of weaker signal of lower molecular weight, and sometimes weak bands larger than GP72 (Fig. 4, lanes 1 and 7). Three independently derived GP72 null mutants from the YN1H strain, and one from the YLH strain, together with the parental and single mutant clones were analyzed by Western blot using WIC 29.26. No GP72 was evident in any of the null mutants (Fig. 4, lanes 4–6, and 9). Similar levels of GP72 were present in the parental and single mutant clones (Fig. 4, lanes 1–3, 7, and 8).

We speculated previously that the broad band of lower molecular weight present in lysates of YN1H may contain degradation products or intermediates in the synthesis of GP72. However, data from the null mutants refute this hypothesis. The broad area of staining of lower molecular weight became stronger in the single and null mutants derived from YN1H and resolved into two distinct bands (Fig. 4, lanes 2–6). In the YLH mutants, these bands lost prominence in the double mutant though the area of staining remained (Fig. 4, lane 9). In contrast, the bands of a molecular weight higher than GP72, visible in the normal and single mutant strains, disappeared in the double mutants (Fig. 4, lanes 4–6, and 9). These may be differently processed forms of GP72.

GP72 Null Mutants Have Abnormal Flagellar Morphology

In culturing the parasite clones involved in this study, we noticed that the null mutant epimastigotes had abnormal mor-
phology. Detailed examination by scanning and transmission EM revealed that the flagellum was detached from the body of the parasite after emerging from the flagellar pocket of the null mutants (Fig. 5), and the overall shape of the epimastigote was altered. The anterior end was much shorter and broader. The abnormal flagellar attachment affected the mobility of the parasite. In liquid culture, the double mutants tended to sink faster than the parental strain. Nevertheless, the doubling time and the parasite concentration reached at stationary phase were unaffected. Microscopic examination of the flagellum, cell membrane, and underlying pellicular microtubules revealed no other obvious changes (Fig. 6).

This unexpected phenotype cannot be attributed to accidental selection for a second mutation affecting flagellar attachment during the cloning of the transfected parasites. We have generated several GP72 double mutants from independent transfections and used two different Y strains. All the null mutant clones have the same abnormal morphology. The chances of selecting for a second mutation affecting flagellar attachment in each case is remote. The abnormal morphology is not correlated with Neo and Hyg expression, because single or double drug-resistant clones that still have at least one GP72 gene have normal flagellar and cell morphology.

**Discussion**

The study of gene function has been aided in many organisms by the generation of null mutations through sexual exchange or homologous gene targeting (Capecchi, 1989). *T. cruzi* has no known sexual stage and, until recently, genetic manipulation by DNA transfection was not possible. Major advances in the field have documented the efficiency of homologous integration of transfected DNA in trypanosomatids, making it feasible to inactivate any chosen gene that is not a member of a large family (Capecchi, 1990). As GP72 is encoded by a single pair of allelic genes, unlike many of other surface antigens of *T. cruzi*, direct genetic manipulation was possible.

Because nothing is known about the location and characteristics of promoters and transcription termination signals in *T. cruzi*, we sought to avoid changes in the sequences surrounding the GP72 coding region. The targeted replacement plasmids, p72neo72 and p72hyg72, differed from the normal GP72 locus only in the coding sequences themselves and the insertion of two restriction sites between the 5' or 3' noncoding sequences and the start and stop codons.

Our results corroborate the efficiency of homologous integration in trypanosomatids (Capecchi, 1990). In the single gene deletions using the Neo construct, all but one of the 10 clones analyzed had the Neo gene integrated homologously. We have not analyzed in detail the unexpected integration events that occurred when the HYG construct was used to delete the second GP72 gene. Our preliminary data indicate that, in these clones, double cross-over events did not take place. Instead, these clones retained a single copy of the GP72 gene and the HYG gene was amplified.

The lack of any GP72 in Western blots using WIC 29.26 provides formal proof that the gene we had previously cloned and characterized as a candidate gene for GP72 (Cooper et al., 1991) does encode the glycoprotein defined by the mAb. A mutation in the glycosylation pathway that synthesizes the glycan epitope on GP72 recognized by WIC 29.26 cannot explain this result as the same epitope is found on other glycoproteins in the mutant parasites. It is interesting to note that the levels of the other glycoproteins with the WIC 29.26 epitope seem to be increased in the single and double mutant clones from the YNH strain. We hypothesize that the parasites may be compensating for the lack of GP72 on the surface by increasing the amount of the glycan epitope on other glycoproteins.

In the Y strain, the glycan epitope present on GP72 is evenly distributed over the entire surface of the epimastigote including the flagellum (Kirchhoff et al., 1984). These results were extended by Harth et al. (1992) who used a mAb, 8G2, that recognizes the same glycan epitope as WIC 29.26. Using both indirect immunofluorescence and immuno EM it was evident that glycan epitope was in the cell surface membrane, flagellar pocket and the cytoosome. There has been no published indication of an exclusively flagellar localization. Hence, the phenotype of the GP72 null mutant was surprising.

Three *T. cruzi* antigens have been localized to the flagellar attachment region (Souto-Padron et al., 1989; Cotrim et al., 1990), but none of them seem to be related in any way to GP72. Two of these antigens, recognized by antibodies to clone 1 and clone 30 (Ibáñez et al., 1987), are antigens of high molecular weight (160->205 kD) and contain tandemly repeated amino acid sequence motifs (Ibáñez et al., 1988). The third is a 300-kD antigen of unknown sequence (Cotrim et al., 1990). In *T. brucei*, the homologous antigen to *T. cruzi* clone 1 has been characterized (Müller et al., 1992), and it is also localized to the flagellar attachment region. Also in *T. brucei*, an 88-kD glycoprotein with a transmembrane association to a unique flagellum attachment region has been characterized as well as 180-, 200- and 300-kD antigens associated with the paraflagellar rod and the flagellar attachment zone (Woods et al., 1989a, b). None of these antigens, either in *T. cruzi* or *T. brucei*, bear any resemblance to GP72 and therefore are unlikely to perform a similar function.

Freeze-fracture EM has provided further insight into the mechanism of flagellar attachment in trypomastigotes. Linear arrays of closely adjacent intramembranous particles denoted "miniature maculae adherentes" have been observed on both faces of the flagellar membrane occurring only at regions of membrane apposition between cell body and flagellum (Hogan and Patton, 1976; de Souza et al., 1978). Vickerman (1969) has suggested that two mechanisms of flagellar binding may exist. The first is weak and easily disruptive by fixation procedures for EM and exists over the whole region of adhesion. The second is strong and operates at discrete locations where the maculae are found. We are unable to say at this time whether the null mutant parasites have maculae or not. However, as GP72 is not localized only in this area, it is unlikely to be involved in maculae structure. Instead, GP72 may be responsible for the weak binding mechanism which need not necessarily be localized.

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