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Modulation of a cytoskeletal calpain-like protein induces major transitions in trypanosome morphology

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Introduction

Much of the premolecular classification of eukaryotic microbes, such as trypanosomes, rested on descriptions of their shape, size, and form. It is therefore axiomatic that the morphogenesis of daughter cells at division must occur with high precision, reproducibility, and fidelity. However, dramatic changes associated with essential life cycle stages (Matthews, 2011) are crucial for proliferation in different host or vector tissues and central to pathogenicity and virulence. In microbes with a cell wall, the underlying cytoskeleton orchestrates changes in shape and form (Piel and Tran, 2009). In nonwalled protists, cytoskeletal arrangements and developmental principles, including cytotaix, are critical (Beisson and Sonneborn, 1965; Moreira-Leite et al., 2001; Morrissette and Sibley, 2002). Trypanosomatid cell shape is defined by a sub–plasma membrane microtubule corset (Sherwin and Gull, 1989). Trypanosomatids are characterized by the emergence of a single flagellum from a flagellar pocket (FP) with the kinetoplast (mitochondrial DNA complex) tethered to the basal body (BB; Ogbadoyi et al., 2003; Gluenz et al., 2011). Hence, definition of shape and form, axis, and polarity comes from flagellum position and orientation of the cytoskeletal arrays. The flagellar attachment zone (FAZ) comprising filaments inside the flagellum, punctate attachments between the flagellum and cell body membranes, and a cytoplasmic FAZ filament seems key to morphogenesis of trypanosomes (Sherwin and Gull, 1989; Vaughan et al., 2008).

Trypanosomatid parasite forms have been categorized historically based on relative positions of the nucleus and kinetoplast along the anterior–posterior axis of the cell and by the location of flagellum emergence (Hoare and Wallace, 1966). The most characteristic cell forms delineated in that nomenclature were the trypomastigotes and epimastigotes of organisms such as Trypanosoma brucei and the amastigotes and promastigotes of Leishmania species (Fig. 1). How do such big developmental changes in the shape and form of these single-celled microbes occur during their life cycle transitions, and what processes have orchestrated the evolution of divergent parasite forms? In particular, are large changes in gene expression patterns responsible for the first and large genome content variations responsible for the second process?

The genome of trypanosomatids possesses an unusually large number of different calpain-like proteins (Ersfeld et al., 2005),
with many of them unlikely to be catalytically active. We now focus on a particular calpain-like protein, ClpGM6, that lacks the catalytic triad and locates to the FAZ. This protein was originally characterized only as a fragment of multiple, near-perfect, 68–amino acid GM6 repeats (Müller et al., 1992).

Here, we report that the striking consequence of ClpGM6 depletion in T. brucei is a shortening of the FAZ with concomitant dramatic transition of cells from a trypomastigote to an epimastigote-like appearance, in which the kinetoplast and associated structures are juxtaposed or anterior to the nucleus. Importantly, and in contrast to other cell morphology mutants reported to date, ClpGM6 RNAi cells maintain their growth in extended culture, and the epimastigote-like morphology is inherited over continuing cell generations.

**Results and discussion**

**ClpGM6**

The ClpGM6 gene is represented in the T. brucei genome as two gene fragments, Tb11.57.0008 and Tb11.47.0036, both contain GM6 repeats with calpain domains (Ersfeld et al., 2005). Bioinformatics and Southern blots suggest that the fragments represent the two ends of the ClpGM6 gene (Fig. S1, A and B). Orthologues were identified in both *Trypanosoma cruzi* and *Leishmania major* genomes (Fig. S1 C).

Our ClpGM6 antibody confirmed the original GM6 study by Müller et al. (1992), by staining the FAZ region (Fig. 2 A and Fig. S1 D). Colocalization of ClpGM6 and FAZ1 (a FAZ filament protein) or ClpGM6 and callagin (flagellar membrane protein) showed ClpGM6 to be located on the flagellar side of the FAZ (Fig. S1, D and E). The antibody recognized several high molecular bands (Fig. 2 B) on blots of cell lysates (Müller et al., 1992). On ClpGM6 RNAi knockdown, the Western blot signal and immunofluorescence (IF) diminished and remained low but detectable (Fig. 2, B and C).

**ClpGM6 RNAi produces dramatic changes in cell form**

Uninduced cells displayed a trypomastigote appearance with an attached flagellum (Fig. 2 D). At 48 h after RNAi induction and thereafter, virtually all cells in the culture displayed a very distinct morphology (Fig. 2 E) with a long free flagellum and kinetoplasts juxtaposed or anterior to the nucleus. These cells were reminiscent of the natural epimastigote forms, and we refer to this phenotype as epimastigote-like. This is a dramatic morphological change uncoupled from the associated differentiation of cell type; for instance, these cells do not express the epimastigote-specific surface marker *brucei* alanine-rich protein (BARP; Fig. S1, F and G; Urwyler et al., 2007). These cell populations were still actively motile, and cells moved in a similar manner to the uninduced population (Video 1).

Scanning EM (SEM) of these cells confirmed that their appearance varied dramatically from the original trypomastigote forms (Fig. 2 F). The flagellum emerged onto the surface much closer to the anterior end of the cell with a much-reduced length of flagellum attached to the cell body (Fig. 2 G). In rare cases, the free flagellum emerged extremely close to the anterior end of the cell (Fig. 2 H), giving rise to an even more extreme external morphology reminiscent of *T. brucei* gametes (Peacock et al., 2014) and *Leishmania* promastigotes.

**The epimastigote-like phenotype is stable and does not affect proliferation**

The ClpGM6 RNAi-induced epimastigote-like culture exhibited a normal growth rate (Fig. 3 A) and essentially normal stages of the cell cycle based on analysis of kinetoplast and nucleus divisions (Fig. 3, B–H). Even after >40 cell generations, the culture was still proliferative with the epimastigote-like morphology. This phenotype contrasts markedly with those produced previously with other FAZ components, the depletion of which causes flagellum attachment errors leading to cytokinesis defects (LaCount et al., 2002; Vaughan et al., 2008; Zhou et al., 2011).

Cells at the start of the cell cycle with a single kinetoplast and nucleus (1K1N) from established RNAi-induced cell cultures displayed a long free flagellum with the kinetoplast anterior to the nucleus (Fig. 3 B). Kinetoplast division preceded nuclear division (Fig. 3, C–E). In postmitotic cells with two kinetoplasts and two nuclei (2K2N), the interkinetoplast (4.7 ± 0.9 µm [mean ± SD]), n = 25 in induced vs. 5.0 ± 0.6 µm, n = 17 in uninduced cells) and internuclear distances (6.0 ± 0.8 µm vs. 5.8 ± 0.7 µm) were not affected by ClpGM6 depletion. Thus, kinetoplast/nuclear division processes were functional but delivered a completely different epimastigote-like nuclear/kinetoplast orientation.

Fig. 3 I and Table S1 summarize morphometric measurements of uninduced and induced 1K1N cells with a single flagellum. They show that the dramatic increase in length of free flagellum (12.2 ± 2.8 µm, in 27 cells with RNAi induced for 72 h vs. 2.7 ± 0.6 µm, in 28 uninduced cells) could not be accounted for by an overall increase in the flagellum length (19.1 ± 1.5 µm...
also seen in 2K2N postmitotic cells (Fig. 3 K and Table S2). (7.5 ± 1.5 µm vs. 9.8 ± 1.6 µm). The changes in morphology were resulting primarily from decreased anterior end–nucleus distance a reduction in cell body length (14.2 ± 2.1 µm vs. 17.2 ± 2.4 µm), anterior to the nucleus (Fig. 3 J). ClpGM6 RNAi induction gave = 28), and the associated kinetoplast was often juxtaposed or n posterior end of the cell body (7.7 ± 1.8 µm, n = 24 vs. 18.0 ± 1.6 µm) but rather by the reduced length of the por... section of new flagellum; the tip of this free flagellum was still attached to the old flagellum by FC (Fig. 4 C). IF showed that a strong, normal ClpGM6 signal was associated with the attached proximal portion of the new flagellum (Fig. 4, D–F). In these cells, the interkinetoplast distance was significantly reduced (3.8 ± 0.9 µm, n = 12 for induced vs. 4.9 ± 0.7 µm, n = 14 for uninduced cells). The internuclear distance was not affected by ClpGM6 depletion. The anterior kinetoplast–anterior nucleus distance was normal, but the posterior kinetoplast was positioned closer to the posterior nucleus (1.3 ± 0.4 µm from the center of the nucleus) than in uninduced cells (2.6 ± 0.5 µm) and typically was juxtaposed to the nucleus. Thus, in transitioning cells, the major change in FAZ structure and therefore cellular organization happens in the portion of the dividing cell where the new flagellum and FAZ are being built and has immediate consequences for BB/kinetoplast positioning. The transition cells show that the new FAZ is affected by ClpGM6 depletion, but the old FAZ and flagellum organization and attachment to the cell body remain. Thus, depending on the penetrance of the RNAi depletion, the transition from one cell type to the other can be made in one division and hence recapitulates the likely normal patterns of complex morphogenesis seen in the T. brucei life cycle (Sharma et al., 2009). When RNAi induction was removed, the population regained a trypomastigote morphology over several days.

**FP regional organization is maintained**

In trypomastigotes, the single Golgi is closely associated with the FP, which is an invagination of the plasma membrane around the proximal end of the flagellum. This kinetoplast, BB, FP, and Golgi region is associated in a high-order organization with cytoskeletal structures (Lacomble et al., 2009) and is positioned in a specific order of kinetoplast, FP, Golgi, and nucleus described from the posterior to anterior end of the cell (Fig. 4 G). In ClpGM6 RNAi-induced cells, transmission EM (TEM) revealed that this architectural unit was unchanged (Fig. 4 H) but now located on the anterior side of the nucleus. Thus, the ClpGM6 RNAi cells have not only a completely...
considerable extent in the total intensity of ClpGM6. In this group, there is not a strong correlation between the amount of ClpGM6 and the length of the FAZ. In the second group, which has lower amounts of ClpGM6 and which is exclusively RNAi-induced cells tending to epimastigote-like morphology, there is a relationship whereby decreases in ClpGM6 amount directly correlate with, and are likely to be causal of, decreasing FAZ length. We then asked whether ClpGM6 depletion affects other FAZ proteins, particularly those in the cytoplasmic FAZ filament, such as FAZ1 (Kohl et al., 1999) or DOT1 (Woods et al., 1989). Epimastigote-like cells displayed a very bright FAZ1 signal with total intensity being essentially the same as in trypomastigotes repositioned BB, but also all the associated organelles have been repositioned.

FAZ length correlates with amount of ClpGM6

To elucidate the role of the ClpGM6 protein in FAZ assembly, we plotted the total intensity of anti-ClpGM6 IF along the FAZ against FAZ length in uninduced and induced cells. The data could be fitted by an exponential function, which approached the plateau value of 19.2 µm and intercepted the y axis at 2.9 µm (Fig. 5 A). In essence, there are two groups of cells. The first contains the cells with a long FAZ (14–22 µm), which vary to a considerable extent in the total intensity of ClpGM6. In this group, there is not a strong correlation between the amount of ClpGM6 and the length of the FAZ. In the second group, which has lower amounts of ClpGM6 and which is exclusively RNAi-induced cells tending to epimastigote-like morphology, there is a relationship whereby decreases in ClpGM6 amount directly correlate with, and are likely to be causal of, decreasing FAZ length.

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We interpret the aforementioned data as ClpGM6 level being the limiting factor in FAZ elongation but not initiation. In *T. brucei* cells, flagellum elongation drives elongation of the FAZ filament (Kohl et al., 2003). The location of ClpGM6 on the flagellum side of the FAZ region suggests that it could be a part of the links that connect the axoneme/paraflagellar rod via the flagellar and plasma membranes to the FAZ filament (Müller et al., 1992). Depletion of ClpGM6 would limit the number of links that can be constructed, resulting in a shorter FAZ region on the flagellum side, leading to the construction of a shortened FAZ filament on the cell body side (Fig. S2). When insufficient ClpGM6 is available, the FAZ is too short to allow for cytokinesis.

Much of the regulation of trypanosome cell type differentiation likely operates via posttranscriptional mechanisms involving RNA-binding proteins, such as RBP6 (Kolev et al., 2012) and ALBA3/4 (Subota et al., 2011). The biological context of our result is emphasized by the fact that ClpGM6 mRNA is the most highly down-regulated transcript in trypomastigote cells differentiating to epimastigotes on depletion of the latter (Nilsson et al., 2010). Our results indicate that accompanying developmental changes in cell form are likely to be achieved by the modulation in the level of only a few key proteins, such as ClpGM6, influencing the length of the FAZ filament, thereby governing the positioning of BBs and associated organelles in the cell and determining the origin and plane of cytokinesis. Thus, complex life cycle and evolutionary changes in trypanosomatid parasite cell shape and form are likely to be brought about by relatively transient changes in a few key proteins that are genetically stably expressed.

We therefore asked whether this was reflected in a modified ultrastructure. TEM analysis revealed that in 17 out of 46 cross sections of cells with ClpGM6 RNAi induced for 72 h, the FAZ filament was indeed widened, producing an expanded gap between the subpelliclar microtubules and the microtubule quartet (Fig. 5 E). This was not observed in any of 24 cross sections of the uninduced cells (Fig. 5 E). The cell body FAZ filament assembly initiates in the correct position in the ClpGM6 RNAi cells but is broader, suggesting that there is no feedback between the length of the FAZ filament and the expression of its constituents.

In this context, we prepared two further cell lines with RNAi targeted to different parts of the *ClpGM6* gene via hairpin constructs with greater penetrance than the original construct (Fig. S1 H), which produced double-stranded RNA from opposing promoters. Both confirmed the specificity of the initial epimastigote-like phenotype (Fig. S1 I). At later induction time points, the level of ClpGM6 was reduced below detectable amounts (Fig. S1 I), and the absence of ClpGM6 led to the accumulation of cells failing division (Fig. S1 I and J), with very short multiple FAZ filaments (Fig. S1 K).
Figure 5. FAZ length correlates with amount of ClpGM6 but not with amount of FAZ filament constituents. (A) A plot of the length of the FAZ versus the total anti-ClpGM6 staining intensity in the FAZ. Each data point represents a single 1K1N cell. Circles, 19 uninduced cells; diamonds, 21 cells with ClpGM6 RNAi induced for 24 h; triangles, 15 cells with ClpGM6 RNAi induced for 48 h; squares, 18 cells with ClpGM6 RNAi induced for 72 h. The red line denotes a fit by an exponential function, which approaches the plateau value of 19.2 µm and intercepts the y axis at 2.9 µm. (B) A plot of the length of the FAZ versus the total anti-FAZ1 staining (L683 antibody) intensity in the FAZ. Each data point represents a single 1K1N cell. Circles, eight uninduced trypomastigote (Tryp.) cells; squares, nine ClpGM6 RNAi-induced epimastigote-like (Epi.) cells. (C) 1K1N cells stained with the L683 antibody recognizing FAZ1 in the FAZ filament. Phase images of an uninduced cell (left) and ClpGM6 RNAi-induced cells overlaid with the IF signal of L683 (green) and the fluorescence signal of DAPI (magenta). (D) 1K1N cells stained with the DOT1 antibody recognizing the FAZ filament. Phase images of an uninduced cell (left) and ClpGM6 RNAi-induced cells overlaid with the IF signal of DOT1 (green) and the fluorescence signal of DAPI (magenta). (E) TEM images of the FAZ region in an uninduced cell (left) and two cells with ClpGM6 RNAi induced for 72 h, illustrating that in induced cells, the electron-dense FAZ filament (brackets) is wider than in uninduced cells. Bars: [C and D] 4 µm; [E] 150 nm.

simple modulations of expression of cytoskeletal regulatory proteins that alter the organization of complex modular units.

Materials and methods

ClpGM6 gene

Sequencing of the genome revealed that the repeats previously described by Müller et al. (1992) existed in a large protein identified initially by the gene identifier Tb11.47.0036 (obtained from TriTrypDB). Subsequent genome releases clarified that the gene was larger, and it became encompassed by two gene identifiers Tb11.47.0036 and Tb11.57.0008 (obtained from TriTrypDB, Fig. S1 A). Tb11.57.0008 does not have a stop codon, and the sequence finishes in the middle of a repeat, and Tb11.47.0036 begins within a repeat and possesses a stop codon. The genome sequence annotation has likely collapsed the repeat region, but our Southern blot analysis is consistent with the ClpGM6 protein being encoded by a single gene on chromosome 11 with calpain-like domains in the N-terminal and C-terminal regions and a large repetitive central core of nearly identical repeats. Furthermore, because of its extreme size, the ClpGM6 gene cannot be easily amplified using available molecular biology technologies.

For Southern blotting, 5 µg genomic DNA was digested with 25 U HindIII and 0.5 M NaCl for 30 min to denature the DNA and soaked in 0.5 M Tris-HCl, pH 7, and 3 M NaCl for 30 min. The DNA was transferred to Hybond membrane (GE Healthcare) by blotting overnight with 20× SSC (3 M NaCl and 0.3 M sodium citrate). A digoxigenin-labeled probe was produced using the DIG High Prime DNA Labeling and Detection kit (Roche). The probe was used to detect the presence of the ClpGM6 gene using the DIG High Prime Labeling and Detection kit. The membrane was probed with the C-terminal probe before stripping and probing with the N-terminal probe. Reagents were purchased from Sigma-Aldrich unless stated otherwise.

Cell growth, transfection, and RNAi induction

T. brucei procyclic cells of the strain 29–13 were grown at 28°C in SDM-79 medium (Gibco) supplemented with 10% of FCS (Brun and Schönberger, 1979; Wirtz et al., 1999). The cultures were maintained between 2 × 10^5 and 2 × 10^6 cells/ml. Cell densities were determined using a CASY Cell Counter (Roche).

To create a ClpGM6 knockdown cell line, a 331-bp gene-specific sequence coding for the GM6 repeats was amplified by PCR and cloned between the opposing tetracycline-inducible T7 promoters of the p2T-177 vector using BamHI and XhoI restriction sites (Wickstead et al., 2002). To prepare hairpin constructs, two fragments (construct #1: nucleotides 4,302–4,960; construct #2: nucleotides 3,069–3,762) located N-terminally of the repeats with no identity to any other sequence in the T. brucei genome were amplified by PCR and cloned into the pQuadra vector (Inoue et al., 2005). To create a cell line inducibly expressing BARP, the open reading frame of Tb927.9.15630 (obtained from TriTrypDB) was amplified by PCR and inserted into the pDEX777 vector (Poon et al., 2012) using HindIII and BamHI restriction sites. All constructs were linearized by digestion with NotI, and 29–13 cells were transfected following a standard protocol (McCulloch et al., 2004). Cells with a stably integrated vector were selected in the presence of 5 µg/ml phleomycin and cloned by limiting dilution. ClpGM6 RNAi or BARP expression was induced by the addition of doxycycline to the final concentration of 1 µg/ml to the medium. Cultures to be analyzed for BARP expression were grown for 16 h before harvesting in the presence of 5 mM metalloprotease inhibitor bathophenanthroline disulfonic acid (Urwiler et al., 2007). The ClpGM6/calflagin double-labeled cell line was created by modifying the Sm.OxP927 cell line (Poon et al., 2012). The EYFP gene was inserted into one of the endogenous ClpGM6 alleles at the 5’ end of the open reading frame, and the mCherryFP gene was inserted into one of the endogenous calflagin alleles at the 3’ end of the open reading frame.

Anti-ClpGM6 polyclonal antibody

The polyclonal rabbit antibody against two peptides within the GM6 repeats (KASDSRSFLDPMPEC and CERRKLIAEDREGN) was prepared by
Covabob and immunopurified using a column with the coupled peptides. The final concentration of the antibody stock was 0.25 mg/ml.

IF

*T. brucei* procyclic cells were grown to densities of 0.5–1.0 x 10⁷ cells/ml. Cells were washed with PBS, resuspended to 2 x 10⁷ cells/ml in PBS, and settled on microscope slides. Cells were then either directly fixed in –20°C methanol or, to extract cytoskeletons, incubated in PFME (100 mM Pipes, pH 6.9, 1 mM MgSO₄, 2 mM EGTA, and 0.1 mM EDTA) + 1% Igepal CA-630 for 4 min. Subsequently, the cytoskeletons were fixed in –20°C methanol, in which they were stored for 20 min to several weeks.

Before incubating with primary antibodies, both whole cells and cytoskeletons were rehydrated in PBS. The polyclonal rabbit anti-ClpGM6 antibody was used at a final concentration of 0.5 µg/ml and detected with goat anti-rabbit IgG (Fc [fragment crystallizable] specific secondary antibody conjugated with TRITC (Jackson ImmunoResearch Laboratories, Inc.). Mouse monoclonal IgM anti-F21 antibody L683 (Kohl et al., 1999) was diluted 1:3 in PBS. Mouse monoclonal IgM antibody DOT1 (Woods et al., 1989) was used neat. Both mouse monoclonal IgM antibodies were detected with a goat anti-mouse IgM–specific secondary antibody conjugated with Alexa Fluor 488 (Invitrogen). Finally, all samples were mounted into 90% glycerol in 50 mM phosphate buffer, pH 6.9, 1 mM MgSO₄, 2 mM EGTA, and 0.1 mM EDTA + 1% Igepal CA-630 for 4 min. Subsequently, the cytoskeletons were fixed in –20°C methanol, in which they were stored for 20 min to several weeks.

Online supplemental material

Fig. S1 shows that Tb11.57.0008 and Tb11.47.0036 fragments are parts of the same ClpGM6 gene, the product of which localizes to the flagellar side of the FAZ region and is important for cytokinesis. Fig. S2 shows a cartoon of a *T. brucei* cell transitioning from trypomastigote to epimastigote-like morphology as a result of depletion of ClpGM6. Video 1 shows an epimastigote-like cell, which is actively motile and moves in a similar manner to uninduced cells. Video 2 shows a dividing epimastigote-like cell with the tip of its new flagellum attached to the side of the old flagellum by the FC. Table S1 summarizes morphological measurements of 1K1N epimastigote-like and trypomastigote cells and is provided online as an Excel file. Table S2 shows morphological measurements of 2K2N epimastigote-like and trypomastigote cells and is provided online as an Excel file. Online supplemental material is available on http://www.jcb.org/cgi/content/full/jcb.201312067/DC1.

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