Activation of Endocytosis as an Adaptation to the Mammalian Host by Trypanosomes

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Immune evasion in African trypanosomes is principally mediated by antigenic variation, but rapid internalization of surface-bound immune factors may contribute to survival. Endocytosis is upregulated approximately 10-fold in bloodstream compared to procyclic forms, and surface coat remodeling accompanies transition between these life stages. Here we examined expression of endocytosis markers in tsetse fly stages in vivo and monitored modulation during transition from bloodstream to procyclic forms in vitro. Among bloodstream stages nonproliferative stumpy forms have endocytic activity similar to that seen with rapidly dividing slender forms, while differentiation of stumpy forms to procyclic forms is accompanied by rapid down-regulation of Rab11 and clathrin, suggesting that modulation of endocytic and recycling systems accompanies this differentiation event. Significantly, rapid down-regulation of endocytic markers occurs upon entering the insect midgut and expression of Rab11 and clathrin remains low throughout subsequent development, which suggests that high endocytic activity is not required for remodeling the parasite surface or for survival within the fly. However, salivary gland metacyclic forms dramatically increase expression of clathrin and Rab11, indicating that emergence of mammalian infective forms is coupled to reacquisition of a high-activity endocytic-recycling system. These data suggest that high-level endocytosis in Trypanosoma brucei is an adaptation required for viability in the mammalian host.

Trypanosoma brucei is a protozoan parasite causing African sleeping sickness in humans and nagana in animals (4). The trypanosome insect vector is the tsetse fly (Glossina spp.) (Fig. 1). Completion of the life cycle necessitates multiple differentiation events to accomplish both maturation within and transition between hosts. In the mammalian bloodstream, trypanosomes exist as proliferative long slender (LS) forms that transform into quiescent short stumpy (SS) forms in a density-dependent manner (36). LS and SS forms express the variant surface glycoprotein (VSG) as a means of immune evasion via antigenic variation (7). The nonproliferative SS forms are thought to be preadapted for survival in the tsetse fly. During a blood meal the fly ingests SS forms that differentiate into the dividing procyclic stage (PCF) in the midgut (48).

T. brucei has a complex developmental cycle within the tsetse fly (47), and the entire process requires at least 2 weeks. Once midgut procyclic forms are established, development progresses in the proventriculus, with subsequent migration to the salivary glands (41, 47, 49). In the proventriculus at least four morphological forms may be distinguished. Procyclic trypomastigotes differentiate into long trypomastigote (LT) forms with an elongated nucleus which undergo further division, producing an asymmetrically dividing epimastigote (AsDE). AsDE division generates one postdivision short epimastigote (PdSE) (41, 47). All forms can be recovered from salivary exudate produced from the foregut and mouthparts (41), but it is likely the PdSE that initiates salivary gland infection by attaching to the epithelium. These attached epimastigotes multiply and differentiate and subsequently detach into metacyclic forms, representing the final stage of the life cycle in the vector (44, 47, 49). Metacyclic forms reexpress VSG from a subset of specific VSG genes and are quiescent until inoculated into a mammalian host. They subsequently differentiate into long slender bloodstream forms (BSFs), completing the life cycle (44).

During trypanosome development, major changes to several cellular systems take place. In bloodstream forms, VSG assembles into a densely packed surface coat (7) which is lost on entering the fly, with procyclin dominating the procyclic cell surface (37–40). Endocytosis is clathrin dependent and developmentally regulated, being approximately 10-fold more active in cultured BSFs than in procyclic cells (1, 15, 29, 31). Increased endocytosis is associated with greater expression of the endocytic coat protein clathrin and the recycling endosome marker Rab11 (24, 29). Changes to expression, function, and location of endocytic proteins indicate significant remodeling of the endocytic apparatus during life cycle progression (17–20, 32). A potential immunological role for high-level endocytosis in BSFs comes from rapid capping and internalization of anti-VSG antibodies (6, 30, 32), but it is not evident whether there is also a role associated with life stages in the insect vector (18) or why the insect stage has reduced endocytic activity. Recently it has been suggested that this latter aspect protects the procyclic form from lytic factors in the blood meal (34).

Most experimental work focuses on cultured bloodstream and procyclic trypanosomes, and little is known about the cell
biology of the many uncultured stages from the insect vector. Bloodstream trypanosomes are capable of differentiating into procyclic forms in vitro (8) but cannot differentiate further into later stages of the life cycle. Here we addressed the role of endocytosis in the life cycle of T. brucei by measuring expression levels of Rab11 and clathrin in insect stages and monitoring changes that occur during differentiation.

MATERIALS AND METHODS

Trypanosomes. Several strains of trypanosomes were used for production of stumpy forms, fly infection, and in vitro culture. Bloodstream and procyclic form Trypanosoma brucei brucei strain Lister 427 was grown at 37°C in HMI-9 and SDM-79, respectively (10, 23). To generate short stumpy forms T. brucei rhodesiense EATRO 2340 (GUP 2962) was grown in rice pretreated with cyclophosphamide. Monomorphic slender forms were harvested 3 days postinfection and short stumpy forms after 7 days of infection when >80% of the population was stumpy. Bloodstream form T. brucei J10 (MCRO/ZM/74/J10 [clone 1]) was used to infect tissue culture. Cryopreserved aliquots were thawed and added to horse blood at 30°C (for clathrin, Rab11, p67, BIP, and ConA and 250 ms for TF) were used for all markers throughout. Fluorescein within a region of interest was quantified using Metamorph V3 (Universal Imaging Corp.). For presentation only, acquired gray scale images were falsely colored, enhanced, and assembled in Adobe Photoshop CS (Adobe Systems Inc.).

Statistics. To test correlation between values representing fluorescence results from different cohorts, the Mann-Whitney U test was used.

Protein electrophoresis and Western blotting. Lysates from 1×106 to 1×107 cells were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and wet blotted onto polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA), blocked with 5% milk in Tris-buffered saline−0.5% Tween 20, and probed with goat anti-rabbit immunoglobulin G (IgG) or rabbit anti-mouse IgG (Sigma) at a 1:10,000 dilution in 1% milk−Tris-buffered saline−0.5% Tween 20. Detection was by chemiluminescence and exposure to X-ray film (Kodak BioMax MR), and quantification was performed using ImageJ software (http://rsb.info.nih.gov/ij/).

In vitro differentiation. Short stumpy forms were harvested from infected mice by cardiac puncture and purified over a DE52 column by use of PEG buffer (0.488 g/liter NaH2PO4, 2.55 g/liter NaCl, 8.08 g/liter Na2HPO4, 15 g/liter D-glucose [pH 7.8]) (27). Purified cells were diluted at ~2×107 cells/ml into SDM-79−6 mM cis-aconitate and incubated at 27°C. Samples were taken at various times and lysed in SDS-PAGE sample buffer (26).

Metabolic labeling. Cells were washed twice in PBS, resuspended at 4×107 cells/ml in Met/Cys-free RPMI 1640 medium−10% dialyzed fetal bovine serum, and incubated in 37°C (BSF) or 27°C (PCF) for 1 h. Cells were pulse labeled with [35S]S-labeled Pro-mix (Amersham Biosciences) at 50 μCi/ml for 1 h and chased by addition of 3 ml complete media. A total of 107 cells were removed at each time point, pelleted, snap frozen on dry ice, and stored at −80°C. Immuno precipitation. Cell pellets were lysed with a mixture of 100 μl 25 mM Trit-Ci (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and complete protease inhibitors (Roche). Nuclei were removed by centrifugation for 20 s in a microcentrifuge, and the supernatant was transferred to a clean microfuge tube, 13 μl 10% SDS was added, and the lysate was heated at 95°C for 5 min. Samples were centrifuged, and the supernatant was diluted with 4 volumes of a mixture of 190 mM NaCl, 50 mM Tris (pH 7.5), 6 mM EDTA, 1.25% Triton X-100, and protease inhibitors and precleared with 30 μl of Pan sorbin at 4°C for 1 h followed by pelleting. To the supernatant, 2.5 μl of
anti-clathrin serum was added at 4°C overnight and 20 μl of protein A-Sepharose beads was added for 1 h at room temperature. Beads were washed twice with a mixture of 150 mM NaCl, 50 mM Tris (pH 7.5), 5 mM EDTA, 0.1% Triton X-100, and 0.02% SDS followed by two washes with a mixture of 1 M NaCl, 50 mM Tris (pH 7.5), and 0.02% Triton X-100 and finally resuspended in SDS sample buffer. Samples were separated by SDS-PAGE and exposed to a phosphorimager plate for 2 days. Radioactivity was quantified by densitometry.

Cycloheximide treatment. BSF cells at 1 × 10^6 cells/ml were incubated with 100 μg/ml of cycloheximide in complete HMI-9. Samples were collected for up to 8 h and analyzed by Western blotting.

RESULTS

Long slender and short stumpy forms have similar endocytic activities. We examined expression of marker proteins at the single-cell and multiple-cell population levels. We used clathrin, Rab5A, Rab11, and p67, well-characterized marker proteins for endosomal vesicle coats, early and recycling endosomes, and the lysosome (13, 25, 29). We included trypanosome BiP to represent an unrelated marker for the endoplasmic reticulum (3). Previous work demonstrated a correlation between endocytic activity and clathrin expression (29) and also between endocytosis and recycling activity for Rab5A and Rab11 expression, respectively (32).

Expression of clathrin, Rab5A, Rab11, and BiP in cultured BSF and PCFs and mouse-derived LS and SS cells was determined by Western blotting. No significant differences in expression levels of clathrin, Rab5A, or Rab11 were observed for any of the bloodstream forms (Fig. 2A). However, a 10-fold decrease in clathrin levels and a 5-fold decrease in Rab11 levels in cultured PCF cells compared to cultured bloodstream form were observed (Fig. 2A) that reflected reduced endocytic-recycling activity in the PCF (1, 24). We found decreased expression of BiP in PCF compared with BSF, with the PCFs expressing approximately 60% of the BSF level (see Fig. S1 in the supplemental material) rather than the threefold difference noted previously (3); this difference is likely ascribable to strain and culturing effects.

To directly quantitate endocytic activity, uptake of ConA and transferrin was assayed in cultured BSF, LS, and SS parasites. At 0 min, ConA and transferrin were seen in the flagellar pocket only (Fig. 3A), but after 10 min similar internal levels of ConA and transferrin were found in all cell types, and these levels remained unchanged for up to 30 min (Fig. 3B). These data suggest no significant difference in levels of receptor-mediated endocytosis between BSF, LS, and SS (Fig. 3B) and together with the Western blot data suggest similar endocytic activities for cultured BSF and in vivo LS and SS forms. Further, no significant alteration to endocytic activity accompanies differentiation of LS to SS forms.

Validation of immunofluorescence for quantification. We compared Western blot quantitation with analysis of the same cell batches by immunofluorescence. We found equal levels of clathrin and Rab11 in in vitro- and in vivo-cultured bloodstream forms and 10- and 5-fold decreases in the levels of clathrin and Rab11, respectively, in PCFs (Fig. 2B and 4B). No significant change in p67 levels was observed by immunofluorescence between life stages, and only a small decrease in BiP expression in the PCF was observed (Fig. 2B and 4B; also see Fig. S2 in the supplemental material). Thus, results showing a high degree of coincidence between endocytic activity and quantitation of clathrin and Rab11 by both Western blotting and immunofluorescence were obtained.

Down-regulation of endocytic activity during differentiation to procyclic forms. Expression of clathrin, Rab11, and p67 was
determined using trypanosomes isolated from tsetse flies 24 h after infection (Fig. 4A). *T. brucei* J10 BSFs express levels of clathrin and Rab11 similar to those seen with cultured BSFs (data not shown), but after differentiation in the insect midgut, expression of clathrin and Rab11 was reduced 10- and 5-fold, respectively, levels similar to those seen with cultured PCFs (Fig. 4A and 4B) and indicating that endocytic remodeling is a rapid event occurring within 24 h.

We exploited an in vitro model to follow the kinetics of differentiation more closely (28, 50) using the well-characterized pleomorphic cell line EATRO 2340. Trypanosomes enriched in stumpy forms (>80%) were harvested from infected mice and placed under in vitro differentiation conditions. Differentiating trypanosomes were analyzed for VSG, CAP 5.5, a stage-regulated cytoskeleton-associated protein induced during differentiation (21, 22, 28), the endocytic markers clathrin and Rab11, and also cell number (Fig. 5). Coomassie staining of total protein lysates revealed loss of VSG after 4 h (Fig. 5A), while parasite numbers remained constant for ~14 h until reentry into the cell cycle (Fig. 5B). CAP5.5 appeared by 8 h of differentiation (Fig. 5C). These data are consistent with prior analyses of this transition (28). Clathrin levels remained unchanged at 14 h but decreased by 24 h, which is consistent with the rapid attainment of PCF expression levels in midgut trypanosomes (Fig. 5A and C). Rab11 expression decreased more rapidly (Fig. 5A and C). Differential loss of Rab11 and clathrin could have resulted from delayed deactivation of clathrin expression or via the intrinsic half-lives of the proteins. The half-life of clathrin was estimated by pulse-chase metabolic labeling and immunoprecipitation, and in both BSF and PCF ~50% of the clathrin was still present at 24 h, indicating a stable protein (Fig. 6A). By contrast, Rab11 levels diminished to less that 30% after only 6 h (Fig. 6B). Therefore, differential loss of clathrin and Rab11 during differentiation can be accounted for by the protein half-life.

**Endocytosis is upregulated specifically in metacyclics.** Multiple life stages of trypanosomes in the insect vector were harvested by dissecting the midgut, proventriculus, and salivary glands of tsetse flies 30 days following infection. Expression of Rab11 and clathrin in 30-day midgut PCFs was similar to that seen with cultured or 24-h midgut PCFs (Fig. 7A). From the proventriculus, LT, AsDE, PdSE, and PdLE stages were obtained. Clathrin and Rab11 expression remained at PCF levels, suggesting no reactivation of endo-
cytosis in trypanosomes during proventricular development (Fig. 7A). Trypanosomes were obtained as epimastigote and metacyclic forms from the salivary glands. Expression of clathrin and Rab11 was increased threefold in epimastigotes compared to midgut stages (Fig. 7A) and was increased fivefold in metacyclics, bringing expression of both antigens close to BSF levels (Fig. 7C). Levels of the lysosomal marker p67 showed no significant change between life cycle stages in the midgut, proventriculus, or salivary glands (Fig. 7B and 7C), but there was a reduction in total levels of BiP in several life stages within the tsetse fly (Fig. 7C). As parasites move from midgut to proventriculus, they become elongated, with reduced cell volume. Decreased volume can account for much of the variation in total BiP levels. We also observed that the positions of clathrin and Rab11 remained anterior to the kinetoplast as the nucleus relocated from the

FIG. 4. Clathrin and Rab11 levels are similar in the cultured and midgut (MG) procyclic forms of T. brucei. (A) Clathrin, Rab11, p67, and BiP in midgut procyclic forms of T. brucei after 24 h of tsetse fly infection. Immunofluorescence of midgut PCFs, showing parasites stained with antibody to clathrin, Rab11, BiP (red), and p67 (green), is presented. Cells were counterstained with DAPI (blue) for DNA. Phase-contrast images are shown below the respective fluorescence images. Scale bar, 2 μm. Note that locations of antigens are highly similar to those of cultured PCFs (Fig. 2B). (B) Quantitation of fluorescence for clathrin, Rab11, p67, and BiP in midgut PCFs 24 h after a blood meal. The levels of clathrin and Rab11 in midgut cells are similar to cultured PCF levels. Numbers in parentheses indicate the numbers of cells analyzed for each life cycle stage. Values show the mean and standard deviations. **, statistically significant difference compared to bloodstream levels at P < 0.001.
DISCUSSION

Endocytosis and recycling are developmentally regulated in trypanosomes, but the role that the processes play in parasite survival remains ill defined (12). High endocytic activity correlates with VSG expression and rapid internalization of surface-bound antibodies (5, 7, 30). Studies have been restricted to cultured BSF and PCF cells and ignored the quiescent stages, i.e., stumpy BSFs and metacyclics, and those life stages present within the tsetse fly. By analysis of tsetse fly-derived material and in vitro differentiation, we have begun to explore these aspects.

Expression of clathrin and Rab11 is rapidly attenuated upon entry of the BSF trypanosome into the fly and is complete after 24 h. Based on the respective turnover rates of these two proteins and the expression profile during in vitro differentiation, it is likely that down-regulation of both Rab11 and clathrin occurs early in differentiation. As protein is lost during the early and nonreplicating periods of differentiation, this must be active turnover and not simply a result of dilution. Expression of endocytic and recycling markers remains low throughout the greater part of the period in the tsetse fly, indicating that high endocytic activity is not required either for defense against insect immune factors or for remodeling of surface coat molecules (46). Reactivation of clathrin and Rab11 is observed in salivary gland stages, particularly metacyclics, and is coincident with reactivation of VSG, but this does not include p67, the lysosomal marker, which is expressed at a constant level (Fig. 8). The relative positions of the early and recycling endosomes with respect to the kinetoplast remain remarkably constant such that these compartments are
FIG. 7. Expression of clathrin, Rab11, BiP, and p67 in T. brucei during development in tsetse flies. Localization of antigens in insect stages of T. brucei after 30 days of infection in flies. Immunofluorescence of PCF in midgut, LTs, AsDEs, PdLE, and PdSE in the proventriculus, and epimastigotes (Epi) and metacyclic forms (Meta) in salivary glands. Phase-contrast images are shown below the respective fluorescence images. Representative images shown here are false colored and exposure enhanced for presentation purposes. Scale bar, 2 μm. (A) Clathrin and Rab11. Parasites are stained for CLH and Rab11 (red) and costained with DAPI (blue) for DNA. (B) BiP and p67. Parasites were stained for BiP (red) or p67 (green) and costained with DAPI (blue) for DNA. (C) Quantitation of clathrin, Rab11, BiP, and p67 fluorescence. Numbers in parentheses indicate the numbers of cells analyzed for each life cycle stage; values show means and standard deviations. * and **, statistically significant difference compared to BSF levels (P < 0.01 and P < 0.001, respectively).
reactivation of high-level expression of clathrin and Rab11 from a restricted set of metacyclic-specific VSG genes (6, 45). To human serum trypanosome lytic factor (9) and express VSG as a possibility.

Down-regulation may simply conserve energy, as the contribution to ATP consumption is considerable, and the endocytosis being a factor that is required for survival within the mammalian host. By contrast, levels of p67, a lysosomal marker, are similar through the entire life cycle. Levels of BiP, an endoplasmic reticulum marker, are higher in the mammalian bloodstream forms and slightly reduced in the insect stages; the lower level of BiP, particularly in the proventricular forms, is due to the smaller size of the parasites. The model assumes that recycling activity is proportional to expression levels of Rab11 and that endocytosis is similarly proportional to clathrin protein levels, an assumption that is supported by published work (29, 32, 33). Shading is used to indicate separate life cycles, with the transition of BSF to PCF arbitrarily considered to be the start or end of a cycle. Clathrin is shown in blue, Rab11 in yellow, p67 in green, and BiP in red. Epi, epimastigote; Meta, metacyclic form.

FIG. 8. Activity of endocytic and recycling systems through the trypanosome life cycle. Endocytic and recycling activity is high in BSF stages; this activity is retained following differentiation to the nonproliferative stumpy form. Both systems are rapidly down-regulated following differentiation to the insect form within 24 h. Both recycling and endocytosis remain at low levels throughout development in the tsetse fly except for the latter stages. In the metacyclic form, expression of Rab11 and clathrin increases, probably as a component of preadaptation to the mammalian host. In support, levels of p67, a lysosomal marker, are similar through the entire life cycle. Levels of BiP, an endoplasmic reticulum marker, are higher in the mammalian bloodstream forms and slightly reduced in the insect stages; the lower level of BiP, particularly in the proventricular forms, is due to the smaller size of the parasites. The model assumes that recycling activity is proportional to expression levels of Rab11 and that endocytosis is similarly proportional to clathrin protein levels, an assumption that is supported by published work (29, 32, 33). Shading is used to indicate separate life cycles, with the transition of BSF to PCF arbitrarily considered to be the start or end of a cycle. Clathrin is shown in blue, Rab11 in yellow, p67 in green, and BiP in red. Epi, epimastigote; Meta, metacyclic form.

Among models to explain why reactivation of endocytosis accompanies reacquisition of mammalian infectivity we envisage three possibilities: that endocytosis represents a component of the immune evasion system, that there is a requirement for high rates of endocytosis and recycling due to the VSG coat, and that there is a nutritional requirement. In endemic areas many of the available hosts may have been previously infected with T. brucei and harbor an antibody response to metacyclic and early expressed VSGs, suggesting a need for avoiding antibody recognition immediately on entering a mammalian host. Association of endocytosis with removal of immunoglobulin from the parasite surface is clearly supportive of such a role (6, 32). The second hypothesis, that VSG expression necessitates rapid endocytosis and recycling, is supported both by the demonstration that interfering with VSG expression compromises the ability of parasites to progress through the cell cycle (42) and by the presence of VSG myristate proofreading activity at the cell surface (11), suggesting a VSG monitoring process. We consider the nutrition hypothesis to be the least likely due to the results showing similar growth rates for BSF and PCF in vitro and to direct evidence that endocytosis in the PCF is not growth rate limiting (33).

In summary, we demonstrate a strong correlation of expression levels of clathrin and Rab11 with expression of VSG and mammalian infectivity, which indicates that remodeling of the endocytic system is a component of the program of BSF-to-PCF differentiation and also of metacyclogenesis in tsetse flies. These data clearly imply that the endocytic and recycling pathways are necessary for survival in the mammalian host.

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