Clathrin-Dependent Targeting of Receptors to the Flagellar Pocket of Procyclic-Form *Trypanosoma brucei*

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In *trypanosomatids*, endocytosis and exocytosis occur exclusively at the flagellar pocket, which represents about 0.43% of the pellicle membrane and is a deep invagination of the plasma membrane where the flagellum extends from the cell. Receptor molecules are selectively retained at the flagellar pocket. We studied the function of clathrin heavy chain (TbCLH) in the trafficking of the flagellar pocket receptors in *Trypanosoma brucei* by using the double-stranded RNA interference approach. It appears that TbCLH is essential for the survival of both the procyclic form and the bloodstream form of *T. brucei*, even though structures resembling large coated endocytic vesicles are absent in procyclic-form trypanosomes. Down-regulation of TbCLH by RNA interference (RNAi) for 24 h rapidly and drastically reduced the uptake of macromolecules via receptor-mediated endocytosis in procyclic-form trypanosomes. This result suggested the importance of TbCLH in receptor-mediated endocytosis of the procyclic-form trypanosome, in which the formation of large coated endocytic vesicles may not be required. Surprisingly, induction of TbCLH RNAi in the procyclic *T. brucei* for a period of 48 h prohibited the export of the flagellar pocket-associated transmembrane receptor CRAM from the endoplasmic reticulum to the flagellar pocket, while trafficking of the glycosylphosphatidylinositol-anchored procyclin coat was not significantly affected. After 72 h of induction of TbCLH RNAi, procyclics exhibited morphological changes to an apolar round shape without a distinct structure of the flagellar pocket and flagellum. Although trypanosomes, like other eukaryotes, use similar organelles and machinery for protein sorting and transport, our studies reveal a novel role for clathrin in the secretory pathway of trypanosomes. We speculate that the clathrin-dependent trafficking of proteins to the flagellar pocket may be essential for the biogenesis and maintenance of the flagellar pocket in trypanosomes.

African trypanosomes are unicellular eukaryotic flagellates causing sleeping sickness in humans and related diseases in livestock. These diseases are endemic in large parts of tropical Africa and are generally fatal if left untreated. *Trypanosoma brucei* has a biphasic life cycle alternating between a mammalian host and its transmitting insect vector, the tsetse fly (41, 42). The bloodstream form of *T. brucei* is covered with a dense surface coat, made of the variant surface glycoprotein (VSG). By undergoing antigenic variation of the VSG coat, the parasite escapes attack by the host immune system (6, 10). When the parasite is ingested by a tsetse fly, it differentiates into the procyclic form, and its VSG coat is shed and replaced by a coat of procyclin (procyclic acidic repetitive protein).

*T. brucei*, an extracellular organism, depends on its host for nutrients for growth and development. The presence of subpellicular microtubule sheath underneath the plasma membrane has prohibited pinocytosis through the cell surface as means of nutrient uptake (2, 4, 41, 45, 46). This subpellicular microtubule sheath is absent in the flagellar pocket, a deep invagination of the plasma membrane where the flagellum extends from the cell. Thus, the flagellar pocket is the only site where endocytosis and exocytosis take place in trypanosomatids (2, 4, 9, 11, 18, 25, 26, 31). In the secretory pathway, membrane proteins are exported from the endoplasmic reticulum (ER) to the flagellar pocket. From the pocket, the surface coat proteins and some invariant surface glycoproteins are continuously moved onto the surface covering the cell body and the flagellum while receptors for the uptake of macromolecules are exclusively retained at the flagellar pocket (7, 25, 26, 31, 48). In the endocytic process, endocytic vesicles pinch off from the pocket membrane and then the cargo proteins are either delivered to the endosomal/lysosomal compartment or recycled to the surface. The flagellar pocket, representing ~0.43% of the pellicle membrane, exhibits a relatively high internalization rate. Accordingly, the pocket membrane is internalized every 2 min in the bloodstream form trypanosome (9). Currently, the key factors controlling the biogenesis and maintenance of the flagellar pocket are not clear and mechanisms involved in bidirectional trafficking via the flagellar pocket remain to be determined.

In *T. brucei*, glycosylphosphatidylinositol (GPI)-anchored surface coat proteins dominantly cover the plasma membrane. Thus far, only two receptor proteins located at the flagellar pocket of *T. brucei* have been characterized: (i) the GPI-anchored bloodstream form transferrin receptor complex (12, 21, 37, 39), and (ii) a cysteine acidic repetitive transmembrane protein, CRAM, a putative lipoprotein receptor of procyclic trypanosomes (19). CRAM is a transmembrane receptor protein and is abundantly expressed in procyclins. Molecular determinants involved in the exocytosis and endocytosis of these membrane proteins have not yet been characterized.

In higher eukaryotes, trafficking between organelles, either on the secretory pathway or during endocytosis, is mediated by membrane-bound vesicular structures. Individual proteins are...
dependent on intrinsic signals dictating their trafficking fate (5, 16, 35). Little is known about the signaling processing of the protein trafficking in trypanosomes. Ultrastructural analysis and the identification of protein factors associated with transport vesicles suggest that machinery similar to that of higher eukaryotes may operate in trypanosomes (4, 23, 25, 26). Endocytosis in bloodstream form trypanosomes occurs by formation of spiny-coated vesicles of 100 to 150 nm in diameter, resembling clathrin-coated vesicles, which pinch off from the flagellar pocket membrane (1, 18, 24, 44, 47). Receptor-mediated endocytosis also takes place in procyclic-form trypanosomes, although structures resembling large clathrin-coated endocytic vesicles are rarely seen (18). Procyclic trypanosomes may take up macromolecules in smooth vesicles associated with the flagellar pocket (18).

Clathrin-based vesicular trafficking is a conserved mechanism in higher eukaryotes; proteins and lipids are transported from the plasma membrane or trans-Golgi network (TGN) by this mechanism (17, 32, 38, 40). The clathrin-coated vesicles range in diameter from 700 to 900 Å. Cytosolic clathrin forms a triskelion structure, which consists of a trimer of 190-kDa heavy chains radiating from a central hub, each with an associated ~25-kDa light chain. During vesicle formation, clathrin interacts coordinately with other coat proteins, such as adaptors, and selectively recruits specific cargo molecules into budding vesicles (16, 38). In the secretory pathways, COPII-coated vesicles drive selective export from the ER to the Golgi and COPI vesicles mediate retrograde transport within the Golgi and between the Golgi and the ER (35).

To facilitate the study of protein trafficking in trypanosomes, several laboratories initiated the study of adaptor complexes and clathrin in T. brucei (24). T. brucei Clathrin heavy-chain (TbCLH) is highly expressed in the bloodstream form, which correlates with the need for a relatively high rate of endocytosis in this stage of the parasite. In contrast, TbCLH is expressed at a relatively low level in the procyclic form, suggesting that endocytosis in procyclics may be relatively inefficient. TbCLH is distributed throughout the posterior end of the bloodstream form cell, being found on large vesicles and tubular structures. By immunoelectron microscopy, Morgan et al. demonstrated that clathrin is localized to collecting tubes at the flagellar pocket and is also associated with the TGN (24). The subcellular localization suggested that TbCLH most probably has conserved functions—mediating endocytosis and transport from the TGN to the endosome/lysosomal compartment in trypanosomes. Apparently, down-regulation of TbCLH in bloodstream form trypanosomes resulted in an enlarged flagellar pocket, suggesting that TbCLH may be essential for the maintenance of the dynamics of the flagellar pocket membrane (1).

We present a functional study of TbCLH in procyclic trypanosomes, in which the function of large coated vesicles may not be required for endocytosis. Surprisingly, we found that down-regulation of TbCLH selectively prohibits the export of receptors from the ER to the flagellar pocket. These data represent the first example of the functional importance of clathrin in the secretory pathway in trypanosomes. We speculate that TbCLH plays an essential role in the bidirectional trafficking at the flagellar pocket.

MATERIALS AND METHODS

Trypanosomes. The procyclic form of T. brucei was maintained at 25°C in SDM-79 medium supplemented with 10% fetal bovine serum (8). Bloodstream form trypanosomes were maintained in HMI-9 medium at 37°C (14). The procyclic trypanosome cell line 29–13 and the bloodstream form cell line 13–90, which harbor the T7 RNA polymerase gene and the tetracycline repressor gene, were obtained from G. Cross’s laboratory and were used as the parental strains for transformation using tetracycline-inducible double-stranded RNA (dsRNA) expression constructs (49). For all transformations, early- to mid-log-phase trypanosomes (procyclic trypanosomes at a cell density of 5 × 10^6 to 8 × 10^6 cells/ml and the bloodstream form at a cell density of 2 × 10^5 cells/ml) were used. For induction of dsRNA, trypanosomes were cultured in medium containing 1.0 µg of tetracycline per ml.

Antibodies. Polyclonal antibody against ESA66 was a gift from the laboratories of Peter Overath and Piet Borst (27, 39). Monoclonal antibody p67 and the rabbit-derived polyclonal anti-Bip antibody were gifts from Jay Bangs (3, 15). Anti-CRAM antibody was as previously described (19). The rabbit-derived anti-TbCLH polyclonal antibody was raised against a glutathione-S-transferase–TbCLH recombinant protein containing the TbCLH coding region spanning amino acids 761 to 1007. The monoclonal anti-EP procyclin (containing glutamic acid [E]-proline [P] repeats) antibody was purchased from Cedarlane.

Stable DNA transformation. Linearized plasmid (10 or 20 µg) was electroporated into trypanosomes using a BTX electroporator as previously described (36). At 36 and 16 h after electroporation of the procyclic form and the bloodstream form, respectively, G418 (20 µg/ml for procyclics and 2 µg/ml for the bloodstream form), hygromycin B (40 µg/ml for procyclics and 2 µg/ml for the bloodstream form), and phleomycin (2 µg/ml for procyclics and 1 µg/ml for the bloodstream form) were added to select stably transformed trypanosomes. The individually cloned drug-resistant trypanosomes were obtained by limiting dilution using microtiter dishes. To clone procyclic transformants, 2 × 10^5 to 5 × 10^5 wild-type trypanosomes per ml were added to each well to facilitate cell growth.

RNA isolation and Northern blot analysis. RNA samples were isolated by guanidine thiocyanate lysis and purified by centrifugation through CsCl cushions. RNA samples were separated in 1% formaldehyde–agarose gels and transferred to nitrocellulose filters. Northern blots were hybridized with ^32P-labeled probes. Following hybridization, filters were washed to a final stringency of 0.1 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate at 65°C.

Western blot analysis and ELISA. Total-cell lysates of 2 × 10^7 trypanosomes were size separated in polyacrylamide gels and electrophoretically transferred to nitrocellulose filters. The nitrocellulose filters were blocked with 5% nonfat dry milk in TBST (50 mM Tris-buffered saline, 0.5% Tween and 0.1% sodium dodecyl sulfate). The nitrocellulose filters were incubated overnight. After three washes, the filters were reacted with an enhanced chemiluminescence detection system (Amersham Life Science). The protein level was quantitated by enzyme-linked immunosorbent assay (ELISA) analysis. Each well of 96-well ELISA plates was coated with 100 µl of total protein lysates of 1.5 × 10^7 trypanosomes, incubated overnight at 4°C, and then blocked with 3% nonfat milk in phosphate-buffered saline (PBS)–0.1% Tween 20 (PBST) for 1 h at room temperature. Following three washes, the plates were incubated for 1.5 h at room temperature with primary antibodies (100 µl/well) which were diluted in PBST–3% nonfat milk. After the wells were washed, 100 µl of horseradish peroxidase-conjugated secondary antibody (diluted 1:1000 in PBST–3% nonfat milk) was added to each well, and the wells were incubated for 1.5 h at room temperature and then washed. To develop the plates, 100 µl of 1:3,5,5’,tetramethylbenzidine (TMB) solution (Sigma Co.) was added to each well. The reaction was stopped by the addition of 50 µl of 2 M H_2SO_4 per well, and the optical density was measured at 450 nm with an ELISA photometer (MR5000; Dynatech).

Immunofluorescence microscopy. Trypanosomes were harvested by centrifugation for 10 min at 400 × g and washed once with PBS. The cells were suspended in 4% parafformaldehyde in PBS (pH 7.4) for 10 min at room temperature. Following fixation, the cells were dotted onto 12- well slides and fixed in cold methanol for 5 min. After rehydration in glycine (0.14 M in PBS), the slides were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h. Then the first antibody reactions were performed with PBS–0.1% Tween 20–3% BSA for 1 h and then slides were washed three times with PBS. The slides were then reacted with various fluorophore-conjugated goat-derived anti-rabbit, anti-rat, or
anti-mouse immunoglobulin G (IgG) for 1 h. After being washed with PBS, the slides were mounted with mounting medium containing 4′,6- diamino-2-phenylindole (DAPI) (H-1200; Vector Laboratories Inc.). The cells were viewed and photographed under a Nikon or Leica fluorescence microscope. The images were directly captured by a charge-coupled device (CCD) camera and analyzed by the MetaMorph program of Universal Imaging Co.

**Serum lipoprotein purification.** Different lipoprotein fractions from bovine serum were isolated by sequential ultracentrifugation (13). The range of densities recovered for high-density lipoprotein particles (HDL) is 1.063 to 1.21. Protein concentrations were determined using the bicinchoninic acid protein assay reagent (Pierce). Lipoproteins were 125I-labeled using IODO-GEN iodination reagent and precoated tubes (Pierce).

**Uptake and degradation.** For each assay, 1 or 0.5 ml of cells (10^6 cells/ml) were used. For the uptake assay, 125I-labeled ligand (IgG or HDL) was incubated at 28°C for 2 to 4 h with trypanosomes in SDM-79 serum-free medium–5% BSA in the absence or presence of a 20-fold excess of unlabeled ligands. Following incubation, the trypanosomes were washed three times with SDM-79 medium–0.2% BSA. They were then transferred to a new set of tubes and centrifuged, and the amount of radioactivity associated with the cell pellets was measured in a gamma counter and referred to as the amount of uptake.

For the degradation assay, following the incubation of trypanosomes with 125I-ligand at 28°C, trypanosomes were centrifuged and the supernatants were collected for determination of the amount of chloroform-acetic acid (TCA)-10% wt/vol)-soluble 125I-labeled products. The amount of free 125I was removed from the TCA-soluble fraction by precipitation with 5% (wt/vol) silver nitrate. The amount of radioactivity in the TCA-soluble, noniodide fraction was referred to as the amount of degradation. Total and nonspecific counts were referred to as the measurement of reactions performed in the absence and presence, respectively, of a 20-fold excess of unlabeled ligands. Specific counts were obtained by subtraction of nonspecific counts from total counts.

**RESULTS**

TbCLH RNAi reduces the growth efficiency of trypanosomes. We used the dsRNA interference (RNAi) approach to evaluate the function of TbCLH in *T. brucei*. The original TbCLH probe was obtained from the *T. brucei* genome project. Following the screening of a cDNA library and reverse transcription-PCR, we obtained the full-length TbCLH cDNA clone. We used the pZJM vector, provided by P. Englund’s laboratory, to express dsRNA of TbCLH in trypanosomes (43). A DNA fragment of 738 bp, encoding amino acids 761 to 1007 of TbCLH, was cloned into pZJM vector, resulting in pZJM-CLH, in which the TbCLH dsRNA expression is controlled by two opposing and inducible T7 promoter-tetracycline operators (43). The plasmid is designed to integrate into the rDNA nontranscribed spacer region. The linearized pZJM-CLH was introduced into the bloodstream form cell line 13–90 and the procyclic-form cell line 29–13 which express T7 RNA polymerase and tetracycline repressor (49). Clonal transformed cell lines were established and maintained under noninducing conditions. Integration of the input plasmid at the nontranscribed rDNA spacer region in each cell line was confirmed by Southern blot analysis (data not shown). Then the impact of TbCLH RNAi on cell growth was studied after induction by the addition of tetracycline. We found that in bloodstream form trypanosomes, induction of TbCLH RNAi rapidly reduced cell growth; at 8 to 12 h after induction, most of the cells had rounded up and stopped replicating (Fig. 1A). In the procyclic form, at 24 h after induction of TbCLH RNAi, cell growth rapidly declined, although 97% of the cells remained morphologically unchanged (Fig. 1B; Table 1). At 48 h after TbCLH RNAi induction, procycles started to die and a small portion of them (~18%) adopted a round shape. At 72 h after induction of TbCLH RNAi, the majority of cells (~90%) revealed a round morphology (Table 1). A representative image of the morphological changes of procycles from the polarized shape to a nonpolar round shape is shown in Fig. 2, in which cells were stained with anti-α-tubulin antibody 70 h after induction of TbCLH RNAi. In addition to the cell shape change, these round cells appeared to lack the long extended flagellum (Fig. 2). Currently, we are not certain about the mechanisms involved in the shortening of the flagellum. However, we speculated that TbCLH RNAi might have impaired the biogenesis of the flagellar pocket and the flagellum. To confirm the RNAi effect, we compared the steady-state TbCLH mRNA levels in TbCLH RNAi-induced and -noninduced trypanosomes by Northern blot analysis (Fig. 1, bottom panels). Induction of TbCLH RNAi for 12 h in the bloodstream form and for 24 h in the procyclic form indeed drastically reduced the steady-state mRNA level of TbCLH, while the α-tubulin mRNA level was not significantly changed (Fig. 1, bottom panels).

The rapid and severe lethality of TbCLH RNAi to bloodstream form trypanosomes has hampered our planned biochemical analysis of the bloodstream form TbCLH RNAi cell lines. The impact of TbCLH RNAi on the morphological changes of the bloodstream form trypanosomes was previously described in detail by Allen et al. (1). Therefore, we will not reiterate these observations in this report. However, we were surprised by the rapid and dramatic killing effect by TbCLH RNAi in procycles, in which TbCLH expression is relatively low and the formation of large coated vesicles may not be a prerequisite for endocytosis. We speculated that TbCLH may have unique and essential functions in procycles and subsequently focused our studies on the function of TbCLH in protein trafficking in procyclic-form trypanosomes.

TbCLH RNAi reduces the endocytosis efficiency of the procyclic form of *T. brucei*. We first determined the impact of TbCLH RNAi in endocytosis and protein trafficking in procycles, for which very few studies of the related issues have been documented. We have previously demonstrated that the procyclic form of *T. brucei* takes up lipoprotein particles via receptor-mediated endocytosis (20) and characterized CRAM receptor-mediated endocytosis in procycles by using anti-CRAM IgG as a ligand (22). Therefore, we chose to compare the endocytic efficiency of HDL particles and anti-CRAM IgG in procycles before and after 24 h of TbCLH RNAi induction (at this point, most of the procycles still have the wild-type morphology). It appeared that induction of TbCLH RNAi for 24 h specifically reduced the uptake of 125I-anti-CRAM to 40% (Fig. 3A) and that of 125I-HDL to 35% (Fig. 3C) in TbCLH RNAi procyclic cell lines compared to the uptake observed in noninduced cells. As a control, the uptake ability of the parental host cell line 29–13 in the presence and absence of tetracycline was measured (Fig. 3, bars 1– and 1+). Addition of tetracycline alone did not significantly affect the uptake efficiency of the cell line 29–13 (Fig. 3, bars 1+) . We next determined whether the cell-associated ligands can be efficiently internalized and degraded upon induction of TbCLH RNAi. Following incubation of trypanosomes with 125I-anti-CRAM and 125I-HDL, the supernatant was harvested and the
amount of TCA-soluble noniodide material that represents degradation products was measured. Interestingly, degraded materials could not be detected after induction of TbCLH RNAi for 24 h (Fig. 3B and D). The control experiments using cell line 29–13 showed that addition of tetracycline alone did not significantly affect the degradation efficiency of procyclins (Fig. 3B and D and bars 1/H11001). Thus, down-regulation of TbCLH drastically reduced the uptake of macromolecules and completely abolished the degradation ability of procyclins. Even though trypanosomes retained 35 to 40% of their ability to bind ligand molecules, on induction of TbCLH RNAi, these trypanosomes were incapable of internalizing and/or directing the bound ligands to endosomal/lysosomal compartments. These results suggested that TbCLH plays important roles in receptor-mediated endocytosis in procyclic trypanosomes.

TbCLH RNAi affects the subcellular localization of CRAM in procyclic-form T. brucei. Our data have demonstrated that TbCLH is essential for cell growth and receptor-mediated endocytosis in procyclic trypanosomes, demonstrating its conserved role. We further evaluated the impact of TbCLH RNAi in the trafficking fate of receptor molecules in procyclins. Since currently CRAM is the only characterized receptor molecule at the flagellar pocket of procyclic trypanosomes, we compared the subcellular localization of CRAM before and after TbCLH RNAi induction by indirect-immunofluorescence analysis. It appeared that 24 h after the induction of TbCLH RNAi, ~80% of the cells contained CRAM at the flagellar pocket while ~20% exhibited aberrant localization of CRAM, which spread throughout the cell except for nucleus and kinetoplast (Fig. 4). The uptake analysis described above was performed at this time. Thus, a small amount of the reduction of uptake after TbCLH RNAi induction could be attributed to the lack of receptors at the flagellar pocket in some cells.

Surprisingly, at 48 h after the induction of TbCLH RNAi, only 25% of cells exhibited flagellar pocket localization of CRAM; in 75% of cells, CRAM was no longer concentrated at

![Fig. 1. Effect of TbCLH RNAi on cell growth of T. brucei.](image)

**FIG. 1.** Effect of TbCLH RNAi on cell growth of T. brucei. A cell population derived from an individually transformed drug-resistant trypanosome was used for the growth analysis. The growth curves for the TbCLH RNAi trypanosome cell lines (the bloodstream form [A] and the procyclic form [B]) were determined in the absence (black squares; tet−) or presence (white squares; tet+) of 1 μg of tetracycline per ml (for induction of dsRNA expression). Cells were continuously maintained at log phase. If needed, medium was added to expand the culture. The total number of cells in each culture was calculated at different time points. The bottom panels represent the Northern blot analysis of the effects of RNAi. TbCLH and Tubulin indicate the probes used. 13-90 indicates that the RNA sample was obtained from the parental bloodstream cell line 13-90. − and + indicate that RNA samples were obtained from cells cultured in the absence or presence of tetracycline for 12 h (for the bloodstream form) or 24 h (for the procyclic TbCLH RNAi cell line), respectively.

### TABLE 1. Quantitative analysis of phenotypic changes of procyclic trypanosomes on TbCLH RNAi induction

| Phenotype                                | Phenotype after following induction period of TbCLH RNAi (%) |
|------------------------------------------|------------------------------------------------------------|
|                                          | 0   | 24 h | 48 h | 72 h |
| Round cells (%)                          | 0   | −3   | −18  | −90  |
| Cells containing CRAM at FP* (%)         | >99 | −81  | −25  | <5   |
| Anti-CRAM uptake efficiency              | 100 | 40   | ND   | ND   |
| HDL uptake efficiency                    | 100 | 35   | ND   | ND   |
| Anti-CRAM degradation efficiency         | 100 | 0    | ND   | ND   |
| HDL degradation efficiency               | 100 | 0    | ND   | ND   |

* FP, flagellar pocket.

b ND, not determined.
the flagellar pocket but was spread throughout the cell (Fig. 4). At this point, morphologically, >80% of cells still maintained a flagellated, slender wild-type shape (Table 1). At 72 h after the induction of TbCLH RNAi, >95% of cells exhibited altered CRAM distribution and morphological changes to the round shape (Fig. 4). To determine whether TbCLH RNAi may result in the release of CRAM to the entire cell surface, we subsequently performed staining with anti-CRAM antibody on live trypanosomes at 4°C. We found that induction of TbCLH RNAi for 48 or 72 h did not lead to the escape of CRAM onto the cell surface or the surface of the flagellum (data not shown).

The aberrant CRAM distribution resulting from TbCLH RNAi frequently exhibits a reticulum or tubule structure and a distinct perinuclear staining pattern. This pattern is similar to the tubular network of the ER extending throughout the cell. To better understanding the altered CRAM distribution on TbCLH RNAi induction, we performed colocalization of

FIG. 2. Morphological changes of procyclic trypanosomes on induction of TbCLH RNAi. Trypanosomes were first incubated with a monoclonal anti-α-tubulin antibody and then reacted with rhodamine-conjugated goat anti-mouse IgG. Slides were mounted with mounting medium containing DAPI. The large and small blue dots in each cell represent the nucleus and the kinetoplast, respectively. (A) Procyclic trypanosome with a wild-type shape. (B) Round cells that developed 70 h after the induction of TbCLH RNAi.

FIG. 3. Reduction of the efficiency of receptor-mediated endocytosis in procyclic-form trypanosomes by TbCLH RNAi. Procyclic TbCLH RNAi trypanosomes (10⁷ cells/ml) were incubated at 28°C for 2 h with 60 μg of ¹²⁵I-anti-CRAM IgG per ml (A and B) or with 100 μg of ¹²⁵I-HDL per ml (C and D) in serum-free SDM-79 medium containing 3% (wt/vol) BSA. The cell-associated counts were then measured and referred to as the amount of uptake (A and C). The supernatants were collected for the measurement of degradation products (B and D). The results represent the amount of specific uptake and degradation, calculated as the difference between total (in the absence of unlabeled anti-CRAM IgG or HDL) and nonspecific uptake and degradation, respectively (in the presence of 20-fold unlabeled anti-CRAM IgG or 1 mg of unlabeled HDL per ml, respectively). Results represent the average of triplicate determinations. Bars 1 represent data generated by the host cell line 29-13, and bars 2 represent data generated by the procyclic TbCLH RNAi cell line. – and + indicate experiments performed before and after the addition of tetracycline for 24 h, respectively.
CRAM with the ER-specific marker Bip, with the Golgi-specific dye BODIPY TR ceramide, and with the lysosomal/endosomal marker p67 (Fig. 5). We compared the colocalization of different markers in cells that retained their wild-type morphology after induction of TbCLH RNAi. Representative images are shown in Fig. 5. It was obvious that the majority of CRAM was colocalized with Bip at the ER after induction of TbCLH RNAi for 48 h. The colocalization of CRAM with BODIPY TR ceramide in the Golgi was reproducibly observed in some (~50%) but not all cells after 48 h of induction of TbCLH RNAi. Figure 5B shows an example of cells that have no obvious localization of CRAM in the Golgi. These results indicated that down-regulation of TbCLH may have significantly reduced the efficiency of export of CRAM from the ER. It is possible that TbCLH may be required for targeting of many receptors or flagellar pocket proteins to the flagellar pocket in procysyces. However, this hypothesis cannot be tested until receptors other than CRAM are identified in procyclic trypanosomes.

We also determined whether TbCLH RNAi affects the transport of membrane proteins from the ER to the endosom/lysosomal compartment. The distribution of p67 was compared in procysyces with and without TbCLH RNAi induction (Fig. 5). Overall, p67 remained located at a single specific area between the flagellar pocket and the nucleus on induction of TbCLH RNAi (Fig. 5). This result suggested that the trafficking of p67 most probably is not dependent on TbCLH. Additionally, we did not observe a significant amount of CRAM accumulating in the lysosomal/endosomal compartment.

Since surface coat proteins also travel from the ER to the Golgi and TGN and then to the flagellar pocket membrane and spread over the entire cell surface, as a comparison we compared the distribution of procyclin before and after the induction of TbCLH RNAi (Fig. 5). The procyclic 29-13-derived cell lines predominantly express the EP-procyclin. It appeared that induction of TbCLH RNAi did not significantly affect the distribution of the EP-procyclin over the entire cell surface, even though it prevented the export of CRAM from the ER to the flagellar pocket. This result suggested that at least two secretory pathways may exist in procyclic-form trypanosomes and that they can be distinguished by the requirement of TbCLH.

At 72 h after TbCLH RNAi induction, most of the procysyces had assumed an apolar round shape. The morphological changes have made it difficult to perform a precise evaluation of the colocalization. However, we found that p67 exhibited a highly dispersed distribution in round cells (data not shown). We speculate that at this stage, many morphological or physiological changes of various organelles along the trafficking pathway may directly or indirectly result from TbCLH RNAi.

TbCLH RNAi up-regulates the steady-state CRAM protein level in the procyclic form of T. brucei. Our studies suggested that TbCLH RNAi affected both inbound and outbound trafficking of CRAM in procysyces. These events may ultimately lead to a change of the turnover rate and the steady-state expression level of CRAM. In the subcellular localization experiments, we often observed that on induction of TbCLH RNAi, most cells exhibited a relatively intense staining with anti-CRAM antibody in addition to having an aberrant localization of CRAM. This observation suggests that either CRAM is more accessible to the antibody or the level of the CRAM protein is elevated. We thus compared the mobility and the steady-state expression level of CRAM before and after TbCLH RNAi induction by Western blot analysis. At 24, 48, and 72 h after induction of TbCLH RNAi, total-cell extracts from the same number of trypanosomes were analyzed (Fig. 6A). Surprisingly, we found that the level of CRAM protein expression was drastically increased on induction of TbCLH RNAi while the mobility of CRAM remained unchanged (Fig. 6A). These results indicated that down-regulation of TbCLH up-regulated the CRAM protein level and most probably did not significantly affect the posttranslational modifications of CRAM. In contrast, the protein level of procyclin and α-tubulin is not affected by TbCLH RNAi (Fig. 6A, bottom panels). We further quantitated the CRAM protein level by ELISA, using the level of α-tubulin protein as an internal control (Fig. 6B). We estimated that 24, 48, and 72 h of TbCLH RNAi induction increased the CRAM protein level by 1.3-, 2.5-, and 4.2-fold, respectively (Fig. 6B). To confirm the
FIG. 5. Colocalization of CRAM with different markers in procyclics before and after TbCLH RNAi induction. (A) Colocalization analysis of procyclic cells prior to TbCLH RNAi induction. (B) Colocalization analysis of procyclics 48 h after TbCLH RNAi induction. Staining was performed using slides containing fixed and permeabilized trypanosomes. The slides were first incubated with different sets of primary antibodies and then reacted with various fluorophor-labeled secondary antibodies. After the slides were washed, cells were mounted with mounting medium containing DAPI. The images were captured using a CCD camera and analyzed by the MetaMorph program from Universal Imaging Co. The images are presented in pseudocolors: green for FITC-labeled CRAM, red for other rhodamine-labeled markers, and blue for DAPI staining, which identifies the nucleus and the kinetoplast. Panels p67 are images from cells costained with rabbit-derived anti-CRAM (green) and mouse monoclonal anti-p67 (red) antibodies. Panels Golgi are images from cells stained with the Golgi-specific dye BODIPY TR ceramide (red). Panels Bip are images from cells stained with rat-derived anti-CRAM (green) and rabbit-derived anti-Bip (red) antibodies. Panels EP are images from cells stained with anti-CRAM (green) and the monoclonal anti-EP procyclin (red) antibody. Panels Tubulin are images from cells stained with anti-CRAM (green) and monoclonal anti-α-tubulin (red) antibodies.
TbCLH RNAi effect, we measured the TbCLH protein level. At 24 h after induction, the TbCLH protein level was reduced to 30%, and at 48 and 72 h of induction, it had been reduced to effectively 0% (Fig. 6C).

**DISCUSSION**

Clathrin heavy chain is evolutionarily conserved and ubiquitously expressed in all eukaryotic cells. The high degree of homology of clathrins from evolutionarily distant organisms, such as from trypanosomes to humans, suggests their functional equivalence. In higher eukaryotes, clathrin-coated vesicles mediate the transport of protein and lipids from the plasma membrane or TGN to lysosomal/endosomal compartments (for reviews, see references 16, 17, 38, and 40). Clathrin-coated vesicles that bud from the TGN function mainly in the sorting of newly synthesized lysosomal enzymes, while clathrin-coated vesicles that bud from the plasma membrane facilitate receptor-mediated endocytosis of ligands. Very little is known about mechanisms involved in protein trafficking in trypanosomes. Protein components involved in cytoplasmic vesicle formation in trypanosomes were only recently identified with the aid of information obtained from genome sequencing (23–26).

By ultrastructural analysis, Morgan et al. demonstrated the potential role of TbCLH in the conserved function of endocytosis at the flagellar pocket and vesicle formation at TGN (24). To delineate the function of TbCLH, we performed an analysis of protein trafficking in trypanosomes when the expression of TbCLH was down-regulated.

By using an inducible TbCLH RNAi approach, conditional trypanosome TbCLH knockout cell lines were established. It appeared that TbCLH is essential not only for the bloodstream form but also for the procyclic-form trypanosomes. Unlike trypanosomes, knocking out clathrin heavy chain did not lead to a lethal effect in yeast and *Dictyostelium*, although cell growth was impaired (29, 30, 33, 34). Apparently, in yeast, sorting to the vacuole is normally clathrin dependent, but there is another mechanism that can be switched on if the clathrin pathway is blocked (28, 33, 34). Down-regulation of TbCLH in
bloodstream form trypanosomes rapidly led to major morphological changes (rounding up) and subsequent death. By ultrastructural analysis, M. C. Field’s laboratory demonstrated that the flagellar pocket is massively enlarged in the TbCLH RNAi-induced bloodstream form trypanosome, in contrast to the plasma membrane covering other parts of the cell surface. These trypanosomes with enlarged flagellar pockets were called BigEye cells (1). The molecular detail of mechanisms involved in the formation of BigEye cells is not clear. It was hypothesized that TbCLH RNAi may inhibit the removal of the pocket membrane (endocytosis) and may not affect continued membrane delivery (exocytosis), thus resulting in the enlarged pocket membrane (1). However, this hypothesis did not explain why only the flagellar pocket membrane was expanded, since the flagellar pocket membrane is a part of the plasma membrane, continuing with the membrane covering the flagellum and other parts of cell surface. It seems that TbCLH RNAi, in addition to blocking membrane removal, may severely affect factors controlling the membrane dynamics between the flagellar pocket and other parts of the cell surface.

The expression level of TbCLH in the procyclic form is relatively low, which is consistent with the relatively low rate of endocytosis and the absence of large clathrin-coated vesicles in procyclics. Nevertheless, TbCLH is essential. A short period of induction of TbCLH RNAi in procyclic-form trypanosomes drastically reduced the amount of uptake and completely abolished the ability to degrade endocytosed ligands. This result provided strong evidence for the role of TbCLH in mediating endocytosis at the flagellar pocket of the procyclic-form trypanosome. Further subcellular localization analysis revealed the requirement for TbCLH in the transport of CRAM from the ER to the pocket, whereas TbCLH is not essential for the trafficking of the GPI-anchored procyclin coat protein. These results suggested that in trypanosomes, TbCLH is uniquely involved in the secretory pathway mediating the trafficking of a subset of membrane proteins. Ultimately, a long period (>50 h) of induction of TbCLH RNAi in procyclic trypanosomes resulted in a major morphological change; cells exhibited a round shape, a structure similar to that observed in the TbCLH RNAi-induced bloodstream form trypanosomes as documented by Field’s laboratory (1).

We have previously shown that CRAM is not an essential gene and that altering the subcellular distribution of CRAM does not affect the normal function of the cell (51). Thus, we think that blocking the export of CRAM from the ER in response to TbCLH RNAi should not be the key factor leading to cell death. We hypothesize that down-regulation of TbCLH may have also prohibited the transport of other receptors or flagellar pocket proteins to the flagellar pocket, thus affecting the function and biogenesis of the flagellar pocket and hence trypanosome viability. We were unable to address whether the transport from the TGN to the endosome/lysosomal compartment is impaired when TbCLH is down-regulated, due to the lack of well-characterized markers.

Down-regulation of TbCLH also significantly increased the steady-state level of the CRAM protein. The observation of up-regulated expression of CRAM in response to TbCLH RNAi raised the question whether the altered distribution of CRAM is a result of the overexpression of CRAM. To exclude this possibility, we examined the subcellular localization of CRAM in two previously established CRAM overexpressors in which CRAM expression was up-regulated to two- to threefold (data not shown). The high-level expression of CRAM in one cell line is the result of using the procyclin gene promoter to drive transcription of the CRAM gene, and the expression in the other cell line is the result of altering its 3’-end sequence. In these two cell lines, the localization of CRAM remains at the flagellar pocket (data not shown). Similarly, we previously also demonstrated that CRAM remained at the flagellar pocket of the bloodstream form trypanosomes when its expression was highly up-regulated (50). Thus, we concluded that the altered distribution of CRAM in induction of TbCLH RNAi is not a result of overproduction of CRAM. In contrast, the surface distribution of the transferrin receptor complex in bloodstream form trypanosomes was affected by its expression level (27). Currently we are not certain about the exact causes of the up-regulated expression of CRAM on TbCLH RNAi induction. However, our immunoprecipitation analysis of metabolically pulse-labeled cell extracts suggested that the increase of the CRAM level is most probably due to the increase in the protein half-life but not to the increase in the protein synthesis rate (data not shown).

In summary, our study has demonstrated that clathrin is likely to have unique and essential functions in both endocytosis and secretory pathways in trypanosomes.

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