Experimental Parasitology 127 (2011) 777–783

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Trypanosoma brucei brucei: Endocytic recycling is important for mouse infectivity

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ARTICLE INFO

Article history:
Received 23 June 2010
Received in revised form 8 January 2011
Accepted 10 January 2011
Available online 20 January 2011

Keywords:
Trypanosome
Protein transport
Vesicle trafficking
Endocytosis
Recycling

ABSTRACT

Endocytosis in the African trypanosome, *Trypanosoma brucei*, is intimately involved in maintaining homeostasis of the cell surface proteome, morphology of the flagellar pocket and has recently been demonstrated as a bona fide drug target. RNAi-mediated knockdown of many factors required for endocytic transport, including several small GTPases, the major coat protein clathrin and a clathrin-associated receptor, epsinR, results in rapid cell death in vitro. Rapid loss of viability in vitro precludes meaningful investigation by RNAi of the roles of trypanosome endocytosis in vivo. Here we have sought to address this issue using strategies designed to produce milder effects on the endocytic system than complete functional ablation. We created a trypanosome clathrin heavy chain hemizygote and several lines expressing mutant forms of Rab5 and Rab11, described previously. All are viable in in vitro culture, with negligible impact to proliferative rates or cell cycle. Clathrin hemizygotes express clathrin heavy chain at ~50% of wild type levels, but despite this demonstrate no defect to growth in mice, while none of the Rab5 mutants affected proliferation in vivo, despite clear evidence for effects on endocytosis. By contrast we find that expressing a dominantly active Rab11 mutant led to compromised growth in mice. These data indicate that trypanosomes likely tolerate the effects of partly decreased clathrin expression and alterations in early endocytosis, but are more sensitive to alterations in the recycling arm of the pathway.

1. Introduction

Maintaining the cell surface composition and turnover of surface components is a vital aspect of the cell biology of African trypanosomes. All endocytosis is mediated via uptake at the flagellar pocket, a small invagination at the posterior end of these cells and is by an exclusively clathrin-dependent, AP2-independent mechanism (Allen et al., 2003; Field and Carrington, 2009). Further, in bloodstream stages an efficient mechanism for removal of surface antibody, recognizing the dominant surface antigen, variant surface glycoprotein (VSG), also exists, and which may play a subsidiary role in immune evasion to antigenic variation (Barry, 1979, Field and Carrington, 2009). Antibody uptake occurs via hydrodynamic flow, and probably is independent of direct participation with cytoskeletal elements, elegantly explaining how GPI-anchored VSG-antibody complexes are efficiently and selectively targeted to the flagellar pocket (Engstler et al., 2007). The absence of AP2 is probably a specialization of trypanosomes expressing VSG, as AP2 is present in *T. cruzi* and *Leishmania* spp., but absent from all African trypanosomes. Remodeling of the endocytic system is also an intimate component of development of the parasite as it progresses between hosts (Natesan et al., 2007, 2010). Further, recent studies with an experimental trypanocide directed against *N*-myristoyltransferase that leads to endocytic defects may suggest that endocytosis is essential in vivo as well as in vitro (Allen et al., 2003; Frearson et al., 2010).

Extensive studies based on RNAi-mediated knockdown and immunolocalization have identified a cohort of proteins involved in endocytosis and recycling of VSG and other surface molecules, and together with ultrastructural studies have delineated endocytic pathways for VSG and invariant trans-membrane domain surface proteins (ISGs) (Allen et al., 2003, Hall et al., 2004a,b, 2005, Pal et al., 2003; Gabernet-Castello et al., 2009, García-Salcedo et al., 2004, Chung et al., 2008, Grünfelder et al., 2003). In brief, VSG is taken up via a clathrin/Rab5-dependent mechanism, sorted at an intracellular location that has yet to be fully defined, and recycled back to the flagellar pocket via Rab11-dependent recycling endosomes. ISGs (invariant Surface Glycoproteins) appear to be less efficiently recycled, and are extensively ubiquitylated and targeted to the lysosome for degradation (Chung et al., 2008, see Field and Carrington (2009) for review). Further, using ectopic expression of mutant isoforms of Rab4, Rab5A, Rab5B and Rab11 we have shown that Rab5A and Rab11 mediate the major pathways for uptake and recycling of VSG-bound immunoglobulin (Pal et al., 2003).

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doi:10.1016/j.exppara.2011.01.001

Despite these significant advances in determining the cell biology of the endocytic system, there is little information on the role of this pathway in vivo. This is unfortunate, as the ability to test the importance of uptake of VSG-antibody complexes and also the role of the pathway in sensitivity to other immune effectors, for example trypanosome lytic factor, remain to be determined in a physiological setting. This failure has been mainly due to the extreme phenotypes that arise following knockdown of many of the endocytic components, including the clathrin heavy chain, actin, epsinR and Rab5A and Rab11 (Allen et al., 2003; García-Salcedo et al., 2004; Gabernet-Castello et al., 2009, Hall et al., 2004a, 2004b, 2005). Each of these factors leads to significant loss of viability in vitro, confounding any attempt to separate the influence of the host environment from fundamental cellular functions. Using a clathrin heavy chain hemizygote with very moderate defects in endocytosis and several previously characterized cell lines expressing Rab mutant isoforms, we assessed the importance of endocytosis and recycling in a mouse model of infection.

2. Materials and methods

2.1. Trypanosomes, in vitro cultivation and mouse infections

Bloodstream form *Trypanosoma brucei brucei* MITat 1.2 (Lister 427) and procyclic form *T. b. brucei* MITat 1.2 were grown at 37 °C in HMI-9 or at 27 °C in SDM-79 respectively as previously described (Brun and Schönenberger, 1979; Hirumi and Hirumi, 1994). To estimate their ability to infect mice in vivo, wild type and manipulated BSF parasites were grown in ICR mice. Mice infected with wild type BSF were culled for humane reasons when parasitaemia was greater than 1×10^8/ml. The level of parasitaemia was determined by tail bleed and counting parasites under a microscope over a period of two to seven days post-infection using a haemocytometer. All procedures involving animals and the housing of the animals were performed in accordance with the ethical guidelines of the University of Glasgow or Edinburgh.

2.2. Recombinant DNA manipulations

To overexpress clathrin heavy chain (CLH) in BSF and PCF cells, we PCR amplified the 5112 bp CLH ORF Tb10.70.0830 from wild type 427 genomic DNA using primers TbCLHFNdeI, GCCATATGATGGA TAATCCACTAACCTCTGC, and TbCLHREcoRI, GCGAATTCTCAG TATGGCATCATGTTAGGG. Restriction sites are underlined. The PCR product was blunt cloned into pCR2.1-TOPO vector, and the CLH ORF released by digesting the pCR2.1-TOPO vector using NdeI and EcoRI and cloned into pXS5 and pXS2 to generate pXS5-CLH and pXS2-CLH respectively. Both pXS5-CLH and pXS2-CLH were fully sequence verified and linearized with XhoI or BstXI before electroporation with BSF or PCF parasites. Transfected BSF and PCFcells were grown in HMI-9 media containing 50 μg/ml neomycin, to isolate clathrin over-expressing lines. To generate a CLH single allele knockout construct ~1 kb from the 5' UTR of Tb10.70.0830 was PCR amplified using primers TbCLH5'UTRF GCGGTACCTACACATAAGTGAAGG AGGG and TbCLH5'UTRR GCCTGGAGCTTTGTTAGTGTCTGTTCC, and ~1 kb from the 3' UTR using primers TbCLH3'UTR-F GCACTAGTCACA GGGAAGGGAGATGGGA and TbCLH3'UTR-R GCGAGCTCGCAG CATTGGAAAGATGTGAG and blunt end cloned into pCR2.1-TOPO (Invitrogen). The 5' UTR fragment was released from the pCR2.1-TOPO vector by digesting with KpnI and XhoI and cloned into pXS5:NEO or pXS2:NEO to generate pXS5-CLH5'UTR:NEO and pXS2-CLH5'UTR:NEO, respectively. The 3' UTR was released from the pCR2.1-TOPO vector by digesting with SpeI and SacI and cloned into pXS5-CLH5'UTR:NEO or pXS2-CLH5'UTR:NEO to generate pXS5-CLH5'3'UTR:NEO and pXS2-CLH5'3'UTR:NEO, respectively. pXS5-CLH5'3'UTR:NEO and pXS2-CLH5'3'UTR:NEO were used to replace a single allele of CLH in the BSF and PCF genome, respectively. Both constructs were sequence verified and restriction digested with KpnI and SacI prior to electroporation with BSF or PCF parasites. Positive transformats were selected on HMI-9 media containing 50 μg/ml neomycin. All transgenic cell lines described here were cloned by limiting dilution prior to further analysis.

2.3. Quantitative real-time PCR

Total RNA from *T. brucei* BSF and PCF parasites were extracted using the Qiagen RNeasy mini kit. Synthesis of cDNA was performed in a 25 μl reaction volume with 2 μg RNA and oligo dT primers using the superscript II reverse transcriptase kit (Stratagene). Further, PCR amplification of a 125 bp fragment of clathrin (4286–4410 bp) was performed either under standard PCR conditions or in a reaction mixture containing cDNA and IQ-SyBr-green supermix using a mini-opticon instrument (BioRad) using the primers qRTCLHF ATACGTGCCCTCAAAACCTG and qRTCLHR GGATTCGAGGTATGGCAGAA.

2.4. Protein electrophoresis and western blotting

SDS lysates from 1×10^6–1×10^7 cells were separated on 12% SDS–polyacrylamide gels and wet-blotted onto PVDF membrane (Immobilon, Millipore, Bedford, MA), blocked with 5% milk in TBS-T (Tris-buffered saline, 0.5% Tween 20) for two hours at room temperature and probed with antibody to CLH at 1:1000, Rab5A at 1:1000, Rab11 at 1:2000 and BiP at 1:10,000 in 1% milk followed by HRP-conjugated goat anti-rabbit IgG (Sigma) or rabbit anti-mouse IgG (Sigma) at 1:10,000 dilution in 1% milk in TBS-T. Detection was by chemiluminescence and exposure to X-ray film (Kodak BioMax MR).

2.5. Southern blotting

Southern blotting was performed using 5 μg of genomic DNA isolated from BSF or PCF parasites in log phase (Medina-Acosta and Cross, 1993). Genomic DNA was digested with NaeI and NdeI, separated by electrophoresis and transferred to a nitrocellulose membrane and probed with specific probes for CLH and Neomycin. Hybridization and washing was done as described previously (Sambrook et al., 1989).

2.6. Cell cycle progression

Trypanosomes were harvested by centrifugation, washed with PBS and fixed with 4% PFA in ice-cold vPBS. Immunofluorescence was performed as described previously (Field et al., 2004). Specimens were analyzed on a Nikon Eclipse epifluorescence microscope equipped with a Hammamatsu CCD camera and data collected in Metamorph under non-saturating conditions (Molecular Devices). For determination of position in cell cycle cells were stained using DAPI, as described (Field et al., 2004); at least 200 cells were examined for each condition.

2.7. Transferin uptake assay

Mid-log phase BSF WT or BSF CLH-1KO cells from culture were harvested, washed and resuspended in serum-free HMI-9 containing 1% BSA at a concentration of $\sim 1 \times 10^7$ cells/ml. Resuspended cells were incubated for 30 min at 37 °C and 125 μg/ml of Alexa-conjugated transferrin (Molecular Probes) was added. Aliquots were removed at 0, 5, 10, 15 and 20 min intervals after the addition of transferrin and placed immediately on ice. Cells were washed with vPBS, fixed with 1% formalin in PBS and transferrin accumu-