Dileucine signal-dependent and AP-1-independent targeting of a lysosomal glycoprotein in *Trypanosoma brucei*

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Abstract

Sorting of trans-membrane proteins destined for the lysosome is achieved by selective inclusion into post-Golgi transport vesicles. In higher eukaryotes sorting may be mediated by a peptidic motif, principally acidic clusters and tyrosine- or dileucine-based cytoplasmic signals or by inclusion of mannose-6-phosphate (M6P) into the N-glycans of lysosomal proteins. In African trypanosomes a major lysosomal trans-membrane protein is CB-1/p67. The cytoplasmic domain of p67 lacks tyrosine and lysine, but does contain a canonical dileucine sequence embedded within an acidic region. AP-1, -3 and -4 adaptin complexes, which recognise tyrosine- and dileucine-sorting signals, are encoded by the trypanosome genome, but the genes for M6P-receptors or activities required to produce M6P are absent, suggesting that lysosomal delivery of p67 is most likely adaptin-mediated. By construction of p67 reporter constructs we show that the dileucine signal is necessary and sufficient for efficient lysosomal delivery of a trans-membrane protein in bloodstream stage trypanosomes. However, this targeting does not require AP-1, as knockdown of the trypanosome /H9253-adaptin subunit by RNAi has no detectable effect on the location or maturation of p67. These data suggest that p67 is targeted to the lysosome by dileucine-dependent but AP-1-independent mechanisms.

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1. Introduction

Lysosomal targeting of biosynthetic polypeptides in higher eukaryotes is principally mediated via post-Golgi sorting processes. The AP adaptin complexes are responsible for recognition of protein cargo molecules via direct binding to short specific sequence motifs (reviewed in [40]). The adaptor complexes AP-1 to -4 are conserved throughout the eukaryotic lineage[1] and contain two heavy chains (one representative of the closely related /H9252-family and a second designated /H9253, /H9254, /H9255, or /H9256), a medium chain and a small chain. The AP complexes recognise primarily tyrosine, NPXY/YXXØ, and dileucine, [DE]XXXL[LI], based signals in cargo molecules; the \(\mu\) chain appears important in recognition of polypeptide-based sorting signals, while additional interactions serve to integrate the adaptors into coat systems [2]. In higher eukaryotes AP-1 and -2 interact with clathrin and mediate transport from the trans-Golgi network/endosome and plasma membrane, respectively, while AP-3 and -4 function in trans-Golgi network and endosomal trafficking [40]. Additional complexes, including Dab2, stonin, PACS-1 and GGA are specific to animals and yeasts and may also recognise DXXLL-based signals[1,3,40]. A further lysosomal targeting mechanism is the addition of mannose-6-phosphate (M6P) to the N-glycans of soluble proteins, principally lysosomal hydrolases; this can be effectively ruled out for trypanosomatids due to the absence of the relevant open reading frames from the trypanosome genome.

Predominant metazoan lysosomal trans-membrane proteins are the LAMP glycoproteins. LAMPs are extensively N-glycosylated type I membrane proteins with comparatively short cytoplasmic domains and are proposed to provide a mainly
protective function, preventing autolytic lysosomal membrane degradation, although a direct role in lysosomal biogenesis is also suspected [4]. LAMPs are delivered to the lysosome via a tyrosine-based signal, which is strongly suggestive of at least partial routing via the plasma membrane [40] and AP-3 knockouts can disrupt targeting of LAMP glycoproteins [5]. By contrast, the M6P-receptor, which also has a tyrosine-based targeting signal, is sorted via AP-1 [6].

Trypanosoma brucei is evolutionarily distant from the major model organisms [42]. The major lysosomal glycoprotein in trypanosomatids is p67 [38] and while the function is unknown the p67 polypeptide has a similar topology to LAMPs and is extensively N-glycosylated [7]. Extensive proteolytic processing accompanies p67 maturation, reflecting extensive peptidase activity residing within the endosomal system [17]. In mammalian stages p67 likely progresses to the lysosome via post-Golgi sorting, but some evidence suggests an indirect route operates involving initial export to the cell surface [7]. p67 contains a [E/D]EDEL dileucine sequence within an acidic short cytoplasmic domain, but lacks tyrosine or lysine residues; the latter excluding ubiquitylation as a mechanism. Removal of the cytoplasmic domain prevents lysosomal targeting of p67 [17] but this mutation may have several effects on the resulting behavior of the truncated protein.

The T. brucei cell surface is dominated by molecules bearing a glycosylphosphatidylinositol (GPI)-anchor, and maintenance of surface composition is required for cell cycle progression in the mammalian form [8]. Pathways for plasma membrane component trafficking have likely evolved to facilitate maintenance of the surface structure. Internalisation is exclusively clathrin-mediated [9,26] and components are returned to the surface via a highly active recycling system [10,26]. Specific secondary loss of the AP-2 adaptor complex from T. brucei has been reported [11,12], together with an absence of Dab2, GGAs, PACS-1 and stonins [1]. Therefore the adaptor complement in trypanosomes is likely simpler than in higher eukaryotes. AP-1 in the procytic form has been implicated in trafficking of CRAM from in vitro interaction data [13], but the overall impact of AP-1 on transport is not known. Here we examine the role of AP-1 and the dileucine cytoplasmic motif of p67 in lysosomal targeting in T. brucei.

2. Materials and methods

2.1. Cell culture

Bloodstream form (BSF) and procyclic form (PCF) T. brucei Lister 427 cells were maintained in HMI-9 and SDM-79 media, respectively, supplemented with 10% tetracycline-free foetal bovine serum (Autogen Bioclear) as described previously [9]. To maintain tetracycline responsiveness the tetracycline-inducible cell lines BSF 90-13 and PCF 29-13 were cultured in the continual presence of 2 μg ml⁻¹ G418 and 1 μg ml⁻¹ phleomycin (BSFs) or 25 μg ml⁻¹ G418 and 1 μg ml⁻¹ phleomycin (PCFs). For growth curves, triplicate cultures were initiated at 5 × 10⁴ cells ml⁻¹ (BSFs) or 1 × 10⁵ cells ml⁻¹ (PCFs) and maintained within the logarithmic growth range. Cell concentration was determined using a Z2 Coulter counter (Beckman).

Following transfection, expression of double-stranded RNA was induced by the addition of 1 μg ml⁻¹ tetracycline.

2.2. RNAi plasmid construction and transfection

To generate the RNAi plasmid p2T7Tiβ1Ad, a 495bp fragment of the Tbβ1Ad gene was PCR amplified from T. brucei genomic DNA using the primers 5'-AAGCTTCTGGGCACTTCAATCTCG-3' and 5'-GGATCCAGTACGAAACCGGC-3', which contain the restriction sites for Hind III and BamHI, respectively, and inserted into the tetracycline inducible RNAi vector p2T7Ti [14]. This region was selected with the RNAi algorithm [15] to minimise the likelihood of off-target effects in the RNAi. A 526bp fragment of the TbyAd gene was also PCR amplified from the same genomic DNA, using the primers 5'-CATCTCGAAGTCGCCCTCCTAGTGCCC-3' and 5'-GGGAAGCTTGTGTGGTGTGTTTGCG-3', which contain the restriction sites for XhoI and Hind III, respectively. To insert this DNA fragment into the p2T7Ti vector, it was subcloned into pPCR-Script (Stratagene) and then excised using the XhoI site at the 5' end of the fragment and the BamHI site in the multiple cloning site of the vector. The resulting 537bp XhoI–BamHI fragment was inserted into p2T7Ti to generate the RNAi plasmid p2T7TiγAd. Both of these RNAi constructs were transfected into PCF 29-13 and BSF 90-13 cell lines as previously described [9].

2.3. Antibody production

TbyAd antisera were generated against an expressed protein fragment (residues 376–571) amplified from T. brucei genomic DNA using high fidelity Herculase DNA polymerase (Stratagene) with the following primers: 5'-GATGTTATCGGACGACCGTGCGCCT-3' and 5'-AGCGAATTCCGGGAAAGCATTCAAGATG-3'. The PCR product was digested with BamHI and EcoRI and inserted into the expression vector pGEX-2TK (Amersham Biosciences). Polyclonal rabbit antibodies were raised against TbyAd-GST recombinant fusion protein, which was SDS polyacrylamide gel purified due to insolubility when expressed in Escherichia coli, mixed with RIBI adjuvant (Sigma) and used to immunize rabbits on at least four immunizations spread over a period of 5 months. At least 0.5 mg of recombinant protein was used per immunization course. The resulting antibodies could not be affinity purified due to the insolubility of the recombinant GST fusion protein. All subsequent assays were therefore performed using the antiserum raised against the fusion protein.

2.4. Production of p67/BiPN reporter constructs and site-directed mutagenesis

Full length p67 together with 279bp and 216bp fragments corresponding to the extreme C-terminus of p67 (for constructs BiPNp67L and BiPNp67S, respectively) were amplified by PCR from T. brucei genomic DNA using the following
primers (enzymes in parenthesis): p67; 5'-ACGAAAGCTTATGATGTTAACCAGAATTCAACACCGTTCTC-3' and 5'-AGGGGCCGCTTGGGATGATTTGAGCTCTATAAGACCTT-GTCAGCTCTCTCGAAGAAGAGCAGCCCT-3' (Nhel and EcoRI) and BiPNp67L; 5'-ACGCTGAGGATCTATAAGACCTTCAGCCTCTTCGGGAGCAGCCTC-3' and 5'-ACGGCTAGCGGAAGCTTATGATGTGACCGCAACCGTTCTC-3' (Nhel and EcoRI). Fragments were inserted into the pXS5HA or pXS5BiPNHA vectors to produce pXS5p67HA, pXS5BiPNp67L and pXS5BiPNp67S, respectively.

To generate DELL double point mutations (to AALL and DEAA) within the C-terminal sequence of p67, BiPNp67L or BiPNp67S, PCR was performed to amplify the p67 gene from T. brucei genomic DNA using the following primers: p67; DELL to AALL; 5'-ACGAAAGCTTATGATGTTAACCAGAATTCAACACCGTTCTC-3' and 5'-ACGGCTAGCGGAAGCTTATGATGTGACCGCAACCGTTCTC-3' (Nhel and EcoRI) and DELL to DEAA; 5'-ACGAAAGCTTATGATGTTAACCAGAATTCAACACCGTTCTC-3' and 5'-ACGGCTAGCGGAAGCTTATGATGTGACCGCAACCGTTCTC-3' (Nhel and EcoRI). Products were inserted into the pXS5HA or pXS5BiPNHA vectors to produce pXS5p67HA, pXS5BiPNp67L and pXS5BiPNp67S, respectively.

2.6. Immunofluorescence microscopy

Indirect immunofluorescence microscopy was performed on BSF and PCF trypomastigotes harvested at log phase growth. BSF cells were washed in vPBS (136.9 mM NaCl, 3 mM KCl, 16 mM Na2HPO4, 3 mM KH2PO4, 45.9 mM sucrose, 10 mM glucose) then fixed for 1 h at 4°C in 4% paraformaldehyde. Fixed cells were adhered to poly-L-lysine coated polyprop slides (Sigma) for 20 min at room temperature, then permeabilised with 0.1% Triton-X 100 (Sigma) in PBS for 10 min. Slides were blocked with 10% goat serum in PBS then incubated with the primary antibody at the appropriate dilution in blocking buffer at room temperature for a further hour. Unbound primary antibody was washed off with PBS/0.05% Triton-X 100 prior to incubation for 1 h at room temperature with secondary antibodies; Texas Red-conjugated goat anti-rabbit IgG or Oregon-Green labelled goat anti-mouse IgG (Molecular Probes), diluted according to manufacturer’s instructions. Slides were washed and stained with DAPI (present in the mounting buffer at 0.5 µg ml⁻¹) to visualise the nucleus and kinetoplast. Essentially the same procedure was used for immunofluorescence microscopy of PCF trypomastigotes, except that these cells were fixed in 4% paraformaldehyde for 30 min at room temperature prior to adhesion to the slides. Cells were examined using a Nikon Eclipse E600 microscope and images were captured using a Photometrics CoolSNAP FX camera. Digital images were captured and false coloured using MetaMorph 5.0 software (Universal Imaging Corporation). Primary antibodies were used in the following dilutions: rabbit-anti-TbCLH [11], 1:500, rabbit-anti-TbBiP [16], 1:1500, mouse anti-p67 mAb280 [17], 1:500 and rabbit anti-TbRAB1 [18], 1:100. Rabbit and mouse anti-HA9 epitope IgG (both from Santa Cruz Biotechnology) was used at 1:1000. To quantify surface expression of p67 constructs, the p67HA cell lines were stained with anti-p67 antibody following the method above (which detects an externally disposed epitope), but omitting the permiabilisation step. The fluorescence intensity in at least 20 randomly selected cells of each strain was measured from MetaMorph images captured under non-saturating conditions and at identical exposures.

2.7. Electron microscopy

Transmission electron microscopy was performed as described previously [9].

2.8. VSG export assay

VSG export was monitored exactly as described [9]. Briefly, 5 x 10⁷ mid-log phase BSFp2T7¹VAd cells grown in the presence or absence of 1 µg ml⁻¹ tetracycline for 16 h were washed once in labelling medium (Met/Cys free Dulbecco’s Modified Eagle’s medium (DMEM) (Sigma) supplemented with 10% dialysed fetal bovine serum and 25 mM Hepes (pH7)), centrifuged and resuspended in 1 ml labelling medium (±tetracycline) and incubated for 15 min at 37°C. Cells were pulse labelled for
7 min at 37 °C with [35S] labelled Pro-mix (Amersham Biosciences) at 200 μCi ml⁻¹, then chased for up to 1 h (37 °C, 5% CO₂) by diluting the cells 1:10 with complete HMI 9 (±tetracycline). 1 ml aliquots of cells were removed at intervals during the chase period and placed on ice. Following centrifugation the cell pellets were washed in 1 ml ice-cold PBS/BSA, resuspended in 920 μl of hypotonic lysis buffer, then incubated for 5 min on ice, followed by 10 min at 37 °C to enable endogenous GPI-phospholipase C (GPI-PLC) to convert susceptible membrane form VSG to soluble VSG. Centrifugation isolated soluble VSG in the supernatant of the lysates. The pellet fraction (containing membrane bound VSG) was washed with ice-cold hypotonic lysis buffer then resuspended in 1 ml ice-cold sample lysis buffer and incubated on ice for 25 min to lyse the membranes and release the VSG. NP-40 was added to the supernatant fraction to bring all of the samples into the same buffer. Lysates were cleared by centrifugation and the supernatants retained for VSG recovery by ConA binding. ConA-sepharose 4B (Sigma) was added to the supernatants in the presence of MnCl₂ and CaCl₂ to aid binding, and mixed at 4 °C for 1 h. The ConA-sepharose with bound glycoproteins were then washed with ConA wash buffer, after which they beads resuspended in SDS-PAGE sample buffer and resolved on 10% SDS-PAGE gels loaded at 1 × 10⁶ cell equivalents per lane. To detect labeled VSG, stained gels were fixed then treated with En3Hance (NEN Life Sciences), before exposure to X-ray film (Kodak). The VSG bands were quantitated, together with background subtraction (adjacent area from the same lane) using ImageJ software.

2.9. p67 processing assay

Processing of p67 was followed as described previously [19,20]. Briefly, BSFp2T7γTiAd cells grown in the presence of 1 μg ml⁻¹ tetracycline for 16 h were harvested, washed twice in Met/Cys free DMEM (Sigma) and resuspended to a final concentration of 1 × 10⁸ cells ml⁻¹ in the same medium supplemented with dialysed fetal bovine serum and incubated for 15 min at 37 °C. Cells were pulse labelled for 15 min at 37 °C by addition of [35S]-labelled Pro-mix (Amersham Biosciences) at 200 μCi ml⁻¹ then chased by 1:10 dilution in complete HMI9 medium. 1 ml samples were withdrawn at 0 and 30 min, 1 h, 2 h and 4 h into the chase period and placed on ice. Cells were washed in PBS and then lysed in ice-cold radioimmune precipitation (RIPA) buffer [19,20]. The lysates were precleared by mixing with 10 μl of a 50:50 slurry of protein A-sepharose beads (Amersham Biosciences) in RIPA buffer for 1 h at 4 °C. The supernatants were then mixed with 10 μl of anti-p67 mAb280 coated protein-A sepharose beads for 1 h at 4 °C. After washing the beads twice with RIPA buffer, twice with high salt RIPA buffer (500 mM NaCl) and twice with TBS, the immunoprecipitates were resuspended in 50 μl of loading buffer and run on 12% SDS-PAGE gels. Fixed and stained gels were treated with En3Hance (NEN Life Sciences), dried and exposed to X-ray film (Kodak). The intensity of metabolically labelled protein bands was quantified using ImageJ software.

3. Results

3.1. Targeting of p67 depends on the presence of a DELL motif in the cytoplasmic domain

The T. brucei genome encodes two near identical copies of the p67 protein (Fig. 1A). Both retain a canonical [D/E]XXL[L/I] putative dileucine adaptin—interaction motif, also partially conserved in the Trypanosoma cruzi orthologue. A second degenerate dileucine motif is present C-terminal to the canonical signal. The cytosolic domains are acidic, with pIs of 2.7 for Tb927.5.1830 and 4.2 for Tb927.5.1810. Deletion of the entire cytoplasmic portion of p67 results in partial mistargeting of p67 in BSF trypanosomes, broadly consistent with a trafficking role for the cytoplasmic domain [17]. We generated a panel of p67 mutants and chimeras using sequence derived from Tb927.5.1810 (p67B) and based around BiPNHA [21], containing the trans-membrane and cytoplasmic domains of p67 together with a junction region from the p67 ectodomain (Fig. 1B). Replacement of the p67 ectodomain by BiPN has the advantage of eliminating both targeting information and protein–protein interactions; specifically this allows the criterion of sufficiency for the transplanted region to be addressed (discussed in [21]). All constructs were transfected into BSF trypanosomes and transformants were obtained expressing an HA-tagged polypeptide of the expected size (Fig. 2); at least two clones for each construct were analysed with essentially identical results in each case.

An HA-reactive band of apparent molecular weight ~100 kDa was obtained for p67HA—some heterogeneity was also detected, likely due to processing of the extensive p67 N-glycans (Fig. 2A). Faint additional lower molecular weight bands were detected on overexposed blots and likely correspond to proteolysed fragments of p67 (data not shown) [17]. p67HA with point mutations within the DELL motif expressed at similar levels to the native p67HA sequence as a predominant immunoreactive band of ~100 kDa (Fig. 2A). By immunofluorescence it was clear that the HA-tagged wild type version of p67HA was faithfully targeted to the lysosome as there was excellent colocalisation of p67 and HA in a perinuclear compartment (Fig. 2B). The absence of mislocalised HA or p67 immunoreactivity resulting from the transgene demonstrated that the HA tag did not interfere with targeting.

BiPNp67S and BiPNp67L were also expressed at the correct size, as demonstrated by Western blot (Fig. 2A). However, BiPNp67S was predominately recovered as a lower molecular weight species (P; processed) rather than as full length (F). By contrast BiPNp67L was mainly expressed as the full-length product, with only a minor processed population. The predicted sizes of the processed forms are consistent with cleavage C-terminal to the HA epitope, presumably within the ectodomain derived from p67 and mimicking the processing described for p67 itself [17]. This difference in processing efficiency can be accounted for by the locations of the two constructs. Specifically, BiPNp67L was effectively retained in the ER, likely protecting the polypeptide from peptidases present in post-ER portions of the transport pathway [17]. BiPNp67S was targeted to the
Fig. 1. AP-1 consensus interaction sequence in the cytoplasmic tail of p67. (Panel A) ClustalX alignment of the predicted amino acid sequences for the C-terminal region of the two p67 ORFs from *Trypanosoma brucei* (Tb927.5.1810/1830, p67A and p67B) together with the *Trypanosoma cruzi* orthologue. “*” indicates residues identical in all three sequences, “:” highly conserved, “.” conserved, “-” gaps introduced into the alignment. The predicted position of the *trans*-membrane domain is underlined. The location of a sequence that conforms to the dileucine AP-1 consensus ([D/E]XXXL[L/I]) is also indicated in red in the *T. brucei* sequences and in green in the *T. cruzi* orthologue. (Panel B) Diagrammatic representation of p67-derived constructs used. p67HA is a full length version of p67 containing an HA-tag at the C-terminus; other constructs are based on BiPNHA [21] and contain the indicated regions of p67 derived from the C-terminal region of the ectodomain, the *trans*-membrane domain and the cytosolic domain. Mutagenesis was performed by mega-primer PCR and all constructs were contained within pXS5 [39]. Sequence numbering is based on Tb927.5.1810.

We examined if the EEDELL sequence was necessary by localisation of the two mutagenised versions of the construct, BiPNp67S-AALL and BiPNp67S-DEAA. In comparison to the respective wild type versions of the constructs, some targeting to the lysosome was retained but with considerably reduced efficiency, and with a prominent population of the HA-tagged protein at the cell surface (note that the HA epitope is positioned on the luminal/external side of the membrane) (Fig. 2B). We confirmed this result by analysis of the mutant forms of p67HA, with equivalent alterations to the DELL motif. These constructs appeared by immunofluorescence to be widely distributed across the cell surface, rather than exhibiting highly specific targeting to the lysosome, similar to the BiPNp67HA mutants (Fig. 2C). We probed for surface mislocalisation by analysis of cells by immunofluorescence using antibody against p67, with a clear increase in surface p67 immunoreactivity in the mutated forms (Fig. 2C and D). Hence these data suggest that the presence of an intact DELL sequence is required for lysosomal targeting, and moreover that disruption of any one motif, i.e. DE or LL, is sufficient to interfere with targeting.

Taken together these observations indicate that the DELL sequence in p67 constitutes an authentic lysosomal targeting signal that is both necessary and sufficient for correct and efficient trafficking. Interestingly, mistargeting of BiPNp67S-AALL and p67HA-AALL suggests that the targeting signal in p67 may be more extensive than the canonical dileucine signal as defined in higher eukaryotes, and includes acidic residues N-terminal to the dileucine.

3.2. Expression of AP-1 subunits in trypanosomes

Adaptin complexes mediate the major dileucine-dependent transport pathways in higher eukaryotes. AP-1 components are constitutively expressed in trypanosomes, based both on Western analysis of the β-subunit and microarray transcriptome analysis [11] (Koumandou and MCF, unpublished data), and interaction with CRAM, a type I membrane protein and the AP-1 μ subunit has been demonstrated in vitro [13]. AP-1 mediates a major trafficking pathway from the TGN to lysosomes.
in higher eukaryotes, and disruption of AP-1 leads to mistargeting of many lysosomal proteins. Hence we selected AP-1 for analysis of potential function in p67 transport. We previously identified and described a β1-subunit, likely part of the AP-1 complex [11]. However, as β-subunits are closely related between complexes we selected a more specific AP-1 subunit sequence, the γ-subunit, for further investigation.

Antisera raised against the recombinant γ-subunit expressed in E. coli recognised a band of the predicted molecular weight (87.3 kDa) in lysates from both BSF and PCF stages of the parasite (Fig. 3A), indicating that, similar to the β1-subunit, the protein is expressed throughout the life cycle [11]. We used RNA interference (RNAi) to address AP-1 function (Fig. 3). In the bloodstream form RNAi of the β1- or γ-subunit resulted in cessation of growth following induction after 2 days, with a loss of cell viability in the cultures. Similar results were obtained in the insect stage, although as is common in RNAi, the kinetics were rather more extended such that the γ-subunit produced a significant growth defect by day 6 and the β1-subunit by day 7. The similarity of the RNAi effect on growth is consistent with β1- and γ-subunits comprising the same complex.

To confirm the specificity of the knockdown we analysed protein extracts of cells following induction of RNAi (Fig. 4). A clear loss of the γ-subunit was observed following induction in both bloodstream and procyclic forms (top and lower panels). Significantly, lysates of bloodstream stage cells that had been subjected to RNAi for the β1-subunit also demonstrated loss of the γ-subunit signal and in both life stages loss of the protein occurred with similar kinetics, so that <20% of the normal γ-subunit signal was obtained following 48 h induction (middle panel). The protracted appearance of a phenotype in the PCFs therefore suggests a lower requirement for AP-1 expression as the slower phenotype cannot be ascribed as less rapid turnover of the targeted gene product. Taken together this is further evidence that the β1- and γ-subunits form part of the same complex as evidence suggests that incompletely assembled complexes are unstable [22]. Unfortunately the γ-subunit antibody did not recognise specific compartments by immunofluorescence, despite repeated attempts and use of multiple fixation conditions (data not shown).

To confirm specific knockdown of the β1-subunit, which in contrast to the γ-subunit could not be assessed by Western analysis, we used semi-quantitative reverse transcriptase (RT)-PCR. To ensure that the PCR was performed under conditions sensitive to changes to mRNA levels we titred mRNA template concentration and cycle number (Fig. S1). A loss of specific product for the β1-subunit over a 24-h time period in the bloodstream form was obtained, whilst the α-tubulin control product was unchanged following RNAi for the β1-subunit. Neither product was generated when the RT step was omitted. These data confirm that the β1-subunit is specifically targeted by the β1-RNAi and therefore that the gene product is required for robust growth.

3.3. Adaptor AP-1 is required for maintenance of normal morphology and cell cycle progression

In order to analyse function and determine the kinetics of onset of a phenotype, we investigated the effect of AP-1 RNAi on progression through the cell cycle and on cellular morphology. Upon induction of γ-subunit RNAi we observed a slow increase in cells with abnormal morphology and also numbers of nuclei (Fig. 5A–C). Defects included the appearance of vesicular structures within the cytoplasm, rounding of the cells, and also enlargement of the flagellar pocket.

In BSFs, a gradual fall in the number of cells in interphase was observed (nucleus/kinetoplast content 1N:1K). This was initially
Fig. 2. (Continued).
due to an increase in 2N:2K cells, but at later times the 2N:2K incidence returned to normal levels with an increase in multinucleated cells (>2N:2K) (Fig. 5D, left). We also observed cells with enlarged nuclei at later time points, indicating that not only was there a defect in cytokinesis, seen as an increase in 2N:2K and >2N:2K cells, but also nuclear division (Fig. 5D). The phenotype is also consistent with a defect to membrane traffic, either in delivery of membrane to the growing cytokinesis furrow or in the correct trafficking of critical components required for completion of cell division. A significant proportion of BSF cells recapitulated the enlarged flagellar pocket (BigEye) phenotype [9] suggesting an endocytosis defect (Fig. 5E). However, the proportion of cells with this feature was minor (<20%) and onset was comparatively slow, and emergence of the enlarged pocket is likely a manifestation of general disruption to membrane transport, which ultimately affects endocytic activity. Ultrastructural analysis also demonstrated defects within the endomembrane system, principally the accumulation of electron dense structures in the cytoplasm, an enlarged, crenelated flagellar pocket (Fig. 6F–H) similar to Rab5 RNAi [20] and also prominent multivesicular bodies (MVBs). The structure with electron dense deposits at the membrane and the presence of prominent MVBs is similar to a phenotype observed by Williams et al. in starved or cysteine peptidase knockout *Leishmania* promastigotes, and interpreted as part of the autophagic pathway [23], consistent with a defect in endosomal trafficking being induced by the γ-subunit knockdown. Hence a pleotropic defect in morphology and mitosis is obtained by ablation of AP-1 and suggests that AP-1 mediates an important trafficking pathway in BSF trypanosomes.

In procycls a less pronounced morphological defect occurred following knockdown of the γ-subunit. Cells became rounded with a clear accumulation of numerous phase-light vesicles as observed by phase contrast (Fig. 5C). A small decrease in the incidence of interphase cells likely reflects the growth defect in these cells (Fig. 5D), but a prominent block to cell
cycle progression was not observed. In induced cells the most prominent ultrastructural defects were accumulation of vesicles within the cytoplasm, which probably correspond to those seen in the light microscope (Fig. 5C), and also rather large vacuoles (>0.2 mM diameter) with electron dense material on the limiting membrane. The origin of the large vacuole is not clear, but the accumulation of the small vesicular structures is consistent with a defect in post-Golgi transport, and is similar to that observed in the light microscope (Fig. 5C), and also rather large vacuoles (>0.2 mM diameter) with electron dense material on the limiting membrane.

3.4. AP-1 is not required for efficient trafficking of p67 or exocytosis of VSG

To specifically analyse trafficking of p67 we used the assay described by Alexander et al. [17] and used by us to demonstrate trafficking defects associated with knockdown of Rab4 [19,20]. p67 is synthesized as a 100 kDa precursor protein in the endoplasmic reticulum, is matured to a 150 kDa form in the Golgi complex by elaboration of its N-glycans, and is then subject to proteolysis generating first a 72 kDa form and subsequently 42 and 32 kDa species as a late endosomal/lysosomal event; an example is shown in Fig. 7A. Unexpectedly, we observed that formation and disappearance of all five characterised forms of p67 were essentially unaltered by loss of the AP-1 γ-subunit after 18 h induction (this time point chosen as a compromise between loss of expression of the γ-subunit and the emergence of grossly altered morphology at ∼24 h) (Fig. 7A). Hence these data suggest that p67 delivery to the lysosome is largely independent of AP-1 expression.

We also monitored export of newly synthesised VSG as a marker for exocytosis and a validated assay previously used by us to investigate the effect of RNAi on VSG transport [9,18,24,25]. Again, no defect in the kinetics of delivery of VSG to the cell surface could be detected (Fig. 7B). Hence we were not able to detect a defect in progress through the ER, Golgi complex, delivery to the lysosome or alterations in the kinetics of N-glycan elaboration.

3.5. AP-1 knockdown does not alter localisation of p67

We chose to confirm the absence of a clear effect of AP-1 knockdown on p67 trafficking by immunofluorescence. We selected several membrane vesicle markers for our analysis; BiP for the ER [16], clathrin for endosomes [9,11,26] and Rab1 for the Golgi complex [18]. We performed the analysis on aliquots of cultures induced for the AP-1 γ-subunit for various times, and allowed the induction in the remaining cells in the culture to proceed until we obtained morphological defects, to demonstrate that the RNAi induction was effective.

In BSFs we saw the expected BiP reticular staining indicative of the ER (Fig. 8A) which was not perturbed significantly, and only became significantly abnormal after 24 h induction when
Additional bands in the upper panel reflect proteolysis of Tb against either the ER chaperone BiP or probed with anti-Tb leftmost lane of each blot is a control lysate from parental cells. Blots were shown to be monospecific.

Fig. 1 shows the antisera to be monospecific.

In endocytosis consistent with a role for AP-1 in post-Golgi transport and supported by the incomplete mislocalisation of the BiPNp67 mutants and the lack of mistargeting of p67 in the AP-1 RNAi cells, suggesting that an additional sorting system may operate. Signal divergence has been described recently for the G1 cyclin CLN3.

The presence of a subpopulation of mutant p67 chimeras at the cell surface may suggest that p67 is at least partially trafficked via the indirect cell surface route. However, considerable experimental evidence is inconsistent with this model; specifically RNAi knockdown of TbCLH, the clathrin heavy chain...
Fig. 5. Morphology and cell cycle progression of TbrAd knockdown cells. Morphology was monitored by phase contrast microscopy. (Panels A and B) Phase contrast galleries of a representative selection of BSF TbrAd RNAi cells (A) and PCF TbrAd RNAi cells (B), induced with 1 μg ml⁻¹ tetracycline for the indicated times (h = hours, d = days). Scale bar for both panels 2 μm. (Panel C) TbrAd RNAi causes nuclear segregation defects in BSF cells. Gallery of images cultures of BSFp2T7TiAd cells induced for 26 h with 1 μg ml⁻¹ tetracycline showing a range of abnormalities in both phase morphology (top row) and DAPI stained DNA content (bottom row). Scale bar: 2 μm. (Panel D) Knockdown of TbrAd by RNAi blocks cytokinesis. BSF and PCF TbrAd RNAi cells were induced with 1 μg ml⁻¹ tetracycline for the indicated times, then fixed and stained with DAPI. The nucleus (N)/kinetoplast (K) ratios were determined for at least 100 cells at each time point. >2N:2K includes all cells with greater than two nuclei, “zoid” is a cell lacking a nucleus (cytoplasm). (Panel E) Quantitation of kinetics of appearance of cells with and enlarged flagellar pocket in BSF TbrAd RNAi cells. BSFp2T7γAd cells were induced with 1 μg ml⁻¹ tetracycline for the indicated times, fixed with 4% paraformaldehyde and then adhered to slides. One hundred cells were analysed by phase contrast light microscopy for each time point to determine the percentage of cells with an enlarged pocket.

<table>
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<tr>
<th>Days post induction</th>
<th>BSFp2T7γAd</th>
<th>PCFp2T7γAd</th>
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<tr>
<td>8</td>
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<td>18</td>
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<td>5</td>
<td>1N2K</td>
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<td>&gt;2N2K</td>
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<td>2N1K</td>
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<td>Zoid</td>
<td>1N1K</td>
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<td>1N2K</td>
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<table>
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<tr>
<th>Hours post induction</th>
<th>Percent of cells</th>
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<td>0%</td>
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<td>10%</td>
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<tr>
<td>16</td>
<td>30%</td>
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<tr>
<td>24</td>
<td>50%</td>
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Concerning AP-1 function, AP-1 is essential in both trypanosome major proliferative life stages, and the complex is constitutively expressed. Secondly, we demonstrate a clear relationship between the previously described β-subunit [11] and the γ-subunit, indicating they constitute the heavy chains of the trypanosome AP-1 complex. Thirdly, evidence suggests that AP-1 is not required for p67 lysosomal targeting or for VSG exocytosis. Fourthly, AP-1 is required for maintenance of the normal architecture of the endosomal system and for progres-
Fig. 6. Ultrastructural analysis of TbyAd knockdown cells. (Panels A–E) Electron micrographs of PCF cells following 3-day induction of TbyAd dsRNA. Note the abundance of large vesicles (1, arrow in panels B and E), prominent multivesicular bodies (2, insets in panel A), large vesicular structure surrounded by electron dense material (3 in panels A, B and D) and the inclusions in the flagellar pocket (4 in panel C). The Golgi complex profile is normal in these cells (panel C, white arrow). Scale bar on main image (A) 0.5 μm. (Panels F–H) Electron micrographs of BSF cells following 16 h induction of TbyAd dsRNA. The major morphological affect is an accumulation of electron dense vesicles in the cytoplasm (white arrows). There are also abnormalities in flagellar biosynthesis (note flagellum structure in the cytoplasm enlarged in the inset, 5, arrow in panel G) and plasma membrane invaginations (black arrow, panel H; note the presence of the microtubule corset beneath the membrane). The Golgi complex profile is normal (enlarged in the inset box in bottom right panel). Scale bar on main image (F) 0.5 μm. FP, flagellar pocket; N, nucleus; K, kinetoplast; G, Golgi.

sion through the cell cycle. The lack of a clear influence of AP-1 knockdown on p67 targeting was unexpected. AP-1 is constitutively expressed, and considering the absence of several additional trafficking factors from the trypanosome genome, we anticipated a major role for AP-1 in endosomal trafficking. The most probable explanations are either redundancy or that other AP complexes may substitute for AP-1 under conditions of the knockdown. The effects on endosome architecture and cell division likely explain the impact of AP-1 knockdown on viability but these defects are likely to be due to protracted disruption of trafficking pathways resulting in accumulation of mistargeted factors that cannot be tolerated. In essence these are secondary effects, notwithstanding a clear requirement for AP-1 expression for continued survival of the parasite. However, it remains formally possible that residual AP-1 expression in knockdown cells is sufficient to support p67 trafficking, but not continued cellular viability.

In mammalian cells it is AP-2 RNAi that leads to defective LAMP glycoprotein lysosomal targeting, with a considerable LAMP presence on the cell surface, but AP-2 is absent from T. brucei and other data argue against surface p67 trafficking. Knockdowns of AP-1, -3 or -4 components in mammalian cells have little effect on steady state LAMP targeting and there remains a considerable level of LAMP glycoprotein within the
Fig. 7. TbvAd is not required for trafficking of newly synthesized VSG or p67 proteolytic processing. (Panel A) p67 processing is not significantly disrupted by TbvAd silencing. Following 18 h culturing in the absence (closed squares) or presence (open squares) of 1 μg ml⁻¹ tetracycline, BSFp2T7TbvAd cells were pulse-labelled with [³⁵S] methionine, followed by a chase period of up to 4 h. Cells were lysed and p67 was immunoprecipitated with mAb280. The different molecular weight proteolytic fragments produced during p67 processing in different intracellular compartments were separated by SDS-PAGE (data not shown) and the band intensities of the gp150, gp100, gp72, gp42 and gp32 fragments were quantified by densitometry. Results are presented as percentage of the total recovered at each time point and are the mean from two experiments together with the standard error. Top right panel shows an example of a pulse-chase experiment to illustrate the sequential processing. (Panel B) Pulse-chase kinetic analysis of arrival of newly synthesized VSG at the plasma membrane. VSG export in uninduced (closed squares) and 18 h induced (open squares) BSFp2T7TbvAd cells was monitored by recovering soluble VSG (following hydrolysis from the plasma membrane by GPI-specific phospholipase C) from hypotonic lysates taken at the indicated time points following pulse labelling with [³⁵S]-methionine. Pulse labelled soluble VSG recovered following purification with Con-A sepharose was SDS-PAGE separated and quantified by densitometry. Data show the mean of two experiments with error bars indicating the standard error. Results are presented as percentages of the total recovered VSG in the supernatant, following background subtraction.
lysosome, 

albeit with the possibility of an undetected kinetic transport defect [29]. We analysed in some detail the kinetics of p67 processing and it is unlikely that a simple kinetic effect can explain our observations. AP-1 RNAi may lead to activation of a compensatory pathway, as is observed for tyrosinase in mammalian cells where knockdown of one AP complex likely promotes interaction with other pathways [30]. Given cross recognition of dileucine signals by AP complexes, the potential for such behavior is present and likely explains the mild trafficking defects that are frequently observed for specific substrates versus the more severe general effects in other systems. Direct analysis of the AP-3 and -4 pathway, in combination with AP-1 knockdown, are required to determine if p67 is trafficked by these alternate pathways.

The trypanosome AP-1 knockdown phenotype is complex, including accumulation of cells post-S-phase and/or with an enlarged flagellar pocket [9]. Both slow onset and low frequency of the phenotypes imply a secondary defect and a lack of mislocalisation of clathrin in the knockdown cells is consistent with this conclusion. Therefore AP-1 knockdown leads to accumulation of vesicular structures, most likely derived from post-Golgi compartments, and partly parallels similar experiments in higher eukaryotes [31,32]. AP-1 knockdowns/knockouts generate embryonic lethal phenotypes in Mus musculus [43] and Caenorhabditis elegans, suggesting essential developmental roles [33]. However, in metazoan cell culture AP-1 is not required for cellular viability [41]. A mild phenotype is obtained by knockout of AP-1 components in Saccharomyces cerevisiae, and a potent trafficking defect is only apparent in combination with a ts clathrin heavy chain mutation [34]. In Schizosaccharomyces pombe the μ1 subunit is nonessential but cells accumulate cytoplasmic vacuoles [35]. AP-1 knockdowns/knockouts generate embryonic lethal phenotypes in Mus musculus [43] and Caenorhabditis elegans, suggesting essential developmental roles [33]. However, in metazoan cell culture AP-1 is not required for cellular viability [41]. A mild phenotype is obtained by knockout of AP-1 components in Saccharomyces cerevisiae, and a potent trafficking defect is only apparent in combination with a ts clathrin heavy chain mutation [34]. In Schizosaccharomyces pombe the μ1 subunit is nonessential but cells accumulate cytoplasmic vacuoles [35]. AP-1 knockdown leads to accumulation of vesicles, as well as engorgement of Golgi cisternae and impaired growth [36], while Dictyostelium AP-1 mutants produce a severe phenotype, including decreased growth, accumulation of cytosolic vesicles and defects in lysosomal enzyme sorting, but retain correct localisation of the majority of subcellular markers [32]. In Leishmania, the closest system to T. brucei so far analysed, AP-1 is not required for cell proliferation in culture but is required for infectivity and this difference between trypanosomes and Leishmania is highly intriguing [37]. Absence of the GGA proteins, down-regulated expression of the AP-3 complex in the bloodstream stage and absence of an AP-2 complex may explain essentiality in T. brucei as Leishmania retains AP-2. Dictyostelium and T. gondii both lack GGAs [1], consistent with GGA/AP-1 redundancy in mammals and yeast. This must however be offset against the lack of an effect on clathrin targeting; hence despite the essentiality of AP-1 in trypanosomes, there must be additional factors that are able to efficiently target
clathrin to endomembranes, and these could include AP-3, AP-4 or TbEpsinR. Further work is required to determine if this is indeed the case.

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Appendix A. Supplementary data


References


