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A role for the vesicle-associated tubulin binding protein ARL6 (BBS3) in flagellum extension in *Trypanosoma brucei*

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ABSTRACT

The small GTPase Arl6 is implicated in the ciliopathic human genetic disorder Bardet–Biedl syndrome, acting at primary cilia in recruitment of the octomeric BBSome complex, which is required for specific trafficking events to and from the cilium in eukaryotes. Here we describe functional characterisation of Arl6 in the flagellated model eukaryote *Trypanosoma brucei*, which requires motility for viability. Unlike human Arl6 which has a ciliary localisation, TbARL6 is associated with electron-dense vesicles throughout the cell body following co-translational modification by *N*-myristoylation. Similar to the related protein ARL-3A in *T. brucei*, modulation of expression of ARL6 by RNA interference does not prevent motility but causes a significant reduction in flagellum length. Tubulin is identified as an ARL6 interacting partner, suggesting that ARL6 may act as an anchor between vesicles and cytoplasmic microtubules. We provide evidence that the interaction between ARL6 and the BBSome is conserved in unicellular eukaryotes. Overexpression of BBS1 leads to translocation of endogenous ARL6 to the site of exogenous BBS1 at the flagellar pocket. Furthermore, a combination of BBS1 overexpression and ARL6 RNAi has a synergistic inhibitory effect on cell growth. Our findings indicate that ARL6 in trypanosomes contributes to flagellum biogenesis, most likely through an interaction with the BBSome.

1. Introduction

The Arf-like (Arl) proteins are a subfamily of the Arf small GTPases, with diverse roles in vesicle trafficking [1], tubulin folding and microtubule dynamics [2], endosome–lysosome fusion [3] and ciliogenesis [4]. While the functions and effectors of the closely related Arf proteins have been extensively characterised, much less is known about Arl protein interactions and their regulation of fundamental processes within the eukaryotic cell.

Human Arl6 is encoded by *Bbs3*, one of 14 genes in which mutations are implicated in the autosomal recessive clinical disorder Bardet–Biedl syndrome (BBS) [5–7]. The pleiotrophic features of this condition include retinal degeneration, renal abnormalities, polydactyly, obesity, mental retardation and hypogenitalism, many of which are consistent with cilium dysfunction. Comparative genomic analysis has shown that orthologues of Arl6 and the other BBS-associated genes are restricted to ciliated and flagellated organisms, and are absent from species which use flagella only for motility of gametes or zoospores e.g. *Plasmodium falciparum* [7–9]. Expression of Arl6 in *Caenorhabditis elegans* is upregulated in ciliated cells in which the protein is trafficked by intraflagellar transport (IFT) [10]. Recent evidence has shown that GTP-bound human Arl6 can bind BBS1, a subunit of the BBSome complex, and may act in recruitment of this complex to the primary cilium [11]. The BBSome is a stable octomeric complex comprised of 7 Bardet–Biedl syndrome associated proteins (BBS1, 2, 4, 5, 7, 8 and 9) plus a novel protein BBIP10 [12,13]. The exact molecular mechanisms regulated by this complex remain largely unknown but there are marked similarities between the predicted structures of the BBSome components and those of the subunits of the COPII and clathrin cages, indicating a possible function of the BBSome as a coat complex [11]. Further, a recent report indicates that the BBSome

Abbreviations: Arf, ADP-ribosylation factor; Arl, ADP-ribosylation factor-like; Arl6ip, Arl6 interacting protein; BBS, Bardet–Biedl syndrome; BBS1, Bardet–Biedl syndrome 1 protein; BSF, bloodstream form; ConA, Concanavalin A; GEF, guanine nucleotide exchange factor; GPCR, G-protein coupled receptor; HRG4, human retinal gene 4; IFT, intraflagellar transport; ITC, isothermal titration calorimetry; MANT, *N*-methylanthraniloyl; MAP2, microtubule associated protein 2; NES, nuclear export signal; NLS, nuclear localisation signal; NMT, myristoyl-CoA:protein *N*-myristoyltransferase; PCF, procyclic form; PCM1, pericentriolar material 1; PFR, paraflagellar rod; PM, plasma membrane; RNAi, RNA interference; RP2, retinitis pigmentosa protein 2; TAP, tandem affinity purification; TiEM, transmission immuno-electron microscopy

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subunit BBS7 localises transiently to the nucleus where it binds to the polycomb protein RNF2 and may have a direct role in transcriptional regulation [14].

The BBSome in *C. elegans* appears to mediate the IFT machinery, as loss-of-function mutations in *Bbs7* and *Bbs8* orthologues cause abnormalities in localisation and motility of two IFT proteins, OSM-5/Polaris/IFT88 and CHE-11, resulting in truncated cilia and inhibition of chemotaxis [15]. However, in the majority of systems studied to date, the BBSome is believed to be required for specific trafficking events to and from the cilium rather than for ciliogenesis. Two G-protein coupled receptors (GPCR), somatostatin receptor 3 and melanin-concentrating hormone receptor 1, fail to localise to the cilium of hippocampal neurons in BBSome knockout strains of mice [16]. In contrast, the GPCR dopamine receptor 1, which normally shuttles to and from cilia, can still be trafficked to neuronal cilia in *Bbs2* and *Bbs4* null mice but fails to exit the cilium in response to receptor ligand in these animals [17]. Insertional mutants of *Bbs1*, *Bbs4* or *Bbs7* in the green alga *Chlamydomonas reinhardtii* produce normal length flagella but phototaxis is disrupted due to a defect in retrograde transport from the flagellum leading to accumulation of signalling proteins [18]. No reports have been published to date on the functions of the BBSome or associated proteins in parasitic protozoa or any other pathogenic organisms.

Here we describe functional characterisation of Arl6 in the parasitic protozoan *Trypanosoma brucei*, as part of a comprehensive study of ARF/ARL proteins as substrates of the validated drug target myristoyl-CoA: protein *N*-myristoyltransferase (NMT) [19–22]. We provide experimental evidence that *T. brucei* ARL6 is *N*-myristoylated as predicted by protein sequence analysis. The trypanosome orthologue of Arl6 is found on small electron-dense vesicles throughout the parasite body. The protein is able to associate with tubulin and might therefore act as a link between the BBSome complex and the intracellular microtubule network. Knockdown of TbARL6 is not lethal to the parasite but causes a significant decrease in flagellum length, perhaps indicative of defects in protein trafficking to the flagellum. This observation suggests a degree of evolutionary conservation in the role of this protein and possibly of the BBSome complex in trypanosomes.

2. Materials and methods

2.1. DNA constructs

All primer sequences are provided in Supplementary Table 1. For protein expression in *Escherichia coli*, a fragment spanning nucleotides 1–570 of the TbARL6 (TriTrypDB ID: Tb927.8.5060) open reading frame was amplified from genomic DNA using primers ARL6-F1 and ARL6-R1 and cloned into plasmid vector pET-YSBLIC3C (pET28a modified for ligation-independent cloning) [23,24] to produce the construct pET-^{His}ARL6. For tetracycline-inducible overexpression of C-terminal myc-tagged TbARL6 and N-terminal myc-tagged BBS1 (TriTrypDB ID: Tb09.211.2080) in bloodstream form *T. brucei*, the open reading frames were amplified from genomic DNA using primers ARL6-F2 and ARL6-R2 or BBS1-F1 and BBS1-R1. The products were ligated into plasmid vectors pT7-$^{MYC\text{-}C}$ or pT7-$^{MYC\text{-}N}$ [22,25]; (obtained from David Horn and Sam Alsford, London School of Hygiene and Tropical Medicine, London, UK) to produce the constructs pTbARL6^{MYC} and pTb^{MYC}BBS1. Mutations were introduced into pTbARL6^{MYC} using the GeneTailor Site-Directed Mutagenesis System (Invitrogen) and primers ARL6-F3 and ARL6-R3 (G2A) or ARL6-F4 and ARL6-R4 (T21N) to produce the constructs pTbARL6-G2A^{MYC} and pTbARL6-T21N^{MYC}. For RNA interference, a region spanning nucleotides 48–362 of the *T. brucei* ARL6 open reading frame was amplified from genomic DNA using the primers ARL6-F5 and ARL6-R5. The product was digested with *Xba*I and ligated into *Xba*I-digested plasmid vector p2T7Ti (a gift from Doug LaCount, PULSe, Purdue University, West Lafayette, IN, USA) to produce the construct p2T7ARL6. For PTP tagging, the ARL6 open reading frame was amplified from *T. brucei* genomic DNA using primers ARL6-F6 and ARL6-R6, digested with *Apa*I/*Not*I and ligated into digested pC-PTP-NEO (a gift from Arthur Günzl, Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington, CT, USA) to produce the construct pTbARL6^{PTP}.

2.2. Production of recombinant TbARL6 in E. coli

The construct pET-^{His}ARL6 was introduced into *E. coli* BL21 star and expression of recombinant protein induced with 1 mM IPTG for 4 h at 30 °C. For large-scale protein purification, cells from 5 l of culture were resuspended in 100 ml lysis buffer (300 mM NaCl, 20 mM sodium phosphate pH 7.4, 40 mM imidazole and 1× complete protease inhibitor cocktail, Roche). Cells were lysed by three rounds of sonication then centrifuged at 50,000 *g* for 40 min at 15 °C. Protein purification was performed using Ni^{2+}-affinity/size exclusion multi-dimensional liquid chromatography on an ÄKTA Express (GE Healthcare). The clarified lysate was applied at 1 ml/min to a first stage affinity-chromatography column (1 ml HisTrap FF, GE Healthcare) equilibrated in 20 mM sodium phosphate, pH 7.5, 300 mM NaCl and 20 mM Imidazole (Buffer A), before removal of non-specifically bound proteins with 20 column volumes of Buffer A. His-tagged proteins were removed using an isocratic gradient of Buffer A containing 0.5 M imidazole (Buffer B) and the $UV_{280\ nm}$ absorbing material was peak-fractionated and collected into a system sample loop. The contents of the sample loop were subsequently applied at 1 ml/min to a second stage size exclusion-chromatography column (Superdex200 16/60, GE Healthcare) equilibrated in 20 mM sodium phosphate, pH 7.5, 150 mM NaCl (Buffer C) and then eluted using a 1.2 column volume isocratic gradient of Buffer C. Fractions (1.8 ml) of $UV_{280\ nm}$ absorbing material were collected and analysed for specific protein content by SDS-PAGE.

2.3. Antibody production

Polyclonal antibodies were produced from two rabbits (Eurogentech, 87 day Classic protocol). Antibodies were purified using a 1 ml NHS-activated HP column (GE) coupled to 5 mg of recombinant TbArl6 protein. Following column equilibration with 10 ml binding buffer (20 mM sodium phosphate pH 7 and 150 mM NaCl), 15 ml rabbit serum was loaded onto the column at 0.3 ml/min. Unbound sample was removed with a 5 ml wash with binding buffer. Elution was then performed using elution buffer at low pH (0.1 M glycine pH 2.7 and 0.5 M NaCl). Fractions of 0.5 ml were collected directly into tubes containing 50 µl 1 M Tris–HCl pH 9.0 and analysed by SDS-PAGE. Peak fractions were pooled and tested by immunoblotting.

2.4. Parasite culture

The *T. brucei brucei* BSF strain Lister 427 was maintained *in vitro* at 37 °C with 5% CO_2 in HMI-9 medium containing 2 µg/ml Geneticin (Invitrogen) as described [26]. The *T. brucei brucei* procyclic strain 449 was maintained *in vitro* at 26 °C in SDM-79 medium containing 25 µg/ml phleomycin [26]. All culture media contained 10% tetracycline-free fetal bovine serum (Autogen Bioclear).

2.5. Protein analysis

Immunoblotting of total parasite lysates was performed as described previously [22,27]. Primary antibodies used were: rabbit anti-TbARL6 (1:500 dilution); rabbit anti-TbNMT [19] (1:500 dilution); mouse anti-myc (1:2000 dilution, Invitrogen); and mouse monoclonal L13D6 against PFR1/2 (1:500 dilution, a gift from Keith Gull, Sir William Dunn School of Pathology, University of Oxford, UK). For subcellular fractionation, parasites were centrifuged at 800 *g* for 10 min at 20 °C,