



The molecular and cellular characterisation of the ADP - ribosylation factor of African trypanosomes

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Abstract

Small GTP-binding proteins of the ADP-ribosylation factor (Arf) family are essential for vesicle formation, intracellular and membrane trafficking and signal transduction. We have cloned and characterized the homologue of Arf1 from *Trypanosoma b. brucei*, *Trypanosoma evansi*, *Trypanosoma congolense* and *Trypanosoma vivax*. The African trypanosome Arf1 cDNA contains an open reading frame of 546 base pairs, corresponding to a 181 amino acid residue polypeptide. The protein carries conserved motifs in the GTP-binding domains for Arf1 proteins and a myristoylation site in addition to domains specific to trypanosomes. The TbArf1 gene generates a transcript of approximately 0.9 kb that is developmentally regulated. Recombinant TbArf1 expressed in *Escherichia coli* binds GTP. GDP and GTP were efficient competitors of [α^{32} P]- GTP binding to the protein but not ATP or CTP. Polyclonal antibodies raised against the recombinant TbArf1 revealed a major protein band of about 21 kDa in *T. b. brucei* and *T. congolense* lysates and additional weaker binding to proteins of higher molecular weight in both lysates. The protein localizes in endosomal compartments between the nucleus and kinetoplast but in addition it also displays a flagellar localisation. The TbArf1 protein is detected in supernatant of *in vitro* cultivated parasites.

Keywords: Recombinant expression, antibodies, secretion, endocytosis, differential gene expression, GTP-binding.

INTRODUCTION

African trypanosomiasis is a widespread, fatal disease caused by several different species and subspecies of *Trypanosoma*. This disease, commonly known as sleeping sickness in human and nagana in cattle, is both an economic and a public health problem. African trypanosomes undergo a complex life cycle involving a tsetse fly vector and mammalian host (Vickerman, 1985) and appear to secrete factors that may be involved in signalling host cell types and/or other trypanosomes (Olsson et al., 1993; Vassella et al., 1997; Valdy et al., 1997). Such factors have a role in co-ordinating complex parasite-parasite and parasite-host interactions resulting in the control of growth and differentiation during their developmental life cycle.

More intriguingly trypanosome cell surface membrane trafficking is highly unusual in that all exocytotic and endocytotic processes take place through a unique organelle, the flagellar pocket (Bangs et al., 1993; Webster and Russell, 1993; Field et al., 1999; Landfear and Ignatushenko, 2001). Thus, transduction of extracellular signals, such as growth factors and environmental cues from the cell surface to the nucleus, secretion and uptake of nutrients and membrane components in the trypanosomes require complex intracellular pathways to target and focus them to this unique area on the cell surface.

A detailed understanding of protein and membrane trafficking events in trypanosomes may reveal important new mechanisms of how endocytic and secretory events can

can be directed to a single highly specialised area of the cell surface and how sorting is geared within and towards such a restricted region. This could also give new insights on possible mechanisms of intra- and extracellular transduction of signals and other pathways involved in exo- and endocytosis in these parasites.

Essential elements in protein and membrane trafficking, signal transduction, and apoptotic pathways include guanine nucleotide-binding proteins (G-proteins), both the heterotrimeric G proteins (Exton, 1997) and the smaller (≈ 20 - kDa) mono-meric proteins related to the oncogene gene product Ras (Kahn et al., 1992; Takai et al., 2001).

Among these mono-meric GTP-binding proteins are the ADP-ribosylation factors (ARFs). The ARF protein was originally identified as a protein cofactor required for

efficient ADP-ribosylation of the $G_{s\alpha}$ subunit of adenylate cyclase by cholera toxin (Kahn and Gilman, 1984). Recently the regulatory role of ARF proteins in the mediation of vesicular transport and membrane trafficking, which are integral components of secretory and endocytic pathways in eukaryotic cells, has been demonstrated (Stafford et al., 1996; Field et al., 1998, 1999).

ARF family members regulate the assembly of coat components in budding vesicles that transit between the ER and the Golgi complex, between the Golgi stacks and in endocytic pathways (Bowen and Kahn, 1995; Randazzo et al., 2000; Vernoud et al., 2003).

Arf1 is a component of non-clathrin-coated vesicles and is essential for the recruitment of COPI coatmer subunits to Golgi membranes. Arf1 binding to membrane is controlled by GTP hydrolysis and the N-myristoylation of a conserved glycine. Immunological data show that Arf1 is localized to Golgi apparatus in mammalian cells. Arf family members also activate phospholipase D that is a key component in the signal transduction pathway (Shome et al., 1997; Donaldson, 2003).

A number of recent studies have revealed the presence of Rab GTPases, main regulators of vesicular traffic, in trypanosomatids indicating that they may play key conserved cellular functions as in higher eukaryotic cells (Field et al., 1998, 1999; Leal et al., 2000; Jeffries et al., 2001).

The COPI complex of *Trypanosoma brucei*, is biochemically and physically similar its mammalian counterpart (Maier et al., 2001) and Arf1 of the related kinetoplastids *Trypanosoma cruzi* and *Leishmania donovani* have been described (de Sá-Freire et al., 2003; Porter-Kelley et al., 2004). These proteins and Arfs play a major role in recycling vesicles and sorting of cargo in both the endocytic and secretory pathways and remodelling the actin cytoskeleton.

To date very little is known about the function of trypanosome Arf proteins and their involvement in protein and membrane trafficking, signal transduction and actin cytoskeleton dynamics. Here we report the molecular and cellular characterisation of the ADP - ribosylation factor of African trypanosomes.

MATERIALS AND METHODS

Strains of trypanosomes

The different bloodstream forms of *T. b. brucei* ILTat1.1, a pleomorphic strain, *Trypanosoma congolense*, clone IL3000, *Trypanosoma vivax*, clone ILDat1.2 and *Trypanosoma evansi* KETRI 2443c1 were grown and isolated as described (Vickerman, 1971). Procyclic *T. b. brucei* ILTat1.1 (tsetse fly mid-gut stage), epimastigote and metacyclic forms of *T. congolense* IL3000 were cultured *in vitro* and isolated as described (Brun and Scheneberger, 1979). The trypanosomes were used immediately, or stored frozen as a cell pellet at -70°C .

Enzymes and radioactive biochemicals

Restriction enzymes were purchased from New England BioLabs, T4 ligase, random priming kits from Amersham Inc., M-MuLV reverse transcriptase and complementary DNA (cDNA) synthesis kit from GIBCO-BRL. All the enzymes were used according to standard procedures (Sambrook et al., 1989) or as recommended by the manufacturer. Radioactive reagents were obtained from Amersham Inc. Taq DNA polymerase was obtained from Promega.

RNA and DNA preparation and analysis

Total trypanosome RNA was isolated by the guanidinium thiocyanate-phenol-chloroform extraction procedure (Chomczynski and

Sacchi, 1987). Poly (A)⁺ RNA was purified from total RNA by oligo (dT) chromatography (Sambrook et al., 1989) and trypanosome genomic DNA was isolated by the SDS-proteinase K- phenol method (Sambrook et al., 1989). Phage DNA was isolated by the Lambdasorb phage adsorbent (Promega) protocol. Plasmid DNA was purified from overnight bacterial culture, by the Wizard miniprep DNA purification system (Promega). Northern and Southern blot analyses were performed as described (Sambrook et al., 1989; Pellé and Murphy, 1993).

Library construction, plasmids and bacterial strains

A cDNA library in λ gt11 was constructed from total messenger RNA prepared from bloodstream forms of *T. congolense* clone IL3000 and *T. b. brucei* ILTat 1.1 using the GIBCO-BRL cDNA synthesis system.

For cloning of polymerase chain reaction (PCR) products, the pMosT vector (Amersham) was used while pBluescript (Stratagene) and the expression system pQE30 (Qiagen) were used for subcloning. The bacterial strains used were competent *Escherichia coli* JM109 (for pMosT and pBluescript vectors) and *E. coli* strain M15 [pREP4] (Qiagen), for protein expression with the pQE system.

DNA sequence analysis

Nucleotide sequencing of cDNA and genomic clones was carried out on double-stranded templates by the di-deoxy chain termination method (Sanger et al., 1977), using the *f*-molTM DNA sequencing system (Promega). Sequence homology searches of the local and public database as well as protein structure analysis were carried out using DNASIS/PROSIS and BLAST (Altschul et al., 1997) programmes. Multiple sequence alignment analysis was performed using the Clustal X (Thompson et al., 1997).

Cloning of African trypanosome Arf1 homologue

An expressed sequence tag (EST) encoding a portion of *T. b. brucei* Arf1 homologue was identified through the exploitation

of randomly amplified differentially expressed sequences (RADES) display applied to identify expressed genes during the life cycle of trypanosomes (Murphy and Pellé, 1994; Osanya et al., unpublished). The arbitrary 10-mer primer 5'AAG CGA GCC G 3' amplified a 0.35 kb RADES product corresponding to a portion of ARF that was abundant in actively-dividing long slender bloodstream forms of *T. b. brucei*. The fragment was used to probe the λ gt11 library of *T. congolense* and identified a phage clone with 0.9 kb insert containing the trypanosome 5' spliced leader.

Two cDNA fragments encoding 5' and 3' half of the full-length cDNA of the *arf1* gene were generated by PCR amplification of cDNA from actively dividing long slender bloodstream form of *T. brucei* ILTat1.1. The 5' region was cloned by PCR amplification using the primer (5' CTT ATA ATA GAA CAG TTT 3') for minixon and internal primer ARFrev (ILO4952; 5' CAG CCC AAG TTT CTC CGC G 3'). The 3' region was cloned using primer ARFwd (ILO4951; 5' CTA TGG CGC CAC TAC TAC C 3') and oligo (dT) primer (5' TAG GCG CGC CTT (TTT) 6 3'). The primers ARFwd and ARFrev were designed from the *T. b. brucei* EST clones T3632 and T5166 (GenBank accession numbers AA585066 and AI795168, respectively) generated from RADES product (Osanya, 1999).

T. congolense and *T. evansi* Arf1 homologue was cloned by PCR amplification from total ss-cDNA from bloodstream forms of the parasites using primers designed from sequences flanking the *TbArf1* ORF. The *T. vivax* homologue was cloned by PCR using primers designed from conserved amino acid sequences [G1 as forward primer] and [G3 rev] together with spliced leader primer and oligo-(dT) primer. In each case two cDNA fragments corresponding to 5' and 3' half of full length for Arf1 were generated and the corresponding products cloned in the pMos T-vector. Following sequencing of the cloned inserts from each trypanosome species, forward and reverse specific primers were designed and used to PCR amplify ss-cDNA and genomic DNA to generate DNA fragments corresponding to ARF ORF and their DNA sequences.

Expression and affinity purification of His-tagged trypanosome Arf1 protein

Plasmid vector, pBluescript, containing a 0.9 kb *T. brucei* Arf1 cDNA insert was used in PCR amplification to generate a 0.55 kb DNA fragment corresponding to the Arf1 ORF. The 5' (amino terminal) primer sequence was 5' GGG ATCC GGT CA A TGG TGG 3' and 3' (carboxyl terminal) primer was 5' CCA AGC T TC ATA CTC TCT TCT TAA TG 3', with first eight nucleotides being extrinsic to Arf1 gene, introducing underlined unique *Bam*HI and *Hind*III sites at 5' and 3' ends of the amplified DNA fragment, respectively, and deleting the ATG initiation codon.

The PCR product was purified (Wizard PCR purification system, Promega), cloned in pGEM- T vector (Promega), excised with *Bam*HI and *Hind*III and then subcloned into pQE-30 expression plasmid vector.

The resultant recombinant plasmid expressing the wild type Arf1 was transformed into competent *E. coli* M15 [pREP4] cells for over expression.

The expression in *E. coli* M15 [pREP4] cells and purification of the His-tagged recombinant Arf1 by a nickel nitrilotriacetate (Ni-NTA)-agarose resin (Qiagen) chromatography were done as recommended by the supplier. The eluted recombinant protein was dialyzed against phosphate-buffered saline (PBS) and stored at -70°C.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses

SDS-polyacrylamide gel electrophoresis analysis was performed essentially as described (Laemmli, 1970). For total trypanosome

proteins, approximately 10^7 cells were dissolved in 1 x SDS- PAGE sample buffer then sonicated for 2 - 5 s to break genomic DNA, before boiling for 5 min. Gels were then stained with Coomassie Brilliant Blue G-25 to visualize the protein bands. For immunoanalysis, samples were electrotransferred onto nitrocellulose filters (Schleicher and Schuell) immediately after SDS- PAGE. Filters were then stained with Ponceau S as described to monitor the transfer and visualize major protein bands. Following destaining, filters were blocked with 5% skimmed milk in PBS and probed with immune sera. Bound antibody was revealed by an anti-IgG peroxidase conjugate in the presence of hydrogen peroxide (Pellé et al., 2002).

Generation of anti TbArf1 antibody in mouse

Affinity-purified and PBS- dialysed His-tagged Arf1 protein was used to immunise Balb/C mice with 3 intraperitoneal injections at 15 days interval. Each mouse received 3 μ g of antigen per inoculation. Anti-gen was emulsified in Freund's complete adjuvant in the first injection and in Freund's incomplete adjuvant for the subsequent boosts. Sera were collected and tested a week after the second inoculation. The animals were sacrificed and the sera collected and stored frozen in the presence of 0.02% sodium azide. The antisera were used for immunodetection of the native protein as described by Sambrook et al. (1989).

Monoclonal antibodies were generated essentially as described (Goding, 1986; Osanya, 2000).

Supernatant containing secreted trypanosome factors

Parasites were seeded at concentration of 1×10^5 /ml were cultured in a T75 flask in 20 ml of growth medium with serum and allowed to grow to log phase as described by Hurumi and Hirumi (1991). The parasite monolayers were washed twice with serum-free medium, and then incubated in culture media free of serum for 4 hours at 34°C. The conditioned medium was harvested, transferred to eppendorf tubes and clarified by centrifugation at 14,000 rpm for 5 min. Clarified medium was concentrated in a SpeedVac concentrator and dialyzed against PBS, then analysed by immunoblotting with antibodies against Arf1.

GTP-binding assay

In situ GTP binding was carried out as described by Coulter and Hide (1995). The recombinant proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose filter. The filter was incubated for 30 min in 6 M guanidine hydrochloride, 50 mM NaH_2PO_4 , 10 mM MgCl_2 , 2 mM DTT and 0.3% Tween 20, pH 7.5, and the concentration of guanidine hydrochloride reduced from 6 to 0.094 M by sequential 2-fold dilutions. After soaking at 4°C, the filter was incubated in fresh buffer containing 3 μ Ci/ml of [α - 32 P]-GTP for 2 hr at room temperature and washed with 50 mM NaH_2PO_4 , 10 mM MgCl_2 , 2 mM DTT and 0.3% Tween 20, pH 7.5, air-dried and exposed at -70°C.

Immunofluorescence microscopy

Immunolocalization on fixed, permeabilized cytopins of trypanosomes were conducted as previously described (Pellé et al., 2002). Slides were incubated with anti-ARF antibody and labelling was visualised with a goat anti-mouse-Ig-FITC (Sigma). The parasite nuclei were then labelled with 1 μ g/ml 4, 6 diamidino-2-phenylindole (DAPI, Sigma) and viewed using a Zeiss Axiophot 1 fluorescence microscope with an oil immersion X 100 Plan-Neofluor lens.

Table 1. Summary of nucleotide sequence identity and similarity analysis amongst trypanosomes and other organisms.

	Sequence Identity (%)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. <i>T-brucei</i>		96.7	98.4	91.2	79.1	66	62.1	72	69.4	74.2	67.6	75.8	74.7	75.8	75.8
2. <i>T-congolense</i>	98.9		95.1	88.5	78.6	66.5	61.5	72.5	69.4	74.7	67.6	76.4	75.8	75.8	75.8
3. <i>T-vivax</i>	100	98.9		92.3	79.1	64.9	62.1	72.5	70.5	75.3	68.7	76.9	76.4	76.4	76.4
4. <i>T-cruzi</i>	98.9	97.8	98.9		76.4	63.4	59.9	70.9	67.8	72.5	66.5	74.2	74.2	73.6	73.6
5. <i>L-major</i>	91.2	90.7	91.8	90.1		59.7	56	63.7	62.3	67.6	62.6	66.5	67.6	67.6	67.6
6. <i>G-lambia</i>	78.5	78	78	78.5	73.8		58.6	66	65.4	67	62.8	66.5	67.5	67	67
7. <i>T-vaginalis</i>	76.4	76.4	76.9	76.9	75.3	71.2		65.4	65	66.3	62.4	66.9	67	66.3	66.3
8. <i>D-discoideum</i>	83.5	83.5	83.5	83.5	83.5	78	80.2		78.7	84.6	73.1	83.5	83	83.5	83.5
9. <i>T-gondii</i>	86.3	86.3	86.3	84.7	82	80.1	79.8	91.3		82.5	74.9	80.9	81.4	80.9	80.9
10. <i>A-thaliana</i>	86.3	85.7	86.3	86.3	83.5	81.7	81.2	91.8	92.9		76.8	86.2	86.8	88.4	88.4
11. <i>S-cerevisiae</i>	84.6	84.6	84.6	84.1	81.9	79.1	79.6	88.5	88	89.5		77.3	78	76.8	76.8
12. <i>C-elegans</i>	87.4	87.4	87.4	86.8	81.9	79.6	80.7	90.1	88.5	91.2	89.5		94	93.4	93.4
13. <i>D-melanogaster</i>	89	89	89	88.5	83.5	79.6	82.4	91.2	89.1	91.2	89	97.8		95.1	95.1
14. <i>X-laewis</i>	89	89	89	87.9	83.5	80.6	82.9	89.6	89.6	92.8	88.4	97.8	98.4		99.4
15. <i>H-sapiens</i>	89	89	89	87.9	83.5	80.6	82.9	89.6	89.6	92.8	88.4	97.8	98.4	100	
	Sequence Similarity (%)														

RESULTS

Cloning of the trypanosome ADP-ribosylation Factor 1 (*arf1*) gene

The differential display method, RADES-PCR, for the identification of developmentally regulated genes from trypanosomes (Murphy and Pellé, 1994) was used to identify sequences that are amplified selectively in actively dividing long slender form parasites. These PCR products were cloned into plasmid pMos-T vector and sequenced. Blast analysis on the 0.35 kb RADES fragment revealed a high sequence homology to other eukaryotic Arf sequences (data not shown).

Two cDNA fragments encoding 5' and 3' halves of the full-length cDNA of the *TbArf1* gene were generated by PCR amplification of cDNA from actively dividing long slender bloodstream form of ILTat1.1 as described in Materials and Methods.

The PCR-amplified products containing the 5' and 3' regions of the putative *TbArf1* cDNA were cloned and their nucleotide sequence determined. Sequence analysis revealed that the *TbArf1* cDNA contains an open reading frame (ORF) of 546 nucleotides encoding a protein of 181 amino acids with a calculated Mr of 20 580 Da.

Combining heterologous hybridization in combination with PCR amplification, Arf1 homologues were identified and cloned from various species of African trypanosomes including *T. congolense*, *T. evansi*, *T. vivax*, *Trypanosoma b. gambiense* and *Trypanosoma b. rhodesiense*.

Alignment of the predicted *TbArf1* amino acid sequences with the deduced sequences of *T. congolense*, *T. b. gambiense*, *T. evansi* and *T. vivax* Arf1 ORFs, showed

they have over 90% identity (Table 1). African trypanosome Arf1 amino acid sequences were compared with the sequences from those of other kinetoplastids, organisms that had diverged earlier than the kinetoplastids in the eukaryotic lineage and organisms that have diverged later, including humans. This analysis revealed kinetoplastid-specific and African trypanosome-specific domains in their Arf1 protein sequences (Figure 1).

The deduced amino acid sequences of the African trypanosome Arf1 proteins show distinct conserved motifs for phosphate and GTP-binding/hydrolysis (Figure 1, boxes), and a glycine residue at position 2 (Figure 1, asterisk), which are characteristic of all Arf proteins (Valencia et al., 1991; Kahn et al., 1992). The GLDAAGKT motif in the trypanosome Arf1 sequence constitutes the phosphate-binding loop (consensus sequence GXXXXGKS/T). The DXGXGQ motif within the sequence TMVDVGGQD, is present in all Arf proteins and is involved in the interaction with the γ -phosphate of GTP, while the NKQD motif is specific for the guanylyl binding. The African trypanosome Arf1 sequence also displays homologies to sequences important for other functions. The involvement of the putative glycine-myristoylation site at position 2 in the binding of protein to membranes has been demonstrated for bovine brain Arf1 (Kahn et al., 1988). Potential serine/threonine phosphorylation sites by protein kinase C are present at positions 138 and 147 and, by casein kinase 2 at positions 64 and 161.

Taken together, these results show that this gene encodes the Arf1 gene homologue of *T. b. brucei* as well as other species of African trypanosomes. The trypanosome Arf1 protein revealed unusual features, which included a

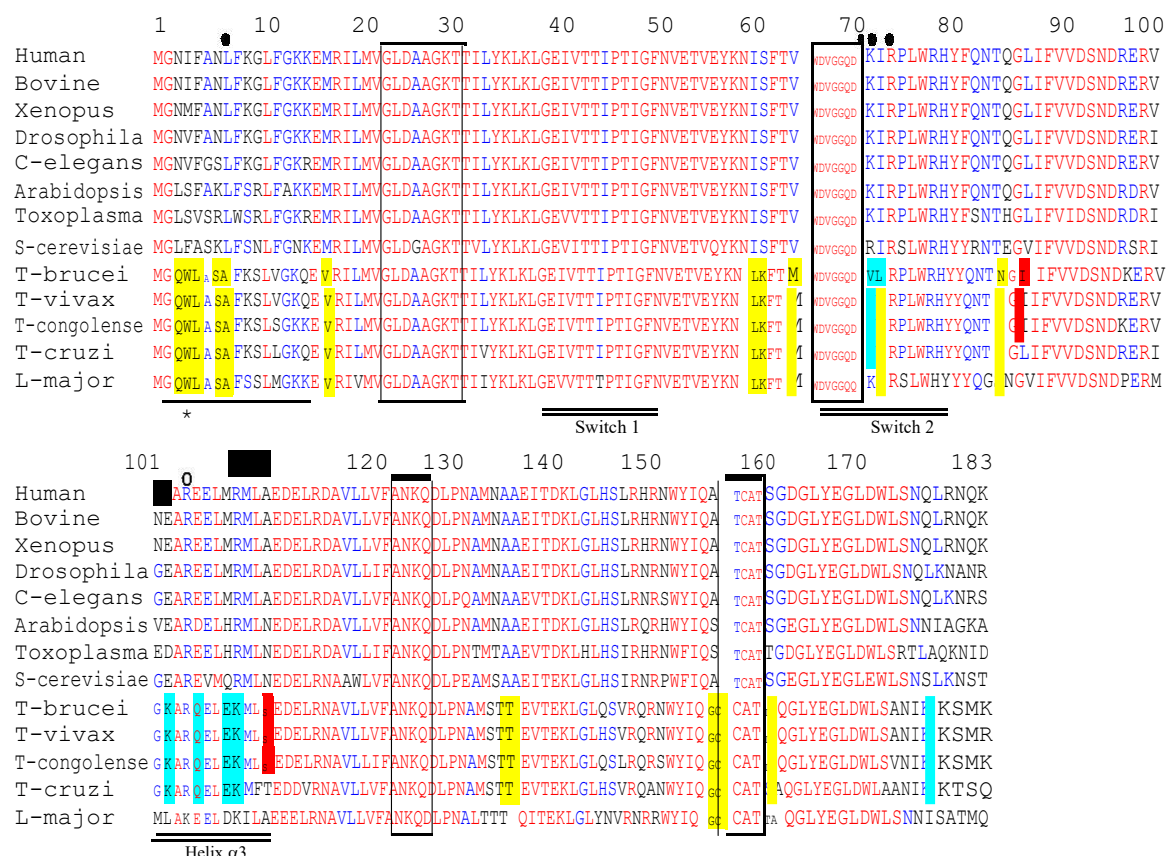


Figure 1. Alignment of Arf1 sequences from African trypanosomes and other eukaryotes. Amino acid identity based on the trypanosome consensus is indicated by the shaded residues. At residues where there is no trypanosome consensus the shading is based on the overall consensus between all sequences. The myristoylated amino-terminal G and N-terminal α -helical domain are marked by an arrowhead and single underline, respectively. The consensus GTP-binding motifs conserved among Ras-family GTP-binding proteins are shown in boxes. Switch 1, switch 2 and α -helix 3 involved in interactions with ArfGEF and ArfGAP effectors is indicated. Closed and open circles above the sequences indicate residues when mutated to alanine (A) result in inviable (closed) or viable (open) cells in yeast (Click et al., 2002).

second cysteine at position 158 and threonine at position 162 flanking the CAT domain; methionine and valine-lysine flanking the DVGQD domain. Other trypanosome specific domains are confined in the helix α 3 region and carboxyl terminal ends of the proteins, respectively (Figure 1, blue shaded residues). These trypanosome-specific modifications could be responsible for unique functioning of the Arf1 in these parasites.

Arf1 is differentially expressed stages of African trypanosomes

during

TbArf1 was initially identified as a developmentally regulated transcript by RADES-PCR (data not shown). Hybridization with DNA of the ORF of *TbArf1* to a Northern blot of total RNA of actively-dividing long slender, intermediate and non-dividing short-stumpy bloodstream forms showed a single transcript of ~ 0.9 kb (Figure 2). The relative amount of RNA loaded in each lane was determined by hybridisation of the same blot to a *T. brucei* 18S

rRNA gene probe. The level of *TbArf1* RNA was higher in the actively dividing long slender forms than in non-dividing short stumpy and procyclic forms.

Northern blot analysis was performed also for mRNA from blood stream, procyclic, epimastigote and metacyclic forms from *in vitro* cultures of *T. congolense* clone IL3000. The *TcoArf1* probe recognised a transcript of ~0.9 kb in all the developmental stages of *T. congolense* examined (Figure 2). The hybridization signal was stronger in the bloodstream and metacyclic forms than in the procyclic and epimastigote forms.

Thus *Arf1* expression appears to be developmentally regulated in both *T. brucei* and *T. congolense* parasites.

The African trypanosome ARF1 protein migrates as a 21.5 KDa protein and localizes to golgi endosomal compartments and flagellar rod

The ORF of the *TbArf1* was subcloned in the bacterial ex-

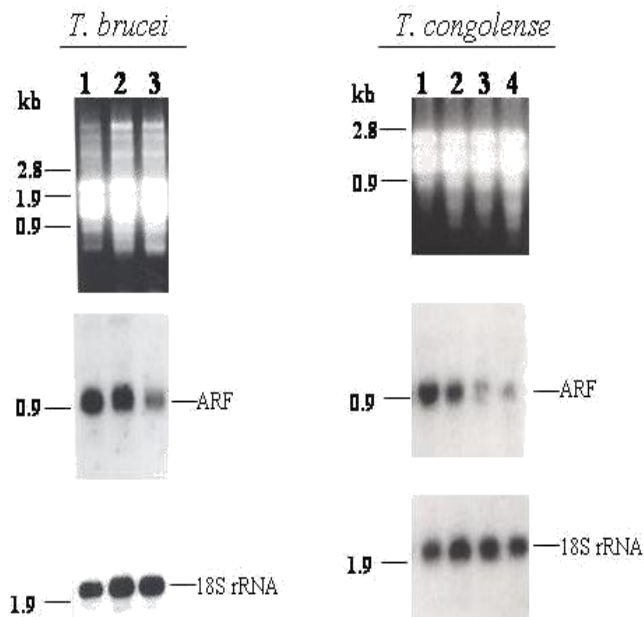


Figure 2. *TbArf1* gene expression is developmentally regulated during the life cycle of trypanosomes. Total RNA prepared from different development stages was denatured and electrophoresed on a 1.4 % agarose gel in 10 mM Sodium phosphate buffer, blotted and hybridised with ³²P-labelled *TbARF1* cDNA and 18S ribosomal DNA probes. Post hybridisation washes were followed by autora-diography. For *T. b. brucei*, actively-dividing long slender (lane 1), short stumpy (lane 2) and procyclic (lane 3) forms of ILTat1.1, were examined. For *T. congolense*, mammalian BSF Nie et al., 2003 (lane 1) and insect metacyclic (lane 2), procyclic (lane 3) and epimastigote forms (lane 4) were examined. The positions of the Arf1 and of the control 18S rRNA transcripts are indicated.

expression vector pQE-30 (Qiagen) and recombinant TbArf1 protein containing a 6xHis-tag fusion at the N-terminus was produced. The recombinant protein was affinity-purified using Ni-NTA resin from bacterial lysates as recommended by the supplier (Qiagen). The recombinant protein migrated by SDS-PAGE with an apparent molecular weight slightly larger than predicted, possibly due to the histidine tag (Figure 3A).

The purified recombinant TbArf1 protein was used to raise antibodies in mice. Total lysates from 10⁶ trypanosomes were resolved by SDS-PAGE, and then electrotransferred onto nitrocellulose filter. The blot was then probed with polyclonal IgG antibodies against TbArf1, which detected a band of about 21.5 kDa in *T. brucei* and *T. congolense* (Figure 3B). The results were in agreement with the molecular weight of deduced amino acid sequences of the different trypanosome *Arf1* proteins. In addition bands of higher molecular weight were detected with the polyclonal antibodies raised against the protein in both *T. brucei* and *T. congolense* lysates (Figure 3B). These bands represent proteins of higher molecular weight with domains recognisable with anti-TbArf1 anti-protein whose 18 kDa carboxyl-terminal domain contains

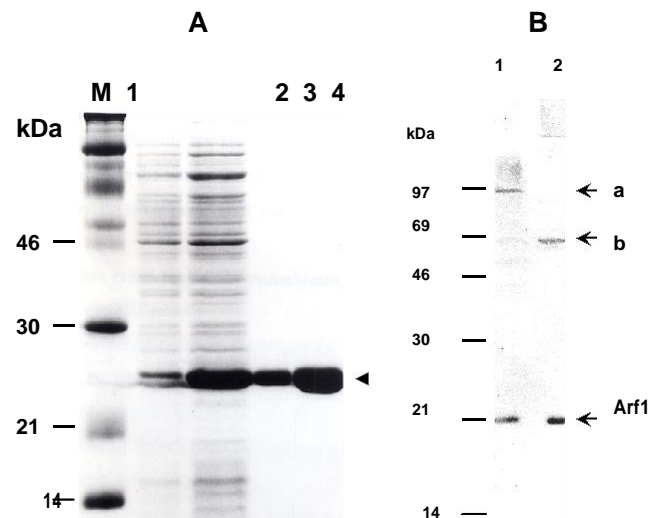


Figure 3. Expression and Western analysis of TbArf1. Panel A; 12.5 % SDS-polyacrylamide gel electrophoresis (PAGE) gel stained with Coomassie BlueR250 showing expression of recombinant TbArf1 in *E. coli*. Sonicated total lysate of IPTG-induced bacteria culture was separated by centrifugation into insoluble (lane 1) and soluble fractions (lane 2). The soluble fraction was chromatographed on Ni-NTA agarose resin (Qiagen) affinity column and bound fractions eluted (lanes 3 and 4). Panel B; Western analysis of native ARF1 from total lysates of *T. congolense* (lane 1) and *T. b. brucei* (lane 2). Bands were detected using mouse anti-TbARF (panel B, lane 2). Arrowhead depicts the native trypanosome ARF whereas the arrowheads reveal proteins of higher molecular weight detected.

Arf1 sequences (Vitale et al., 1996). To examine the spatial distribution of TbArf1, fixed parasites were stained with anti-TbArf1 polyclonal (Figure 4A) or monoclonal (Figure 4B) antibodies which was detected by a bodies or ARF-like proteins. In mammalian cells such proteins include a 64 kDa ARD1fluorescein-labelled goat anti-mouse secondary antibody. Parasites were stained with the kinetoplast in the cells, suggesting that the majority of these compartments were located closer to the flagellar pocket (Figure 4A and B). Electron micrographs of immunogold labelled antibodies revealed similar vesicular and Golgi localisation (Figure 4C).

TbArf1 is a GTP-binding protein

The deduced amino acid sequence of *TbArf1* revealed the presence of domains required for GTP/GDP binding and hydrolysis (Figure 1). We therefore tested whether TbArf1 could also bind GTP/GDP *in situ*. To achieve this, the recombinant protein was resolved by SDS-PAGE, transferred onto nitrocellulose filters and incubated in the presence of [³²P]-GTP described in Materials and methods. This procedure as has been shown to allow the renaturation of small GTP-binding proteins (Coulter and Hide, 1995). An autoradiograph of the filter demonstrated that TbArf1 bound to radiolabeled GTP (Figure 4A). Re-

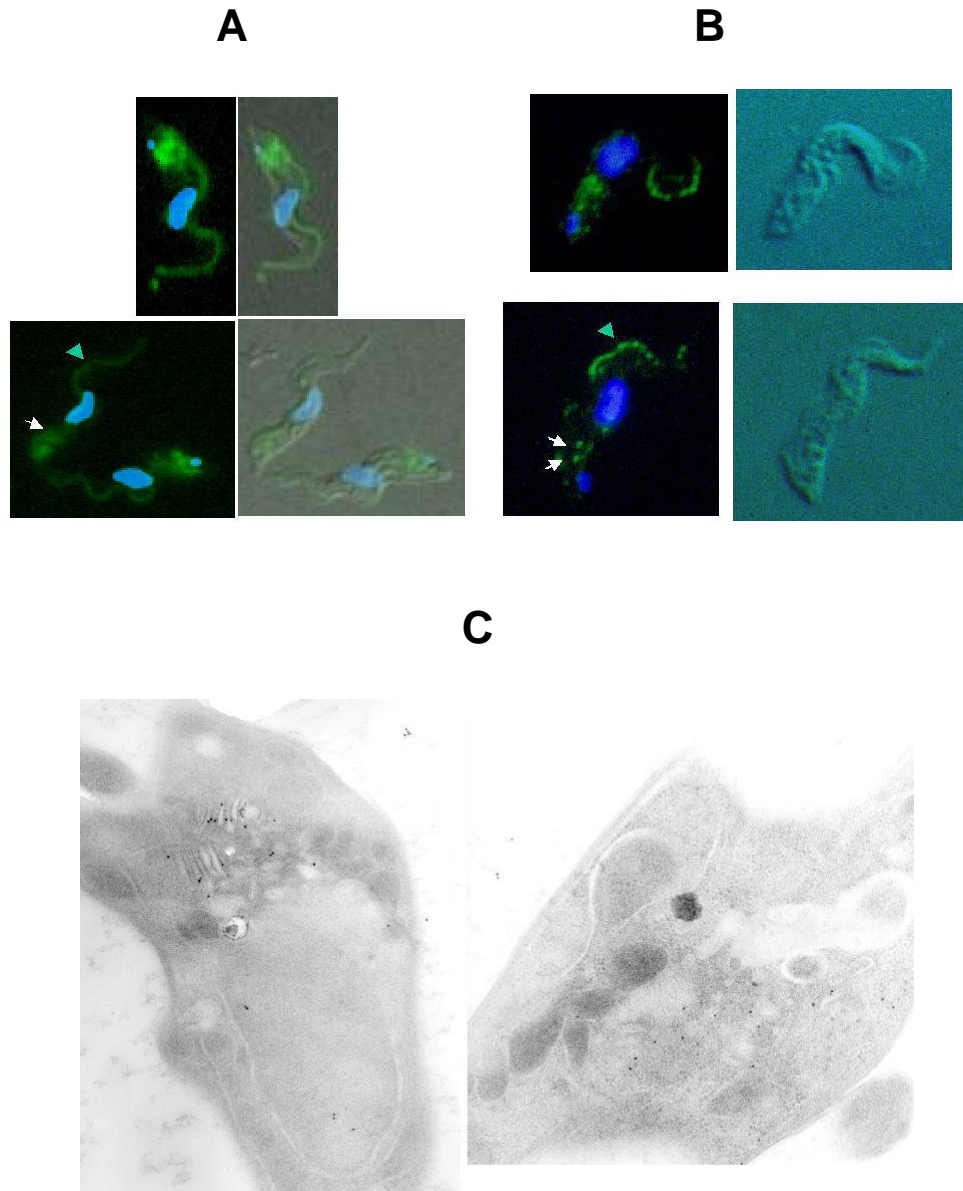


Figure 4. TbArf1 is associated with endosomal compartments and the flagellum. Panels A and B show immunofluorescence localization of TbArf1 in pleomorphic bloodstream forms of *T. b. brucei* ILTat 1.1 with (A) polyclonal antisera against TbArf1 and (B) a monoclonal antibody (MAB), ILA-OL3. Localization of the anti-TbArf1 antibodies was revealed with FITC-labeled goat anti mouse IgG (green) and parasites were stained with DAPI for DNA (blue). Nomarski differential interference contrast (right) and fluorescence (left) images of the cells are shown. Arrows indicate some of the endosomal vesicular staining while the arrowheads show the flagellar staining. The majority of TbArf1 staining with the polyclonal antiserum is in vesicular structures between the kinetoplast and the nucleus, but there is also significant staining along the flagellum. In contrast, the ILA-OL3 MAB staining is punctuated along the anterior flagellum and in endosomal compartments. Panel C shows immunogold labeling of TbArf1 on electron micrographs *T. b. brucei* frozen sections with clear labeling of vesicular and Golgi-like structures.

Recombinant *Theileria parva* Rab1 and p32 sporozoite antigen, expressed, purified using pQE-30 expression system (Janoo et al., 1999) were used as positive and negative controls (Figure 5).

The specificity of GTP-binding to recombinant TbARF

in solution was assessed in the presence of 100- fold molar excess of competing nucleotides (Figure 5B to E). The results revealed that the TbArf1 binds guanine nucleotides (GDP and GTP) as well as UTP but not ATP or CTP

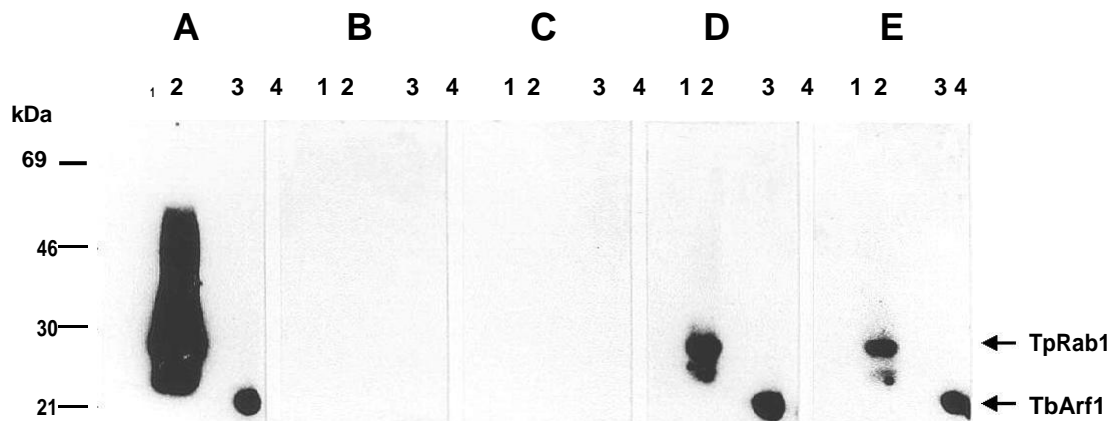


Figure 5. TbArf1 is a guanine nucleotide-binding protein. Expressed recombinant TbArf1 protein purified under native conditions was electrophoresed in a 15 % SDS-PAGE gel, transferred onto nitrocellulose filters and bound to [α - 32 P]- GTP. The lanes containing *E. coli* expressed recombinant proteins are: *T. parva* Rab 4 (lane 1), *T. parva* Rab 1, as a positive control (lane 2), *T. parva* p32, surface antigen as a negative control (lane 3) and TbArf1 (lane 4). Panel A shows binding to [α - 32 P]- GTP alone and panels B, C, D and E show results of binding following addition of excess non-radioactive GTP, GDP, CTP and TTP, respectively.

Antibodies raised against recombinant Arf1 react with trypanosome-secreted factors

Since Arf1 proteins are involved in membrane trafficking, endo/exocytosis and the secretory pathway, we explored whether TbArf1 could be detected in culture medium of *in vitro* propagated BSF and insect forms of African trypanosomes under conditions that ensured healthy parasites with minimal or no lysis. Western blot analysis with anti-TbArf1 antibodies revealed a protein band of about 22 kDa in all the culture supernatants tested (Figure 6). These results suggested that TbArf1 is secreted or released in the medium by African trypanosomes *in vitro*. The antibody reacted also to protein bands of higher molecular weight but weakly and only in the blood stream forms. As a positive control, release of the *T. b. brucei* cyclophilin A protein that has been shown previously to be released into culture media (Pellé et al., 2002) was examined (Figure 6).

DISCUSSION

Adenosine diphosphate-ribosylation factor (Arf) proteins are members of the Arf arm of the Ras superfamily of guanosine triphosphate (GTP)-binding proteins. Arfs are named for their activity as cofactors for cholera toxin-catalyzed adenosine diphosphate-ribosylation of the

heterotrimeric G protein $G_{S\alpha}$. Physiologically, Arfs regulate protein sorting, membrane trafficking and actin cytoskeleton. They function both constitutively within the endo-cytic and secretory pathways, and as targets of signal transduction in the cell periphery. Arfs affect membrane traffic in part by recruiting coat proteins, including COPI and clathrin adaptor complexes, to membranes. However, Arf function involves many additional biochemical

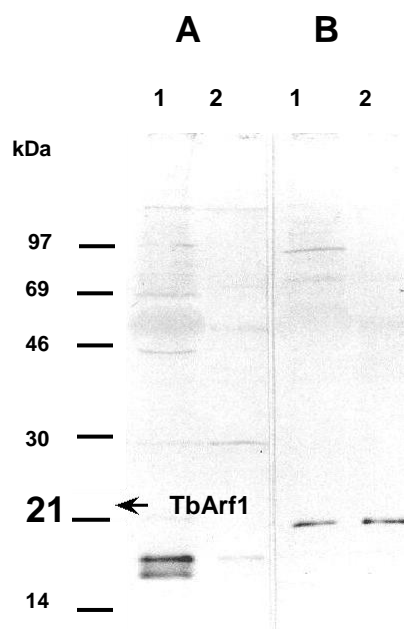


Figure 6. Release of the African trypanosome Arf1 protein into culture medium. Western blots from 15% SDS-PAGE of culture media supernatants of *in vitro* cultivated *T. congolense* (lane 1) and *T. b. brucei* (lane 2) parasites. In panel A, the blots were stained with anti-TSIF (Trypanosome Suppression-Inducing Fraction) which shows released *T. b. brucei* cyclophilin A (Pellé et al., 2002) and in panel B, the reaction with anti-TbArf1. Molecular weights are in kDa.

activities. Arf activates phosphatidylinositol 4-phosphate 5-kinase and phospholipase D with subsequent production of phosphatidic acid (PA) and phosphatidylinositol 4, 5-bisphosphate (PIP₂), respectively providing a means of responding to regulatory signals and a mechanisms to

coordinate GTP binding and hydrolysis. Arf also works with Rho family proteins to affect the actin cytoskeleton. Arf-dependent biochemical activities, actin cytoskeleton dynamics and membrane trafficking are integrally related. Thus understanding Arf's role in complex cellular functions will involve a description of the temporal and spatial coordination of these multiple Arf-dependent events. This is more intriguing and challenging in trypanosome where the endocytic and secretory events are targeted and restricted to the flagellar pocket.

The trypanosome Arf1 homologue was initially identified by RADES-PCR (Osanya, 2000) and shown to be developmentally regulated during the life cycle of the parasite. Southern analyses show a discrete gene copy profile of Arf1 but also reveal the presence of ARF related sequences in the *T. b. brucei* genome. Pays et al. (1997) described an ARL sequence in *T. b. brucei* though not unique to trypanosomes. *T. cruzi* genome has been shown to have at least 2 copies of ARF (de Sá-Freire et al., 2003). Similar sequences have been described in human, yeast, *Plasmodium*, *Leishmania* amongst other organisms. In humans, the ARL protein (ARL 184) is a component of a regulated secretory pathway (Icard-Liepkalns et al., 1997).

The expression of Arf1 gene of both *T. b. brucei* and *T. congolense* is stage regulated. The level of *T. congolense* Arf1 mRNA was found to be developmentally regulated reaching a maximum in the bloodstream forms of the parasite. Similarly, in *T. b. brucei* the mRNA expression reaches the maximum in the actively-dividing LS bloodstream form of the parasite. The high level of expression of the Arf1 gene in these parasites could be indicative of increased vesicular and membrane trafficking activities in certain stages of their life cycles. The mRNA levels of mammalian ARF 2 to 4 and ARLs have been shown to be developmentally regulated (Schurmann et al., 1994). Similarly, developmentally regulated expression of ARF genes has been demonstrated in *Plasmodium falciparum* (Stafford et al., 1996; Troung et al., 1997) and *T. cruzi* (de Sá-Freire et al., 2003). It has been shown that an elevated expression of Arfs occur in the developmental stage of cycle of these parasites where processes such as the uptake of nutrients, extracellular factors and recycling of cell surface proteins are enhanced rate (Coppens et al., 1987; Field et al., 1998; de Sá-Freire et al., 2003). All of these processes require membrane trafficking events involved endocytic and secretory processes in which Arf1 proteins play a vital role (Radhakrishna and Donaldson, 1997; Field et al., 1998). Thus, the elevation of the level of expression of the Arf1 gene might be a prerequisite to the increased cellular activities required for parasite growth and differentiation at that specific phase(s) of the life cycle.

Hence, differential expression of Arf is not unique to trypanosomes, but the stage at which this occurs suggest some intriguing possibilities. In this stage of African trypanosomes Arf1 could possibly be involved in; (1) the

recycling of membrane glycoproteins, (2) uptake of nutrients and extracellular factors and (3) secretion of proteins and other factors involved in host-parasite interaction. In *P. falciparum* Arf1 and ARL mRNA are abundantly expressed in the shizont stage, which is a developmental step characterised by nuclear division followed by cytokinesis. Given the function of ARFs in vesicular-mediated events (Troung et al., 1997) an increase in Arf1 expression is thought to correlate with post-mitotic nuclear vesicle fusion. Thus increased expression of Arf1 in actively dividing bloodstream forms of trypanosome might correlate with increased nuclear and post-binary division vesicle fusion during the multiplication in this stage of the parasite development. However, procyclic and epimastigotes forms of the parasites are actively dividing but do not show increased expression of Arf1. Thus, the increase in protein secretion, uptake of molecules and/or recycling of molecules in actively-dividing bloodstream forms of trypanosomes may be sufficient to explain the up-regulation of Arf1 expression at this stage in the life cycle.

Bacterially expressed *T. b. brucei* Arf1 protein which was purified under non-denaturing conditions bound specifically to GTP and GDP in solution and its GTP-binding was inhibited by UTP. The interaction of ARF with UTP has not been described before. These results confirm that the trypanosome Arf1 is a small GTP-binding protein and its function, like other GTP-binding proteins, are affected and modulated by its interaction with guanine nucleotides (Bourne, 1988; Bourne et al., 1991; Valencia et al., 1991; Exton, 1998). Each GTP-binding protein is a precisely engineered molecular switch that changes its affinities for other macromolecules and it is turned on by binding to GTP and off by hydrolysis of GTP to GDP. The on-off switch mechanism is remarkably versatile, enabling different G-proteins to sort and amplify trans-membrane signals, direct the synthesis and translocation of proteins, guide vesicular traffic, and control proliferation and differentiation of eukaryotic cells (Bourne et al., 1991; Exton, 1998). Thus, conformational changes in Arf1 resulting from *in vivo* GTP binding will influence the functional roles of this protein in trypanosomes. The UTP inhibition of GTP binding to the trypanosome Arf1 protein suggests a possible interaction between UTP and Arf1 but the importance of such an interaction *in vivo* is unclear.

The presence of Arf1 in these parasites was revealed by immunodetection in total protein lysate of trypanosomes with antibodies raised against the recombinant *T. b. brucei* Arf1 protein. Interestingly, the antisera detected other proteins of higher molecular weight (> 60 kDa) in total lysates of both *T. b. brucei* and *T. congolense* parasites. These interactions could be promiscuous or other proteins containing epitopes or domains similar or identical to Arf1. In mammalian cells, a member of the ARF family, ARD1 that differs from other ARFs has been identified (Vitale et al., 1996, 1998). This protein contains an

Arf1 domain and a 46-kDa amino-terminal extension that acts as a GTPase-activating protein for the ARF domain. Therefore, the detection of higher molecular weight proteins in total lysates of these parasites could be indicative of the presence of either trypanosome homologues of ARD1 or ARD1-like proteins. Emerging possibility of the involvement of Arf1 in secretory pathway(s) of trypanosomes include the immunodetection of 21.5 kDa protein in the supernatants of conditioned media in which trypanosomes were grown.

The identification of phospholipase D (PLD) as an effector of ARF raises the possibility that a novel signal transduction pathway may regulate membrane traffic.

The presence of a consensus $G_{\beta\gamma}$ -binding domain of receptor kinases in the trypanosome Arf1 sequences indicates the possibility of a direct interaction between Arf1 and trimeric G proteins. This suggests that Arf1 may be a nexus between heterotrimeric G proteins and downstream effectors such as PLD (Colombo et al., 1995; Exton, 1997).

To elucidate the detailed signal-transduction, protein and membrane trafficking pathways in trypanosomes, identification and characterisation of ARF -interactive proteins or molecules, up-and downstream in these pathways is imperative. To this end localisation studies and identification of interacting molecules and/or proteins are very appropriate. The protein was localized in the Golgi and endosomal compartments in the parasite with unexceptional revelation of presence of Arf on the flagellar compartment. This flagellar localization of Arf underscore the dedicated secretory and endocytic activities of the flagellar membrane (Babler et al., 1990; Landfear and Ignatushchenko, 2001).

In conclusion we have demonstrated the presence of an ADP-ribosylation factor 1 (Arf1) gene in African trypanosomes, *T. b. brucei* and *T. congolense*. Arf1 has discrete polymorphic gene profile in the genome of different species of African trypanosomes and its mRNA expression is developmentally regulated during the life cycle of these parasites. The functional domains of these Arf1 proteins are conserved across the subgenera of African trypanosomes. This suggests the presence of similar protein sorting and membrane trafficking mechanisms in all the species of these protozoan parasites. The trypanosome Arf1 conceivably interacts with other protein components to facilitate membrane recycling and remodelling of the cell surface and other cellular organelles during development of trypanosome. Thus, Arf1 and ARL proteins represent good markers for secretory and membrane trafficking pathways in these parasites. They could also be useful as components of conjugate drugs to facilitate delivery of anti-parasite molecules into the parasites.

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