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Short communication

TbRAB23; a nuclear-associated Rab protein from *Trypanosoma brucei*

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The small GTPases of the Rab family play a central role in the regulation of vesicular trafficking. By interaction with an extensive range of protein partners, Rabs mediate vesicular targeting, membrane fusion and cytoskeletal interactions [1–3]. Rabs comprise the largest subfamily of Ras-like GTPases, and in mammals at least 60 members have been identified (most of which have not been studied at the functional level [4]), 11 in the yeast *Saccharomyces cerevisiae* [5] and 16 in *Trypanosoma brucei*. Rabs function primarily as molecular switches by controlled hydrolysis of GTP; conformational changes between the GTP- and GDP-bound state facilitate a cycle whereby Rabs bind to discrete internal compartments, providing a role in generating specificity in intracellular vesicular trafficking. The precise basis for this specificity remains unknown at present. Rab association with membrane depends on the presence of a prenyl moiety. Prenylation is directed by recognition of a C-terminal CAAX motif; the sequence determines if the protein will receive a C_{20} geranylgeranyl or C_{15} farnesyl isoprene group, but a specialised prenyltransferase, geranylgeranyltransferase II (alternatively called RabGGTase), appears responsible for processing of most Rab proteins [6], although this activity is not represented in the *T. brucei* genome. Whilst some members of the Rab family (e.g. Rab8) possess carboxyl terminal CAAX motifs with just a single Cys carboxyl residue [7], most Rabs have a CAAX motif that contains two Cys residues and are doubly geranylgeranylated. Interestingly, Ran, which is responsible for control of nucleocytoplasmic transport and nuclear envelope assembly [8,9], is not prenylated and has a highly acidic C-terminus [10] whilst proteins of the Ras subfamily are farnesylated.

A global analysis of the Rab family *T. brucei* (Ackers, J., V.D., and M.C.F, unpublished data) identified a number of TbRABs that, by phylogenetic analysis, are implicated to be involved in nuclear functions due to sharing a clade with TbRAN. TbRAN itself is highly similar to the well characterised Ran proteins from *Homo sapiens* and *S. cerevisiae* [11]. This putative nuclear group comprises TbRAB23, TbRAB28 and TbRABX3 and suggests a potential novel function in the nucleus for Rab proteins, which has so far not been reported in any system. We chose to investigate the possible function of these proteins and to confirm the bioinformatics analysis by experimental investigation of TbRAB23.

An alignment of the predicted protein sequences of TbRAB23, TbRAB28 and TbRABX3 with TbRAN shows significant similarities at the amino acid level (Fig. 1A). All four proteins have the common GTP binding domains characteristic of small GTPases, however, none of these putative Rab proteins possess a full compliment of conserved consensus Rab family motifs. Of particular note, TbRAB23 and TbRAB28 have high regions of similarity at the carboxyl terminus. Both contain a sequence of lysine residues and a C-terminal consensus sequence predicted to specify the addition of a farnesyl group rather than the common geranylgeranyl group. Importantly, this sequence is predicted to be efficiently recognised by the trypanosome farnesyltransferase [12]. The lysine-rich domain at the carboxyl terminus of TbRAB23 and TbRAB28 is a feature that has not been previously described in other Rab proteins. The small signalling GTPase, K-Ras, has a similar enriched region of lysine residues, which is required for the correct targeting of the protein to the plasma membrane in mammalian cells [13] (Fig. 1B). The similarity between both TbRAB23 and TbRAB28 proteins extends to within the effector region and suggests that these two TbRABs are the result of an ancient

Abbreviations: FITC, fluorescein isothiocyanate; GST, glutathione *S*-transferase; IFA, immunofluorescence analysis; ORF, open reading frame

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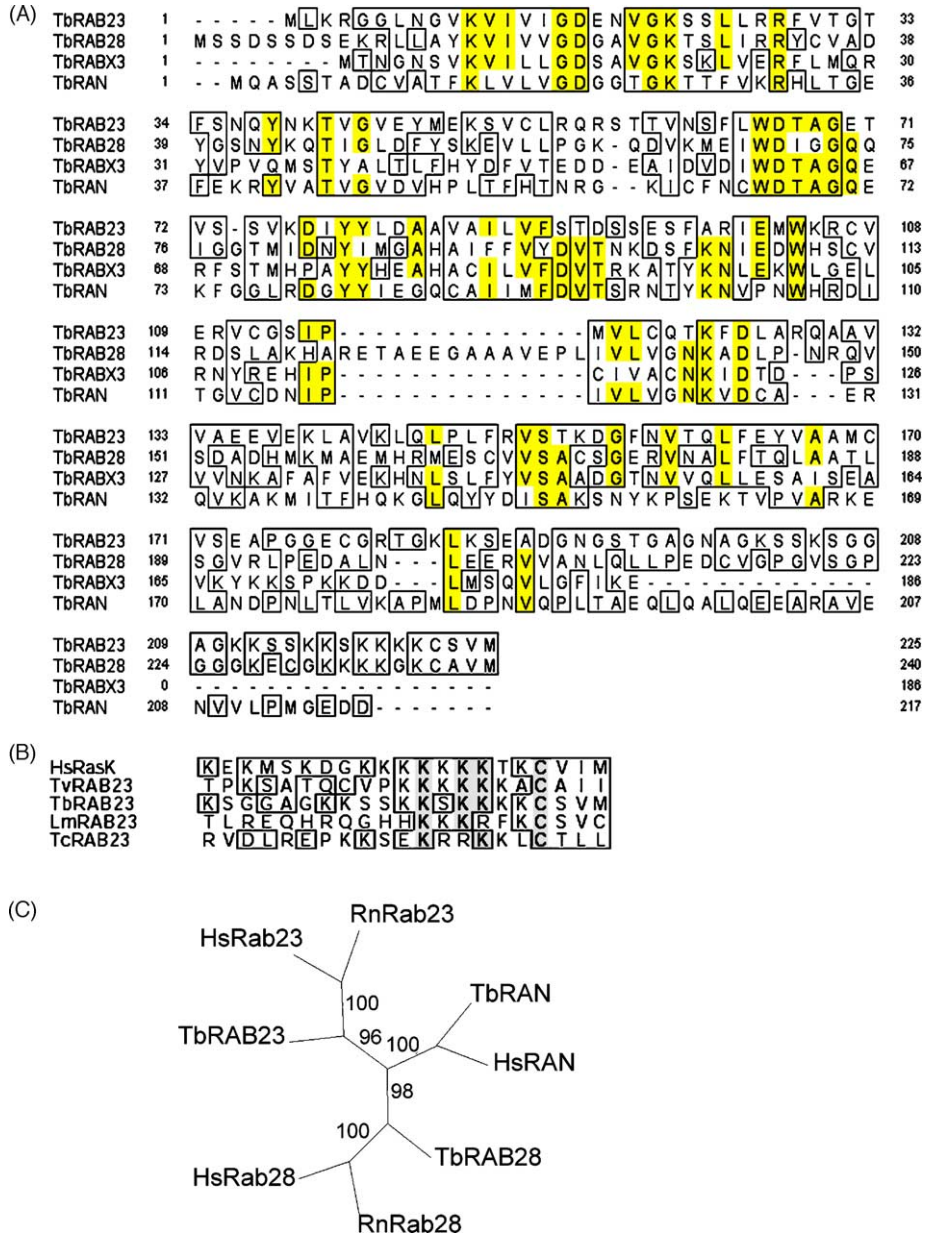

Fig. 1. TbRAB23 and TbRAB28 are atypical Rab proteins. (Panel A) ClustalX alignments of the predicted protein sequences of TbRAB23, TbRAB28 and TbRAN, demonstrating a number of unusual features in this group of proteins.TbRAB23 and TbRAB28 both have consensus sequence for modification by farnesyltransferase at their carboxyl terminus in addition to a lysine-rich region. Similarities are shown boxed, identities are shaded. The alignment was made using default parameters. (Panel B) Alignments of the carboxyl terminus of K-Ras with Kinetoplastida Rab23 homologues. Interrogation of the *Trypanosoma cruzi*, *Trypanosoma vivax* and *Leishmania major* sequence databases suggest that TbRAB23 is represented in at least four species. Each RAB23 homologue shares both the lysine-rich domain and the farnesylation motif at the carboxyl terminus. Note that TcRAB23 does not have a common farnesylation motif at the carboxyl terminus, but recent evidence suggests that the *Trypanosoma cruzi* farnesyltransferase is able to modify ends terminating in CXXL, dependent on the precise identity of the two residues between the C and L. (Panel C) Phylogenetic reconstruction of the TbRAB23 family using sequences from *Trypanosoma brucei* and crown eukaryotes. Analysis was done using PAUP* 4.0 [16] heuristic search option and 1000 bootstraps. Both TbRAB23 and TbRAB28 are distant relatives of their human and rat homologues and the tree topology suggests that both proteins arose from a common ancestor prior to speciation, and hence are ancient members of the Rab family.

duplication, and may have a number of protein partners in common. Interestingly, the closest TbRAB23 homologue in mammalian cells, Rab23, is a negative regulator of the mouse Sonic hedgehog signalling pathway, important in neural development [14]. Hence, Rab23 may be involved in signalling a role not normally associated with Rab proteins.

Interrogation of the *T. cruzi*, *T. vivax* and *Leishmania major* sequence databases suggest that TbRAB23 is conserved between at least four kinetoplastids. Each homologue retains a lysine-rich domain at the carboxyl terminus (Fig. 1B). Interestingly, TcRAB23 does not have a common farnesylation sequence and ends with the motif CTLL, suggesting

that it is most likely to be modified by the addition of a geranylgeranyl group. However, recent evidence suggests that the *T. cruzi* farnesyltransferase is able to modify ends terminating in CXXL, dependent on the two residues between the cysteine and the leucine residues [15]. Phylogenetic reconstruction, using the heuristic search option and 1000 bootstraps in PAUP* 4.0 [16], provides evidence to suggest that both TbRAB23 and TbRAB28 are monophyletic with their human and rat homologues (Fig. 1C) and implies that TbRAB23 and TbRAB28 have arisen prior to speciation separating the mammalian and trypanosome lineages. Therefore, RAB23 and RAB28 are likely part of the core Rab family functionality. TbRABX3 was not included in this analysis as this member of the trypanosomal Rab family has no clear orthologue in higher eukaryotes (Ackers, J., V.D., and M.C.F, unpublished data).

The TbRAB23 open reading frame (ORF) was amplified from trypanosome genomic DNA and inserted into the bacterial expression vector pGEXT2TK for expression in *Escherichia coli* using the primer combination FwdRab23 and RevRab23 (5′-CCG**GGATCC**ATGGAGAAGAGCG-3′ and 5′-CG**GAATTC**CTACATAACACTGC-3′, respectively). Recombinant protein was expressed and purified using glutathione-sepharose [17]. Polyclonal antisera were raised in rabbits and specific antibodies purified by affinity chromatography using immobilised glutathione *S*-transferase (GST)-TbRAB23 following standard procedures [18]. The antisera recognised a band at 26 kDa and this could be competed with recombinant GST-TbRAB23 but not an irrelevant recombinant TbRAB (data not shown).

Analysis of trypanosome lysates prepared from both bloodstream and procyclic cultures by Western blot shows that TbRAB23 is expressed in both stages with an equivalent level of expression in procyclic and bloodstream forms (Fig. 2A). For further analysis both bloodstream and procyclic stage parasites were fixed in 3% paraformaldehyde and stained using affinity purified anti-TbRAB23 antibodies and Texas Red-conjugated goat anti-rabbit secondary antibody (Molecular Probes) [19] and counterstained using DAPI to visualise the nuclear and kinetoplast DNA. Immunofluorescence analysis (IFA) demonstrated that the antibody recognised an antigen in association with the nuclear envelope in both life stages (Fig. 2B) and suggests that TbRAB23 may be involved in a nuclear-located function. A small level of stain was also detected in the kinetoplast in the procyclic stages.

To discern in more detail the location of TbRAB23, trypanosomes were costained with anti-TbRAB23 as before together with monoclonal antibody against NUP1 (gift of Keith Gull), a coiled-coil protein that localises to the inner face of the nuclear envelope [20]. Significant colocalisation was observed for TbRAB23 and NUP1 (Fig. 2C).

In order to gain further insight into the function of TbRAB23, expression of the protein was suppressed using RNA interference (RNAi). The TbRAB23 ORF is not sufficiently homologues to other trypanosome sequences for cross suppression to be expected [21], and was cloned into the RNAi vector p2T7 using *Bam*HI and *Xho*I sites. The vector was transfected into both procyclic and bloodstream stage parasites and stable transformants selected [17]. TbRAB23·RNAi cells were induced for TbRAB23 loss using tetracycline (1 μg/μl) and the effect on cell viability monitored by following growth of cultures for up to 14 days (Fig. 2E). Growth curves were produced by removing aliquots of cultures and diluting with Isoton2 medium (Beckman Coulter), and cells counted using a Z2 Coulter Counter, averaging at least three measurements. Reduction in expression of TbRAB23 was verified by Western blot of bloodstream cultures induced for 48 h (Fig. 2A). Suppression of ∼80% of the TbRAB23 protein was obtained. These data also serve to confirm the specificity of the anti-TbRAB23 antibodies. TbRAB23 does not appear to play an essential role in cell growth since no defects in growth rates were observed in either life stage in the first 7 days of the assay, although it is formally possible that residual expression of TbRAB23 is sufficient for normal growth, in vitro at least. However, in procyclic cell lines a delayed growth phenotype after 7 days was observed, suggesting that there may be a disruption in upstream events resulting in growth inhibition or that the incomplete ablation of TbRAB23 required several generations before a phenotype emerged.

Despite the apparent lack of a strong effect on growth it was observed that the nucleolus, visualised by negative staining with DAPI, became enlarged following 3 days of induction (Fig. 2D). TbRAB23 may therefore function in a manner to provide stability to the nuclear structure, and is consistent with colocalisation of the protein with NUP1, which itself is likely involved in chromatin and higher order nuclear structure [20]. The possibility that NUP1 and TbRAB23 interact in the same pathway is interesting, but will require further investigation. However, taken together, these data clearly implicate that TbRAB23 has a functional role within the trypanosome nucleus.

Sequence and phlyogenetic analysis suggest that TbRAB28 and TbRABX3 may be involved in similar activities to TbRAB23, and that there may exist in trypanosome at least a complex set of nuclear functions that are modulated by small GTPases. Since no expression data are available at present for TbRAB28 and Northern analysis of TbRABX3 suggests a very low abundance of the transcript in procyclic stage parasites and no detectable transcript in bloodstream stages (Jeffries, T., and M.C.F., unpublished data), the possibility that these multiple proteins are expressed at different life stages of the parasites life cycle cannot be excluded. If this is indeed the case, then these GTPases may play some role in the remodelling of the nucleus that appears to accompany differentiation [20]. In conclusion, we have presented the first experimental evidence suggesting that in the divergent parasite *T. brucei* at least one member of the Rab family of GTPases is involved in nuclear functions, supporting the phlyogenetic analysis identifying a subset of the *T. brucei* Rab family with a potential role in the nucleus.

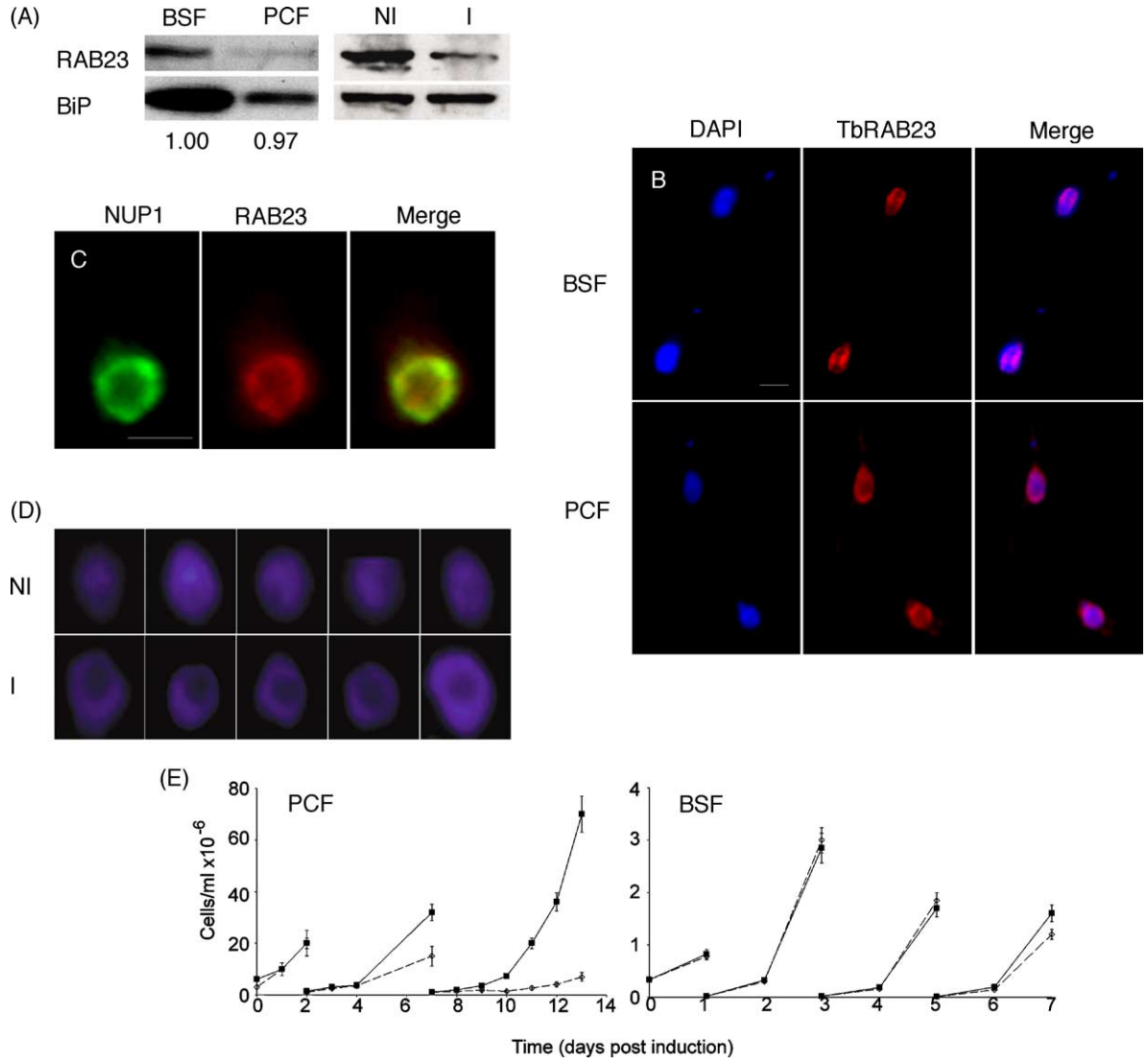

Fig. 2. TbRAB23 is a constitutively expressed nuclear protein with a role in nuclear structure. (Panel A) Left: Western blot analysis of TbRAB23 using affinity purified antibodies. The TbRAB23 band migrates at 26 kDa on reducing SDS–PAGE. TbBiP is used as a loading control, and the numbers beneath the blots indicate the expression of TbRAB23 normalised to TbBiP, with the bloodstream form set arbitrarily as 1.00. (Panel A) Right: Western analysis of expression of TbRAB23 following RNAi in bloodstream form cells after 48-h induction. NI, noninduced; I, induced. TbBiP was used as a loading control. (Panel B) Immunofluorescence microscopy of TbRAB23. Cells were fixed, permeabilised and then stained with primary and secondary antibodies. Left panels show DNA, visualised with DAPI (blue), middle panels TbRAB23 (red) and right panel merge. Note the clear staining of TbRAB23 overlapping with the nucleus in both life stages. BSF, bloodstream form; PCF, procyclic culture form. (Panel C) Immunofluorescence of TbRAB23 and NUP1 in bloodstream stage demonstrates colocalisation. Cells were fixed, permeabilised and then stained with primary and secondary antibodies (fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse and Texas Red-conjugated donkey anti-rabbit; Molecular Probes) as described in the text. NUP1 is shown in green and TbRAB23 in red. A merge is shown at right. (Panel D) Gallery of images of DAPI-stained nuclei from trypanosomes under TbRAB23·RNAi (72 h). Note the presence of an enlarged nucleolus as visualised by weak DAPI staining. NI, noninduced; I, induced. Scale bar = 2 μm. (Panel E) Growth curves of procyclic and bloodstream form cultures following induction of TbRAB23·RNAi with tetracycline. Cultures were monitored daily for cell number by Coulter Counter in triplicate. Noninduced cultures are shown in solid line, and induced in the broken line. Error bars indicate the standard error and in some cases are obscured by the plot symbol. BSF, bloodstream form; PCF, procyclic culture form.

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