GTPases in Protozoan Parasites:
Tools for Cell Biology and Chemotherapy

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Small G proteins belong to a superfamily of GTPases related to the protooncogene ras*, and function as master control elements for a range of cellular functions. This ability is related to their low rate of substrate turnover; GTPases catalyse the conversion of GTP to GDP, but with a rate in the order of one substrate per second, orders of magnitude slower than 'good' enzyme catalysis, but placing the reaction into the temporal frame of many cellular processes including signal transduction, cytoskeletal reorganization and vesicle trafficking. In this article, Mark Field, Bassam Ali and Helen Field describe some recent advances in G-protein studies in the parasite field, concentrating on the protozoan parasites. Because of their numerous roles in cell biology, understanding parasite G proteins has great potential for increasing our knowledge of parasite cellular physiology, as well as providing important inroads into vital processes for potential therapeutic exploitation.*

Several subfamilies of small GTPases are recognized in eukaryotes. Briefly, these fall into the Ras and Rho subfamilies, which are involved in signal transduction pathways from the cell surface to the nucleus[1]. Ras is involved in cell division and differentiation and Rho in cytoskeletal organization and, probably, cell motility[2]. The Rac class of the Rho subfamily is also involved in the oxidative stress response and signal transduction. The Rab, ARF (ADP-ribosylation factor) and Sar subfamilies regulate vesicular trafficking of proteins and lipids[3,4]. ARF and Sar proteins are required for vesicle formation at a donor compartment by recruitment of cytosol-located coat proteins, and individual Rabs are essential for vesicle docking and fusion at an acceptor membrane, probably by priming the docking system. Ran is an essential element in the transport of proteins and RNA across the nuclear membrane. The GTPase motif is also found in numerous larger proteins, including the signal recognition particle receptor and dynamin[5], and homologues have been identified in parasites (M.C. Field and G. Morgan, unpublished), but these will not be dealt with further here. Of particular interest to several laboratories working on parasites have been the Rab proteins, because these GTPases are associated with specific vesicle transport steps along the secretory and endocytic pathways and thus provide much-needed subcellular markers for morphological and biochemical studies. However, there is also considerable interest in the Rho/Ras proteins, as these might provide important reagents for the understanding of cell signalling in these organisms. Because *Plasmodium* Rabs have been reviewed recently[6], this article will not deal directly with the malaria parasite.

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The superfamily of small G proteins all conform to a general structure at the amino acid sequence level (Fig. 1a), with a molecular mass of 20–30 kDa[1]. The high degree of sequence conservation is predicted to result in similar secondary and tertiary structures; indeed, comparison of Ras and Ran X-ray crystallography structures reveals that most differences are confined to loops between helix or sheet elements[7,8]; the loops are probably important in interactions with a plethora of downstream effector and control proteins (Fig. 1b). The enzymatic function of small G proteins is hydrolysis of GTP to GDP (Fig. 1c), achieved at a very slow rate, with typical hydrolysis times being in the order of a second or more. The protein contains four highly conserved GTP-binding regions required for GTPase function (often almost invariant within subfamilies), an effector loop responsible for interaction with GTPase activator proteins (GAPs), other loops important for binding with further modulatory factors and a hypervariable C-terminus, important in conferring specificity of location and function[9,10]. The C-terminus of most small G proteins is modified by prenylation and, in the case of Ras, palmitoylation[11]. Concomitant proteolytic trimming of a few residues and methylation of the C-terminus are frequent. GAPs accelerate this rate by two or more orders of magnitude by providing an 'arginine finger', which protrudes directly into the GTPase active site[8]. In the GTP-bound state, a GTPase is effectively 'on', the most potent example of this being the transforming activity of GTP hydrolysis-defective Ras mutants[12]. Evidence suggests that the slow rate of GTP turnover serves as a clock in vesicle fusion in the mammalian endocytotic system[13] and might be general for G proteins involved in vesicle trafficking[14]. Reactivation following hydrolysis is achieved by simple nucleotide exchange, where again the intrinsic rate is not particularly high but is accelerated by guanine nucleotide exchange factor (GEF). A third factor, guanine nucleotide dissociation inhibitor (GDI) is implicated in targeting and localization, particularly of Rab proteins. The three factors mentioned here are not the only proteins that interact with small G proteins in a nucleotide status-dependent fashion, or influence the status, but this simplistic view serves well enough to highlight the multiple points of interaction that exist in even a simple hydrolytic cycle (Fig. 1).

Methods of identification and genomic complexity

There are several methods that can be considered for identifying and isolating any class of protein *ab initio*, but the evolutionary distances between, for example, *Saccharomyces cerevisiae* and *Giardia lamblia* and the kinetoplastida result in difficulties with approaches based on immunological crossreactivity. Although such methods have been utilized in the identification of trypanosome G proteins[15], a definitive assignment will require sequence data. In the case of nematodes, smaller evolutionary distances make immunological methods more attractive, and such methods have been successful in a study of Ras in *Schistosoma mansoni*[16].

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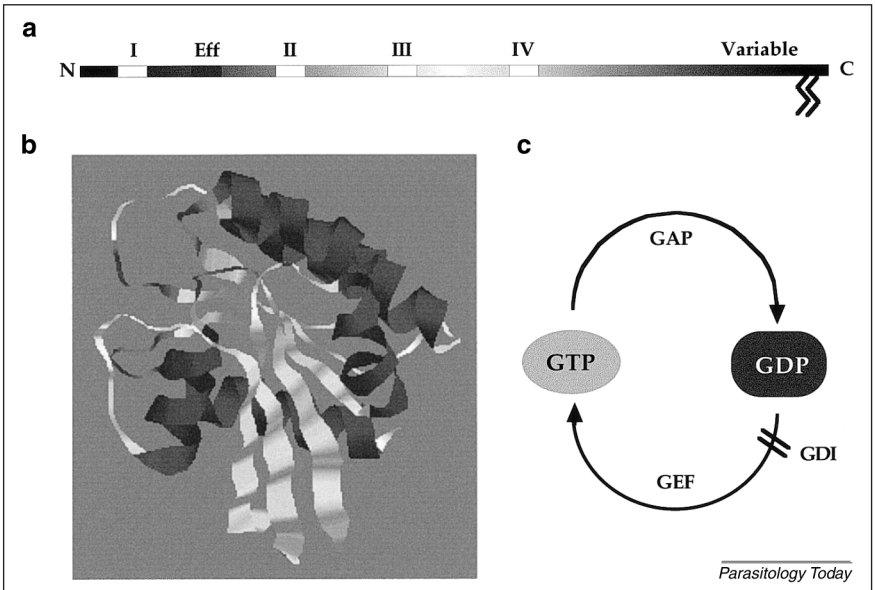

Fig. 1. Highly simplified overview of small G-protein structure and function. Schematic representation of a typical G protein demonstrating the positions of the conserved features (a). The four blocks involved in binding and hydrolysis of GTP are shown as boxes numbered I–IV and the effector loop, primarily responsible for interaction with GTPase activator proteins and other downstream factors, is shown as a shaded box (Eff). The hypervariable C-terminus, important in specifying location is indicated as 'Variable' and the C-terminus is shown modified by two zig-zag structures. Modifications depend on the sequence at the end of the C-terminus, together with some additional specificity encoded by residues more N-terminal, and include prenylation (farnesylation and geranylgeranylation), palmitoylation, proteolytic cleavage and carboxymethylation. In addition, members of the ADP-ribosylation (ARF) family can be myristoylated at the N-terminus. All of these modifications are important for the targeting and function of the protein. General three-dimensional structure of G proteins based on the Ras protein structure (b). Because of the high level of conservation of primary structure and the evidence from crystallographic analysis of additional small G proteins, it is expected that most small GTPases will adopt a conformation similar to Ras. The α-helix is shown in dark, the β-sheet in midtone and the loops in white. Note that the structure is highly compact. A portion of the C-terminus has been omitted as this was not resolved by crystallography. The figure was generated using coordinates deposited in the Brookhaven database[7], using RasMol for visualization and was manipulated using Adobe Photoshop for presentation. Catalytic cycle (c). G proteins are 'on' in the GTP-bound form and 'off' when GDP is bound. Conversion is by hydrolysis (GTP to GDP) or nucleotide exchange (GDP to GTP). The low intrinsic turnover rate is increased by GAPs (GTPase activating proteins) that raise the turnover by >1000-fold, deactivating the G protein. Reactivation is enhanced by GEFs (guanine exchange factors) and blocked by GDIs (guanine dissociation inhibitors). Numerous other factors, including parts of the prenylation machinery and membrane receptors, are also known to influence the nucleotide status of G proteins.

Small G proteins have sequence conservation in short blocks, form a large family, are comparatively well understood biochemically and have short open reading frames (ORFs), all of which make them attractive targets for molecular biological approaches. To emphasize the evolutionary distance problem, low stringency screening with *Trypanosoma brucei* G-protein sequences failed to identify positive clones in *Leishmania major*, and it is quite clear that conservation at the nucleotide level is very poor compared with amino acid sequence (M.C. Field and A. Ivens, unpublished). This suggests that PCR based on high homology regions, in particular block II (Fig. 1a), could be a useful method; this technique has been used successfully in *T. brucei* and *Paramecium tetraurelia* to obtain a collection of sequences, and in *Plasmodium* to obtain a specific G protein[17–19]. Advances in the genome programmes of several parasites might render initial identification somewhat easier in the near future, and, indeed, several GTPases have been identified by random sequencing[20]. However, as most G proteins are found at extremely low levels, it is probable that expressed sequence tag (EST) sequencing must be supplemented with genomic sequence data before significant advances are achieved by this approach.

An estimate, albeit very approximate, of the complexity of G-protein pathways can be obtained by consideration of the 13 Mb *S. cerevisiae* genome, where we have complete information. There are >30 small GTPases in this organism, with 11 members of the Rab subfamily, ie. ~30% of the total. In *T. brucei*, with a genome of ~30 Mb, we currently know of 15 Rabs, two TbARF homologues and TbRan, which indicates a strong bias in the current databases towards Rab G proteins, largely because of direct efforts to obtain Rab ESTs[17], and is unlikely to be biologically relevant (Table 1). There are similar biases for other parasites, and it is probable that numerous GTPases involved in functions distinct from vesicle trafficking await discovery. For *Caenorhabditis elegans*, the sequencing of which has recently been completed (www.sanger.ac.uk/Projects/C_elegans), there are over 100 small GTPases[21]. Most of the yeast genes have several close nematode homologues, whereas many of the worm genes have no apparent equivalent in yeast, clearly indicating the vast increase in complexity in the metazoan.

Characterized small G proteins

• *Trypanasoma brucei*. Table 1 presents the current database of sequenced or sequence-tagged G proteins from several protozoan parasites. Trypanosome G proteins are about 30% identical and 50% similar to their most probable mammalian functional homologues[22,23]. They contain the expected sequence motifs for GTPases and are in the main dispersed in the genome. Two exceptions are the *Tbrab2–31* locus and a cluster of G proteins 3′ to the glucose transporter genes (see below)[22,23]. Of the *T. brucei* Rab proteins characterized so far, four are expected to be involved in endocytosis (TbRab4, -5A, -5B and -7) and three in exocytosis (TbRab1, -2 and -8). TbRab5A and -5B co-localize in the procyclic form and identify numerous small vesicular structures in the flagellar pocket region[20,24] (Fig. 2). In the bloodstream form (BSF), TbRab5B co-localizes with ISG_{100}, a protein recently characterized as being localized to lysosomal and endocytic structures[25]. Interestingly, in the BSF there is substantially less co-localization between TbRab5A and -5B, suggesting developmental modifications in the endocytic pathway[24]. Furthermore, TbRab5B exhibits developmental regulation,

Table 1. Protozoan small G-protein sequences identified to date[a]

Name[b]	Homologue	Location[c]	Refs[d]
Trypanosoma brucei			
TbRan	Ran	Nuclear	41
TbRab1	Rab1	ER	23
TbRab2	Rab2	ER	22
TbRab4	Rab4B	Endosomal	20
TbRab5A	Rab5	Endosomal	20
TbRab5B	Rab5	Endosomal	24
TbRab7	Rab7	Late endosomal	*
TbRab31	Rab31	Golgi	22
Rtb3			17
Rtb5			17
Rtb6			17
TbRab23	Rab23	GenBank	
TbARF	ARF	GenBank	
TbARL3	ARL3 (ARF)	GenBank	
Leishmania			
LmYpt1	Ypt1/Rab1	ER–ERGIC	28
LmRab7	Rab7	Late endosomal	†
Giardia lamblia			
GlRan	Ran	Nuclear	32
GlARF	ARF		33
Entamoeba histolytica			
EhRas1	Ras		35
EhRas2	Ras		35
EhRap1	Rap		35
EhRap2	Rap		35
EhRacA-D	Racs		35
Toxoplasma gondii			
TgRan	Ran	Nuclear	‡
TgRab1	Rab1		e
TgRab5	Rab5		e
TgRab6	Rab6	Golgi	#
TgRab7	Rab7		e
TgRab11	Rab11	AOC–Golgi	#
TgARF	ARF1		e

[a] These data are restricted to G proteins where sequence data are available, and G proteins identified solely by antibody crossreaction or other biochemical criteria are excluded. Eleven members of the Rab subfamily are known in *Trypanasoma brucei* and two in *Leishmania*. Ran is shown for several organisms. Location data are based on experimental determinations or are predictions based on the locations of very close homologues. Numerous expressed sequence tags (ESTs) remain to be characterized in more detail.

[b] We have adopted the following nomenclature: ESTs are given arbitrary names and used until sufficient sequence, and if necessary additional functional data, are available to allow assignment as a homologue (this may not always be possible *sensu stricto*) when the designation of Tb (for *T. brucei*) etc., followed by the mammalian homologue name/number, is applied. In the case of multiples, for example, TbRab5, A is given to the first reported sequence, then B and so on.

[c] Abbreviations: ER, endoplasmic reticulum; ERGIC, ER–Golgi intermediate compartment; AOC, apically oriented compartment.

[d] *, Klöckner *et al.*, 1995; †, Lewis *et al.*, 1998; ‡, Stedman and Joiner, 1996; #, Stedman and Joiner, 1997. See footnotes to the text.

[e] T. Stedman and K. Joiner, unpublished.

as revealed by northern analysis, raising the possibility that it is responsible for the downregulation of endocytotic processes in the procyclic form. TbRab7 has also been localized to endosomal compartments (T. Klöckner *et al.*, Abstract)*. Curiously, during mitosis, TbRab4, -5A and -5B vesicles re-position so that most of them appear anterior to the nucleus; functionally, this might indicate cessation of endocytosis during cell cytokinesis[24].

* Klöckner, T., van den Bogaard, M. and Boshart, M. 'Conserved endosomal localisation of a *T. brucei* small GTP-binding protein homologous to Rab7', Vth Molecular Parasitology Meeting, Woods Hole, MA, USA, 1995

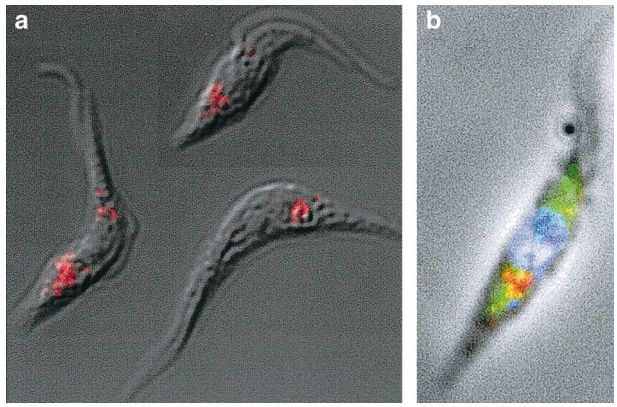

Fig. 2. Light microscopy level localization of TbRabs. Composite image showing Nomarski phase image of *Trypanosoma brucei* procyclic form together with false-colour red fluorescence of TbRab5A, as visualized using antibodies prepared against a synthetic peptide corresponding to the C-terminus of the protein (a). The flagellum is clearly visible for two of the cells and, in particular, in the cell to the left of the field, it can be seen entering the cell body. Note that TbRab5A is localized to small vesicular structures; most of these lie in the flagellar pocket region, with a minority anterior to the nucleus. Based on colocalization with ISG_{100} and sequence homology with mammalian Rab5, TbRab5A is believed to be an important component of the trypanosome endocytic pathway and, in particular, is localized to endosomes. Procyclic *T. brucei* triple stained for TbRab4 (green), TbRab5A (red) and DNA (blue) superimposed on a phase contrast image of the cell body (b). Note that there is a considerably greater proportion of TbRab5A stain between the nucleus and kinetoplast compared with TbRab4, consistent with a role for TbRab4 in control of membrane recycling through early endosomal–*trans*-Golgi network-related structures.

These markers have shown that the trypanosome endocytic pathway is of similar complexity to the higher eukaryote system. Interestingly, the endosomal compartments are present in the procyclic stage, despite downregulation of endocytic activity, suggestive of a sophisticated, but uncharacterized, regulatory system.

TbRab2 co-localizes with immunoglobulin heavy chain-binding protein (BiP), demonstrating that it is located on the endoplasmic reticulum (ER) or an ER-related compartment[26]. Sequence analysis shows that TbRab2 is most closely related to mammalian Rab2, which is mainly localized to the intermediate compartment (ERGIC) and ER transit vesicles; indeed, TbRab2p produced in mammalian cells also localizes to this structure. Overproduction of the protein in procyclic form parasites results in the formation of large vesicular structures, which we believe are an accumulation of ER transit vesicles and hence provide evidence for an intermediate compartment between the ER and Golgi in kinetoplasts[26]. By means of electron microscopy it has been shown that there is a complex vesicular network on the *cis* face of the Golgi complex, which probably corresponds to a trypanosome ERGIC. TbRab31 co-localizes with β-COP, a component of the Golgi coatamer, to a discrete compartment close to the flagellar pocket, indicating that it is probably a Golgi protein (H. Field *et al.*, unpublished; Fig. 2). TbRab31 has some unusual sequence features in the GTP-binding motifs, which result in the loss of GTPase activity while retaining GTP-binding, and in the C-terminus, which has an extremely unusual sequence that might not function as an efficient

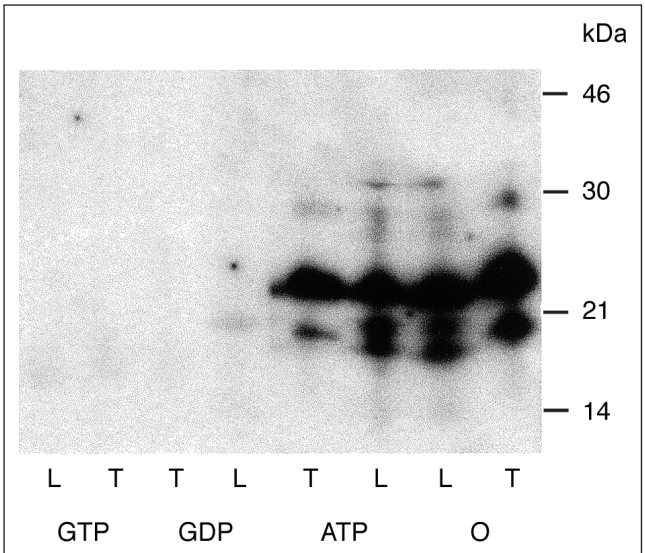

Fig. 3. Similar profiles of GTP-binding activities observed in both *Leishmania major* and *Trypanosoma brucei*. GTP overlays of extracts from protozoa. Detergent lysates from 5×10^7 *L. major* Friedlin promastigotes (L) or 2×10^7 *T. brucei brucei* 427 bloodstream form cells (T) were resolved by reducing SDS-PAGE, renatured and electrotransferred on to nitrocellulose paper. GTP-binding activity was visualized by incubating the blot with α[32P]-GTP followed by autoradiography. Specificity of the interaction was verified by including excess GTP, GDP or ATP in the α[32P]-GTP solution. The overall profiles for both parasites are quite similar, with GTP-binding activity restricted to the 20–30 kDa range; however, it is also clear that there are subtle differences in the profiles, reflecting non-identical GTPase families and expression patterns in the two parasites. The predominant band at 25 kDa is most probably the highly abundant GTPase Ran/TC4, involved in nuclear envelope transport.

prenylation signal (although TbRab31p is membrane bound). TbRab2, in contrast, shows none of these deviations from canonical sequences and is a highly active GTPase[26]. It is not yet known whether TbRab31 is distributed throughout the Golgi stack, or is localized predominantly to one subcompartment. Availability of anti-TbRab31 antibodies has allowed detailed documentation of the behaviour of the trypanosome Golgi during the cell cycle. Electron microscopy data suggested that the Golgi replicated by binary fission, rather than vesiculation at mitosis (K. Gull, pers. commun.). By means of indirect immunofluorescence microscopy and by following cells through mitosis, using basal body and kinetoplast replication as benchmarks, we have found that the trypanosome Golgi replicates very early, probably at the same time as the basal bodies and before the kinetoplast, and remains as a discrete entity throughout the division process. Re-positioning of the Golgi complex after replication is coordinated, with the microtubule array being responsible for the relocation of the basal body/kinetoplast system during replication[27]. In contrast, studies of PfRab6 have indicated that the *Plasmodium* Golgi has an unstacked structure[19] and, therefore, a non-conventional Golgi structure might be a protozoan theme.

Recent studies of the glucose transporter locus have identified three further G-protein ORFs downstream of the transporter genes[23]. The closest to the transporter cluster is a *Tbrab1* gene, closely related to *Tbrab2*, the product of which might have a similar functional redundancy with TbRab2 in ER-to-Golgi transport as do Rab1 and -2 in higher eukaryotes. The subcellular location of TbRab1 has not been investigated and therefore functional assignment is equivocal at present. The gene immediately downstream of *Tbrab1* is more cryptic; this ORF encodes a protein of ~500 amino acids; the N-terminal 50% is related to Ypt7, while the remainder is highly acidic. The sequence is highly degenerate and an important motif no longer contains the conserved IWDTAGQE sequence; the protein might have poor GTPase activity or not even bind GTP, and might have some unique function in *T. brucei*. There are some parallels to the *Tbrab2–31* locus, where one canonical G protein (TbRab2) and a second divergent one (TbRab31) have arisen by a tandem duplication, although, in the former case, divergence is far more extreme[22]. The third G-protein gene in this cluster encodes an ARL3 homologue, a member of the ARF family. All of the genes are expressed constitutively at the mRNA level and, by means of Southern blotting, the locus has been found to be conserved in *T. congolense* and also *T. cruzi* and *L. donovani*. The *T. cruzi Tcrab1* gene implied by these mapping studies would be the first small G-protein sequence from *T. cruzi*.

• *Leishmania*. Two Rab homologues have been identified in *Leishmania*. One is highly homologous to *S. cerevisiae* Ypt1, suggesting a role in ER-to-Golgi transport, which is supported by EM localization studies showing the protein present on the parasite Golgi complex[28]. A second, a homologue of Rab7, was identified by random screening of *L. major* expressed sequences and is under study (S.M. Lewis *et al.*, Abstract)†. In addition, two genes containing GTP-binding motifs have been identified recently by genome sequencing of *L. braziliensis*[29], but these are most probably not small GTPases. The sequence of *L. major* chromosome 1, recently completed, contains no small GTPase genes[30]. As this corresponds to only 1% of the genome, full sequencing is required to obtain the full picture. By GTP-overlay analysis it is apparent that the *Leishmania* complement of small G proteins is likely to be of similar complexity to *T. brucei* (Fig. 3).

• *Giardia lamblia* and *Entamoeba histolytica*. Both small G-protein sequences and a family of ~21 kDa prenylproteins (likely to be small G proteins) have been reported in *Giardia*[31,32]. In particular, both Ran and ARF homologues have been identified by molecular biological approaches and both characterized as authentic GTPases. The Ran homologue was shown to localize to both nucleii. The ARF protein has not been localized[32,33] but was found to complement ARF1 function in yeast[34]. In *Entamoeba*, two Ras and Rap homologues have been identified; EhRas1 has 91% similarity to EhRas2, but only 47% similarity to human Ras. The two Rap sequences, EhRap1 and EhRap2 are similarly related to each other and higher eukaryotes[35,36]. Interestingly, the predicted sequences of the C-termini suggest that the EhRas proteins might be geranylgeranylated rather than farnesylated, as are most human Ras proteins. Samuelson and colleagues[37,38] reported the isolation of a number of Rac homologues from *E. histolytica* and that a Rac homologue is involved in phagocytosis of bacteria and erythrocytes by the parasite.

† Lewis, S.M. *et al.* 'Identification and characterisation of a novel Rab homologue in *Leishmania major* Freidlin', VIIIth Molecular Parasitology Meeting, Woods Hole, MA, USA, 1998

• *Toxoplasma gondii*. Using reverse transcriptase and rapid amplification of cDNA ends PCR strategies, Stedman and Joiner isolated cDNAs from *T. gondii* encoding five Rabs (1, 5, 6, 7 and 11) and one ARF homologue (T. Stedman and K. Joiner, Abstract)‡. TgRab6 and TgRab11 are closely homologous to *P. falciparum* PfRab6 and PfRab11, respectively. In tachyzoites, TgRab6 was localized to the *T. gondii* Golgi whereas TgRab11 was found in a punctate distribution within an uncharacterized apically oriented compartment, in addition to Golgi staining (T. Stedman and K. Joiner, Abstract)#. Overproduction of TgRab6 resulted in enhancement of secretion of soluble bacterial alkaline phosphatase while mutants deficient in GTP hydrolysis reduced secretion of the same protein in transfected parasites. The effects of overproduction of TgRab6 and its GTP-hydrolysis mutants on the secretion of a GPI-anchored reporter protein were less clear, possibly because of the slower turnover of the reporter in *Toxoplasma* than the bacterial alkaline phosphatase (T. Stedman and K. Joiner, pers. commun.). However, overproduction of TgRab11 and TgRab11 GTP-hydrolysis-deficient mutants resulted in discernible phenotypes in GPI-anchored protein localization, possibly implicating TgRab11 in secretion of the important SAG1-related surface protein family.

• Ran/TC4. The abundant small G protein essential for nuclear transport has been identified in numerous parasites, including *P. falciparum* independently at least twice[39,40], *G. lamblia*[32], *T. brucei*[41] and *T. gondii*‡. This is probably a reflection of the high abundance of this protein and hence mRNA levels. No functional analysis on the role of Ran in cell cycle or nuclear import/export processes in protozoa has been published to date.

Heterotrimeric G proteins

• Kinetoplastida. An early investigation into the presence of GTPases in *T. cruzi* and *L. amazonensis* exploited two homology blocks in the GTP-binding region for the design of PCR primers. The *Leishmania* sequence was probably an elongation factor, but the *T. cruzi* clone most probably was derived from a *rab* gene[42]. Unfortunately, no further data have been reported and, apart from the putative Rab1 homologue (see above), this remains the only GTPase sequence for *T. cruzi*. Somewhat more progress in *T. cruzi* has been made by using toxins and antibodies as probes for heterotrimeric G proteins. A developmentally regulated 45 kDa cholera toxin substrate was identified in amastigotes and epimastigotes, which was also recognized by antisera against G_s α-subunits. Using a similar approach, these authors also tentatively identified a G_i/G_o subunit using pertussis toxin[43]. In a further study, antisera to transducin and Ras were utilized to identify putative antigens in *T. cruzi* lysates with molecular weights consistent with their assignment as G proteins. The anti-Ras antibody was also used for expression cloning[44]. Convincing functional data using a number of mechanisms to stimulate the adenylate cyclase signalling cascade (which comprises heterotrimeric G proteins in higher eukaryotes) also suggest the presence of these proteins[45]; for example, an N_s G protein was implied by both cholera toxin-mediated radioadenylation of a 45 kDa band and reconstitution of adenylate cyclase signalling in a cell fusion assay[46]. A putative G_i protein has been isolated from epimastigote membranes; this protein appears to bind GTP and, on the basis of co-chromatography, to block glucagon stimulation of adenylate cyclase. Importantly, the inhibitory activity was sensitive to pertussis toxin[47]. Toxins have also been used to probe for heterotrimeric G proteins in *T. brucei*. A putative G_o subunit that bound GTP was detected by pertussis toxin ribosylation and was present in BSF cells only[15]. GTPase activity present in trypanosome membranes was also increased when cells were exposed to foetal calf serum, epidermal growth factor and bombesin[48]. Similar to *T. cruzi*, there are good functional data concerning adenylate cyclase activity in *T. brucei*, and activation of adenylate cyclase has been found to occur during transformation from the BSF to the procyclic form[49]. Clearly, this has important implications for signalling and response of the parasite to altering host conditions.

In our laboratory, we have been unable to detect GTP-binding activity in proteins with SDS-PAGE estimated molecular weights in excess of 30 kDa in *T. brucei* or *L. major* (Fig. 3). We take this to suggest that either GTP-binding subunits from kinetoplastida heterotrimerics do not re-nature under the conditions developed for mammalian proteins, or that they are present at extremely low levels, or absent. The lack of convincing sequence data makes it impossible to conclude at present whether trypanosomes do contain heterotrimeric G proteins and, if so, how important they are in signalling and other processes. It is worthwhile mentioning that the adenylate cyclase families in *T. brucei*, some of which are present as part of the variant surface glycoprotein expression site, are rather different in structure from their mammalian counterparts[50], and therefore there is the possibility that portions of the kinetoplastida signal transduction pathways are unique to the parasite.

• Apicomplexa. There is also evidence for the presence of heterotrimeric G proteins in *Plasmodium* and *Toxoplasma*. In a study using fluorescence microscopy and western analysis, crossreactivity was detected in *Toxoplasma* tachyzoites. In particular, reactivity with anti-G_α antibodies was observed in the cytoplasm in a region of the cell highly active for secretion, and the position of the antigen altered during invasion[51]. Significantly, aluminium fluoride, a potent activator of heterotrimeric G proteins in most eukaryotes, induced secretion from tachyzoites. In *Plasmodium*, a G_α subunit has also been detected; production of this protein is stage specific. In addition, both pertussis and cholera toxins can affect progression through the life cycle; pertussis toxin increased growth in *in vitro* cultures, whereas cholera toxin resulted in rapid killing, implicating heterotrimeric G proteins as important mediators of *Plasmodium* development[52].

Small G-protein modifications as drug targets

Most small GTPases in eukaryotes are post-translationally modified by prenyl group(s) to facilitate their membrane attachment and their specific localization to cellular membranes. Protozoan parasites are no exception, for example *T. brucei* incorporates prenyl group(s) into at least 20 different proteins. The prenyl groups are of the farnesyl and geranylgeranyl types, similar to

‡ Stedman, T. and Joiner, K. 'Identification of Rab GTPases in *Toxoplasma gondii*', VIIth Molecular Parasitology Meeting, Woods Hole, MA, USA, 1996

Stedman, T. and Joiner, K. 'Expression and localisation of epitope tagged Rab6 and Rab11 GTPases in *Toxoplasma gondii*', VIIIth Molecular Parasitology Meeting, Woods Hole, MA, USA, 1997

other eukaryotes[53]; farnesyltransferase activity has been demonstrated in *T. brucei* and has been partially purified[54,55]. In addition, the presence of the signal for prenylation (a CAAX motif at the C-terminus end of a protein) is present in most small GTPases sequenced so far from protozoan parasites. Inhibition of Ras protein farnesylation has been exploited as a means of controlling and treating tumour cells in mammalian systems (see Ref. 56 for more details) and at least one farnesyltransferase inhibitor is being tested in clinical trials for the treatment of various human cancers[57]. Because some protozoan parasites are rapidly dividing cells in comparison with the host, it is hoped that these strategies might be extended to parasitic pathogens. In addition, evolutionary distances between host and parasite prenyltransferases might provide additional therapeutic selectivity; this prediction has been supported by the observations that the subunits of the trypanosome farnesyltransferase have a much greater molecular weight than those reported in any other eukaryote, and that activity towards peptide acceptors does not mirror the mammalian enzyme, providing real promise of therapeutic potential[55]. Some farnesyltransferase inhibitors inhibit the growth of *G. lamblia*[31] and are toxic at low micromolar concentrations *in vitro* towards *T. brucei*, *T. cruzi* and *L. major*[55,58] (B. Ali and M.C. Field, unpublished), and *S. mansoni*[16]. Therefore, understanding the diverse and vital functions and the processing of G proteins in protozoan parasites will allow the development of new therapies for the diseases caused by these parasites.

Directions

The small G-protein family is numerous, making it amenable to study by direct EST approaches such as PCR. Some of the more general approaches, particularly EST sequencing, have not been particularly informative because of the low abundance of most of the transcripts from these genes. Although in some ways this is a banal observation, it does highlight a fundamental limitation of EST analysis. Out of a current database of several thousand ESTs only three TbRabs have been detected in this way. Reassuringly, TbRan, which is unusual in being a highly abundant small G protein, has been identified[41]. For *Leishmania*, where there is both a well-developed genome sequencing effort[59] and EST project[60], the completed sequence of chromosome 1 (~1% of the *Leishmania* nuclear genome) has not made additional GTPase sequences available. The vital need for a complete data set is highlighted by functional redundancy; for example, in endocytosis it is quite likely that the Rab5 family is at least partly redundant, with overlapping functions, and this indicates both the importance of the system to viability and a confounding problem in simple genetic approaches to function.

Clearly, there are still a large number of small G-protein genes awaiting discovery in parasites. The potential usefulness of these as reagents for the manipulation of signalling pathways, cell cycle, cytoskeleton and protein trafficking is vast, and we are only beginning to glimpse what might soon be possible. Absent from the sequences currently in hand for the kinetoplastida are members of the Ras and Rho family, and an unequivocal demonstration of heterotrimeric G proteins. The potential involvement of these proteins in signal transduction pathways will have fundamental bearing on the understanding of both cell growth and differentiation. In particular, development of therapies based on small G-protein modification, specifically prenylation and acylation, will depend on a good appreciation of the overall roles of G proteins in individual organisms.

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