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Biochemical and genetic evidence for a family of heterotrimeric G-proteins in *Trichomonas vaginalis*☆

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Abstract

We have cloned a single copy gene from the human parasite *Trichomonas vaginalis* that encodes a putative protein of 402 amino acids with ∼35% sequence identity to known α subunits of heterotrimeric G-proteins. It contains the characteristic GTP binding domains G-1 to G-5 with the key residues conserved. The new sequence has an unusual N-terminal extension of ∼70 residues that cannot be aligned to reference G-proteins and which is characterised by proline-rich repeats. To investigate the expression and cellular localisation of the protein we produced specific antisera against a recombinant fusion protein. The antisera recognised a protein of an apparent molecular mass of 51 kDa in protein extracts from *T. vaginalis* and immunofluorescent microscopy established that the protein is localised to discrete endomembranes. Using a protocol designed to purify mammalian heterotrimeric G-proteins incorporating a GTPγS binding assay, we isolated two proteins from *Trichomonas* that are recognised by an heterologous GA/1 antisera raised to a peptide of the conserved G-1 domain of G-protein α subunits. These two proteins have an apparent molecular mass of 61 and 48 kDa, respectively, larger and smaller than the translation product of the cloned gene. Consistent with these results, the GA/1 antisera did not cross-react with the fusion protein produced from the gene we have cloned. These data suggest *T. vaginalis* possesses more than one heterotrimeric G-protein α subunit. Based on the sequence features of the cloned gene and the biochemical properties of the purified proteins, we suggest that these α subunits are likely to be part of classic heterotrimeric G-protein complexes.

Keywords: *Trichomonas vaginalis*; Parasitic protozoa; Heterotrimeric G-proteins; Alpha subunit; Immunocytochemistry; Protein purification; Molecular modelling

1. Introduction

Heterotrimeric G-proteins have been shown to link extracellular signals to intracellular responses in a broad range of eukaryotes [1,2]. In yeast, *Dictyostelium*, plants and animals, they regulate a wide range of cellular processes including cell movement, proliferation and differentiation, upon stimulation by numerous extracellular cues [3–6]. Heterotrimeric G-proteins have also been shown to regulate multiple steps in membrane trafficking and can be localised to endomembranes such as the rough endoplasmic reticulum (RER), Golgi and endosomes [7–9], where they may integrate endocytosis and signal transduction [10].

Heterotrimeric G-proteins comprise α (Gα), β (Gβ) and γ (Gγ) subunits that together form a Gαβγ functional complex; the integrity of this complex is regulated by the nucleotide bound to the Gα subunit [1,3]. In the "inactive" state, Gα is bound to GDP (Gα·GDP) and is part of the Gαβγ complex. When a G-protein-coupled receptor (GPCR) is activated, it binds to the Gαβγ trimer and acts as a guanine exchange factor (GEF), leading to the replacement of GDP by GTP in Gα (Gα·GTP), inducing the Gα "active" state. The conformational changes induced in Gα

Abbreviations: bp, base pairs; EST, expressed sequence tag; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; GPCL, G-protein coupled receptors; IFA, immunofluorescence analysis; ORF, open reading frame; PRR, proline-rich repeat; RER, rough endoplasmic reticulum

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™ database (accession number: AY138840).

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by GTP reduces its affinity for the Gβγ-dimer, leading to the dissociation of Gα·GTP from Gβγ and an increase in the affinity of the freed units for their respective effector molecules leading to cellular responses. The intrinsic GTPase activity of Gα, often up-regulated by other proteins such as the regulators of G-proteins (RGS [11]), leads to a return to the Gα·GDP state and re-association with Gβγ, closing the functional cycle.

Because of their roles in signal transduction and membrane trafficking in model systems, heterotrimeric G-proteins are likely to be involved in the molecular and cellular basis of pathogenesis by parasitic protozoa [12,13]. There is already evidence for heterotrimeric G-proteins in several human parasitic protozoa, including *Plasmodium*, *Trypanosoma*, *Toxoplasma* and *Entamoeba* [12–14]. However, much of the data are biochemical, with as yet little published molecular, structural, cellular and functional characterisation [14].

The protozoan *Trichomonas vaginalis* is the most common, non-viral, sexually transmitted disease, infecting approximately 200 million people worldwide annually [15]. It is a non-limiting pathogen and symptoms include severe vaginal inflammation. Infection is also linked to cervical cancer, a predisposition to HIV infection, infertility and pre- and postnatal complications [15]. Upon contact with host tissue, either to vaginal epithelial cells [16] or extracellular matrix proteins [17], *T. vaginalis* undergoes a drastic cellular transformation. The free swimming pear-shaped flagellated form changes into an amoeboid stage, drastically increasing the surface contact to host tissue and creating a micro-environment controlled by the parasite [18]. This change in cell morphology is thought to be important for establishing long-term infection in the female urogenital tract. There is experimental evidence indicating that these interactions are mediated by specific cell surface receptors [16,17] and these are thought to initiate signalling cascades leading to the morphological transformation. Heterotrimeric G-proteins are good candidate molecules for mediating this cellular signalling given their versatility in model systems. However, nothing is currently known concerning the presence or function of heterotrimeric G-proteins in *T. vaginalis*. Here we present the first molecular, biochemical and cellular localisation data for Gα proteins in *T. vaginalis* and provide evidence for the presence of classic heterotrimeric G-proteins in this important human parasite.

2. Material and methods

2.1. Cell culturing

T. vaginalis G3 cells [19] were grown in MDM [20] with 10% (v/v) heat inactivated horse serum (referred to as 'complete medium'), as previously described [21]. The cells were grown to late log phase 1–2 × 10^6 cells ml^{-1} and harvested by centrifugation (200 × *g*) for all further manipulations.

2.2. Cloning of a T. vaginalis gene encoding a Gα protein

We identified an expressed sequence tag (EST) encoding a putative Gα protein in *T. vaginalis* strain G3 using BLASTX analyses [22]. A 1 kbp PCR fragment generated from the cDNA clone was labelled using the Multiprimer Labelling Kit (Amersham Pharmacia Biotech) and used to probe a genomic library and for Northern and Southern blots. A *T. vaginalis* (strain NIH-C1, ATCC#3000) genomic library (*Sau*IIIA partial digests cloned into Lambda ZAP® express, Stratagene), was kindly provided by Drs. J. Logsdon and A. Roger (Dalhousie University, Halifax, Canada). Several positive clones were identified among 100,000 plaques from the NIH-C1 genomic library using standard methods. A complete open reading frame (ORF) and ca. 200 bp of the 5′ and 3′ flanking regions were sequenced from one clone.

2.3. Sequence comparison and protein structure modelling

Blast searches and retrieval of reference Gα protein sequences were performed using the NCBI http://www.ncbi.nlm.nih.gov/ web interface. For additional sequence analyses we used the SMART [23], Pfam [24], and PROSITE [25] databases and the ExPASY Molecular Biology Server http://www.expasy.ch/. A published Gα protein alignment [2] containing 60 sequences was extracted from the EMBL alignment database (accession number: DS15369). Additional Gα protein sequences from a broad taxonomic sample were added to a total of 100 sequences.

A model was built for the conceptually translated *T. vaginalis* Gα protein sequence, based on the solved structure of the G-protein heterotrimer transducin and related models [26–28]. The structural model was constructed using the program Modeller [29] running on a Silicon Graphics workstation. The program Swiss PDB-Viewer [30] was used to visualise and display the comparative model.

2.4. Expression of a recombinant Gα protein and antibody production

We designed primers to amplify the complete Gα ORF for in-frame cloning into the pET-30a expression vector (Novagen) using *Not*I and *Nco*I restriction sites. The resulting recombinant His- and S-tagged (at the N-terminus end) fusion protein was expressed in *Escherichia coli* BL21(DE3) cells (Novagen). The recombinant protein was extracted from cell pellets using Bugbuster lysis buffer (Novagen), purified using Quick 900 Nickel columns (Novagen), followed by a further gel purification step. The purified, gel eluted, recombinant protein was used to raise antisera (named M006) in rabbits at Harlan Sera Labs, UK. Pre-bleeds were used for controls of the specificity of the M006 antisera for both Western blots and immunocytochemistry. To increase the affinity and titre of the M006 antisera for

immunocytochemistry, we purified it (APM006) using purified recombinant protein coupled to CNBr activated sepharose.

2.5. Western blot analysis

T. vaginalis total protein extracts were obtained from cell cultures directly boiled in sample buffer to reduce proteolysis. Proteins from fractions obtained from purification columns (see below) were concentrated using YM-10 filters (Amicon). All samples were reduced (DTT), alkylated (IAA) and separated on 4–12% Bis-Tris NuPAGE gradient gels (Invitrogen) or standard 10% SDS–PAGE gels using the Protean II gel system (BIORAD). Following electrophoresis, proteins were transferred onto PVDF membranes (Amersham, UK) and probed with M006 antisera (1/5000–1/20,000 dilutions) or GA/1 [31] (NEN-Dupont) (1/1000–1/5000 dilutions). For visualisation, we used a horse-radish peroxidase conjugated goat anti-rabbit antibody (1/50,000 dilutions) (Sigma) and an Enhanced Chemi-Luminescence detection system (Amersham Pharmacia Biotech).

2.6. Immunocytochemistry and endosome labelling

For indirect immunofluorescence analysis (IFA), *T. vaginalis* cells were washed twice in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 7.0) at 37 °C, fixed with either 4% formaldehyde at room temperature, or 70% ethanol at −20 °C, both for 10 min, and then washed twice with PBS pH 7.2. Fixed cells were adhered to poly-L-lysine coated slides for 1 h in a humidifying chamber. Formaldehyde-fixed cells were further incubated with 50 mM NH_4Cl to quench protein side groups exposed by formaldehyde treatment, washed three times in PBS and cellular membranes were permeabilized with 0.2% Triton X-100 in block buffer (3% BSA, 5% non-immune rabbit serum in PBS pH 7.2). Non-specific protein binding sites in the cells were blocked by incubation with block buffer for 15 min. Slides were incubated with the APM006 antisera diluted in block buffer (1/5000) for 2 h and washed three times with PBS pH 7.2 for 5 min each. Cells were then incubated with the secondary antibody, goat anti-rabbit Alexa Fluor 488 (Molecular Probes) (1/2000 diluted in block buffer), for 1 h in the dark. Slides were washed three times with PBS for 5 min each. Coverslips were applied using the mounting fluid Mowiol, including the anti-fading agent DABCO (1,4-diazabicyclo[2.2.2]octane), with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) at 100 μg ml^{-1} added as a nuclear stain. Coverslips were fixed with nail varnish. Slides were stored in the dark at 4 °C prior to further manipulation. Images were captured using a Nikon Eclipse E600 epifluorescent microscope (Jencons pls). To visualise endocytic vesicles, *Trichomonas* cells were incubated with the fluid phase marker Dextran Texas-red (10,000 MW, lysine-fixable, Molecular Probes), at 1 mg ml^{-1} in complete medium for 15 min at 37 °C. Cells were subsequently washed three times in warm PHEM buffer and processed for IFA as described above.

2.7. Biochemical isolation of heterotrimeric G-proteins from T. vaginalis

Total membrane fractions were prepared by homogenising cells with a Teflon-glass potter in 25 mM sucrose at 4 °C as previously described [32]. Cell lysis was monitored by cell counts and samples with >90% cell lysis were further processed. A post-nuclear fraction (5 min, 700 × *g* supernatant) was centrifuged at 100,000 × *g* for 1 h to provide total membranes (pellet) and cytosolic fractions (supernatant). Since *T. vaginalis* cells are rich in cysteine proteinases [21], the E-64 protease inhibitor (Sigma) was added (2 μM) to the initial cell suspension and to the resuspended 100,000 × *g* pellet. Fractions were immediately frozen and stored at −70 °C prior to protein purification or gel analysis.

Heterotrimeric GTP-binding proteins were purified from total membrane fractions by adaptation of established methods for bovine brain Gαβγ purification [33,34]. Gαβγ purification was monitored with a GTPγS binding assay as described in [33] and used mammalian Gαo and Gαi1 as positive controls. Membranes were extracted with 0.4% Genapol-C100 (Calbiochem) in TEDP buffer (20 mM Tris, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, pH 8.0). After centrifugation (100,000 × *g* for 1 h), the supernatant was loaded onto a 50 ml Q-sepharose (anion exchange) column, equilibrated with CHAPS (0.2%) made up in TEDP. The unbound material was removed by washing with the same buffer and a salt gradient (0–500 mM NaCl) was applied to elute bound proteins. The fractions (4 ml) were assayed for the presence of GTPγS-binding activity using [S^{35}]GTPγS (NEN-Dupont). The major peak in activity was pooled, concentrated on YM-10 (Amicon) membranes and loaded onto a G-75 Superdex (gel filtration) column equilibrated with TEDP containing 0.2% CHAPS and 100 mM NaCl. The fractions (1 ml) containing the major GTPγS-binding activity were combined and diluted to bring the CHAPS concentration to 0.05%. The sample was supplemented with GDP (10 μM final), brought to 500 mM NaCl and then loaded onto a phenyl-Sepharose HP (hydrophobic interaction) column. The column was eluted using a double-gradient system in TEDP buffer, commencing with 500 mM NaCl and 0.05% CHAPS and finishing with 50 mM NaCl and 1.5% CHAPS. The fractions were sampled and assayed for GTPγS-binding activity and analysed by SDS–PAGE and Western blotting.

3. Results and discussion

3.1. Cloning of a T. vaginalis gene encoding a Gα protein

Data from a *T. vaginalis* (strain G3) EST was used to isolate a genomic clone from strain NIH-C1 encoding a

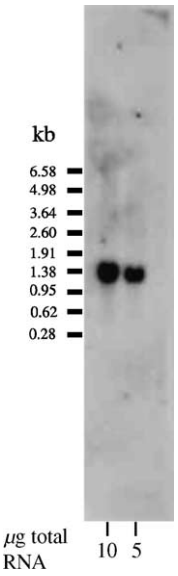

Fig. 1. Expression analysis of the characterised Gα protein coding gene using Northern blotting. Total RNA was isolated from *T. vaginalis* late log phase cells and 10 and 5 μg were size fractionated by electrophoresis on a denaturing agarose gel. An mRNA of about 1.3 kbp was detected by autoradiography using a ^{32}P labelled probe derived from the cDNA clone. The membrane was washed with high stringency conditions prior to exposure.

putative Gα protein of 402 amino acids. A PCR fragment of the equivalent genomic region from strain G3 was identical to the NIH-C1 sequence (see GenBank entry AY138840 for DNA sequence features). There is a potential transcription initiator element (TCAATATTTTGAA) 36 base pairs (bp) upstream of the putative start codon that corresponds to the *T. vaginalis* consensus sequence [35]. The base composition difference (A + T%) between the ORF (52%) and non-protein coding flanking regions (72% for 237 bp at the 5′-end and 79% for 185 bp at the 3′-end) and the codon usage are all consistent with previously cloned *T. vaginalis* genes, as is the apparent absence of any introns [35–37]. The length of the mRNA demonstrated by Northern blot (Fig. 1), is consistent with an ORF of 1206 bp. A Southern blot analysis indicated that this gene is found as a single copy in the G3 strain (data not shown).

3.2. Sequence features of the conceptually translated Gα protein

3.2.1. The GTPase and helical domains

BLAST searches using the *T. vaginalis* sequence identified a *Dictyostelium* Gα protein, GBA2 (accession number: P16051) as the top hit with 35% sequence identity and 60% similarity. Blast searches on the SMART and Pfam databases identified the *T. vaginalis* sequence as a member of the Gα family (SMART entry SM0275, Pfam entry PF00503), hence we named this protein TvGα402. The characteristic GTPase and helix domains of Gα proteins are both present in TvGα402 (Fig. 2). Notably, TvGα402 has relatively conserved helical and GTPase domains (Fig. 2) when compared to more divergent, but functional, Gα sequences from *Dictyostelium* (GBA7 and GBA8) [38], *Arabidopsis* (AtXLG1) [39] and some animal sequences [40,41] (with some examples shown in Fig. 2). Furthermore, 72% of the unusual substitutions found within the TvGα402 helical and GTPase domains are shared with at least one other Gα in the protein databases. The nucleotide binding motifs (the G-boxes) that are crucial for nucleotide binding and exchange, Mg^{2+} coordination, GTP-dependent conformational change and GTP hydrolysis, are all present [42,43] (Fig. 2). Boxes G-1 to G-3, which are involved in Mg^{2+} coordination and GDP/GTP phosphate binding, are the most conserved [43,44]. To facilitate sequence comparisons between G-boxes, we compared concatenated G-1–G-5 sequences for different Gα (36 residues combined, as shown in [43]). The TvGα402 G-boxes have 50% identity and 69% similarity to the corresponding *Dictyostelium* GBA2 sequence. Interestingly, the concatenated G-1–G-5 sequences from TvGα402 are most similar to plant sequences, with 56% identity and 78% similarity to the *Arabidopsis thaliana* GPA1 sequence [45]. The divergent TvGα402 G-5 shares three identical residues and one conservative substitution with GPA1, among the seven amino acids which comprise G-5 (Fig. 2). Pairwise and multiple alignments as well as structure-based alignments (see below) indicate that TvGα402 Ala377 (Fig. 2) corresponds to the universally conserved G-5 alanine, the only residue of G-5 that is in direct contact with GTP [46]. Mutagenesis of this conserved alanine reduces the affinity of Gα for GDP [47].

The modulated interaction between Gα and the Gβγ units upon the bound nucleotide (Gα·GDP or Gα·GTP) is crucial for the function of heterotrimeric G-proteins [42]. In Gαs there are three ‘switch regions’ that undergo major structural changes upon GTP binding causing the heterotrimer to dissociate [28] (Fig. 2). The TvGα402 switch II region showed the highest level of sequence conservation with 11 identical residues and one conservative change over 21 residues (Fig. 2). The important arginine (Arg178 in Gsα1), glycine (Gly203 in Gsα1) and glutamine (Gln204 Gsα1) residues in switch II are all conserved (Arg226, Gly251 and Gln252 in TvGα402, Fig. 2). In mammalian, yeast and *Dictyostelium* Gα, mutagenesis of these residues either dramatically reduces the intrinsic GTPase activity of Gα or abolishes Gα activation upon GTP binding [3,43,47]. The conserved arginine (Arg226 in TvGα402) also corresponds to the target for ADP ribosylation by cholera toxin in some Gα [3]. In contrast, a potential ADP-ribosylation site for pertusis toxin is absent from TvGα402.

In the Gα·GDP/Gβγ state, there are 20 Gα residues in Gtα that are known to be in contact with Gβ located in switch domains I and II and in the αN helix [26] (Fig. 2). The 15 residues located in switch regions I and II are well conserved in TvGα402 (nine identical and one conservative change, 67% similarity, Fig. 2). The residues located at the Gtα αN helix are not conserved (Fig. 2). However, this is

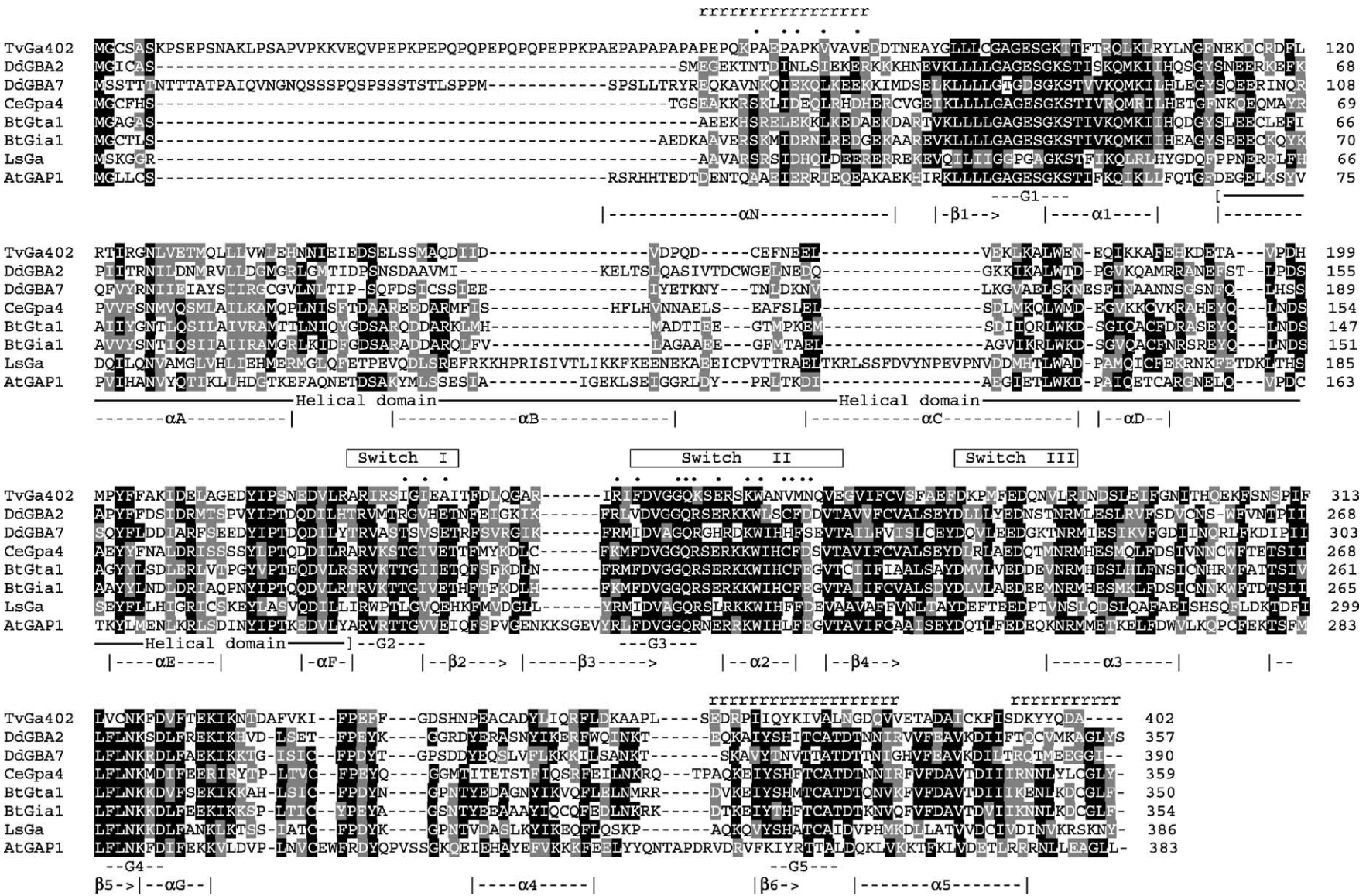

Fig. 2. Sequence features of TvGα402 compared to reference Gα sequences. The conceptually translated sequence of TvGα402 was aligned to selected Gα proteins. GTP binding domains G-1–G-5 are highlighted below the alignment (-Gx-). Secondary structures known for mammalian sequences [26] are shown below the alignment. The helical domain is indicated below the alignment ([—], brackets inclusive). The three switch domains I, II and III, are indicated above the alignment. Other known structural and functional features are highlighted based upon the structural data published for the bovine Gtα [26] and rat Giα [28,47]. The residues involved in contacts with the Gβγ subunits (●) and cognate receptor binding (r) are indicated above the sequence [26]. Taxonomic abbreviations: TvGα402, *Trichomonas vaginalis*; DdGBA2 and DdGBA7, *Dictyostelium discoideum* (accession numbers: P16051 and P34045); CeGpa4, *Caenorhabditis elegans* (Q9BIG5); BtGta1 and BtGia1, *Bos taurus* (P04695 and P04898); LsGa *Lymnaea stagnalis* (pond snail) (CAA87631); AtGAP1, *Arabidopsis thaliana* (AAA32805).

consistent with previously observed levels of conservation and variation among αN helixes [47].

Phylogenetic analyses (not shown) of a Gα alignment indicate that TvGα402 does not cluster with any of the well established classes defined for mammals (Gαo, Gαt, Gαx, Gαi, Gαq, Gα12 and Gαs) [2]. It is part of the so-called GPA group [2] containing sequences from plants, fungi, *Dictyostelium* and some divergent invertebrate paralogues, within which its position is not well resolved.

To gain further insights into the structure–function of TvGα402, we built a model for the core Gα domain (residues 84–402) using the structural data from mammalian heterotrimeric G-proteins (Fig. 3). The sequence alignment and the positions of small insertions/deletions are consistent with the arrangement of secondary structure elements (Fig. 3A), giving confidence in the model. In regions with relatively few amino acid identities to the template, for example, the C-terminal β6 and α5 segments, the protein interior generally consists of non-polar side-chains (see Fig. 2). For β6 and α5, which have been implicated in receptor binding, this suggests that the same framework and therefore possibly comparable function, is maintained in this part of TvGα402. Differences in the amino-terminal regions indicate a degree of alteration in potential Gα–Gβ interactions. However, panels B and C of Fig. 3 show that the polarity of a major Gβ-interacting face in the Gα component of the crystal structure heterotrimer is likely to be maintained in TvGα402, consistent with it forming part of a Gαβγ complex. Fig. 3D emphasises what is apparent from the sequence alignment, that the GDP-binding site is largely conserved in TvGα402. Of the amino acids in the G-1 to G-5 segments that directly neighbour GDP, all but three are identical. One of these three is a conservative change, T97(S). The remaining two, V376(C) and L378(T), are in contact in the template structure, with a potential weak hydrogen-bond between the cysteine and threonine side-chains. With minor structural re-arrangement within TvGα402 this contact can be replaced with a non-polar interaction between the valine and leucine side-chains, maintaining the shape for this part of the GDP-binding pocket. The G-5 box of the *A. thaliana* GPA1, which is known to be functional [6], also has a leucine corresponding to TvGα402 L378 (Fig. 2).

3.2.2. The N-terminal extension

In contrast to the extensive level of sequence conservation observed for the GTPase and helical domains, the N-terminal extension (residues 1–77) of TvGα402 is highly

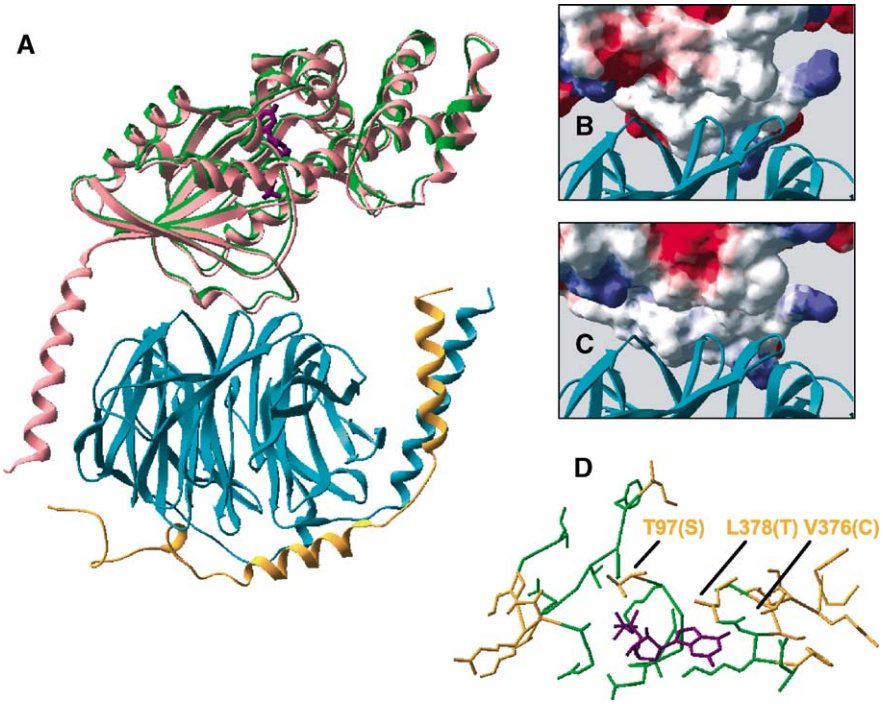

Fig. 3. Comparative modelling of TvGα402 in the context of a Gαβγ heterotrimer. (A) The subunits of a heterotrimer (protein structure data bank file entry 1GOT [26]) are coloured: Gα salmon, Gβ cyan, Gγ gold. Superimposed in green is the modelled TvGα402. The amino-terminal helix of Gα from the crystal structure is partially missing in TvGα402. The crystal structure GDP is drawn in purple, near the centre-top of the panel. (B) A charge potential surface (blue positive, red negative, white non-polar) is shown for the crystal structure of 1GOT Gα at its docking site on the upper face of Gβ in the orientation of panel A. (C) The equivalent and hypothetical docking face to that in panel B is drawn for modelled TvGα402. (D) Amino acids of the G-1 to G-5 regions (see text) are drawn for modelled TvGα402. All side-chain atoms and the main-chain Ca atoms (with Cα–Cα links) are shown, colour-coded according to identity (green) or non-identity (gold) with template Gα. Most residues immediately adjacent to the crystal structure GDP site (purple) are identical in TvGα402. Three non-identical residues that would be in contact with an equivalent site in TvGα402, are labelled with TvGα402 numbering (1GOT amino acid in parenthesis).

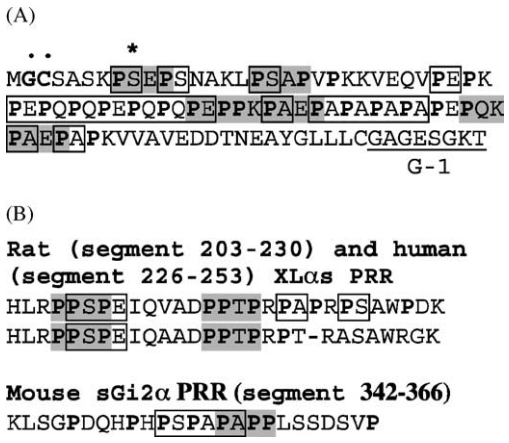

Fig. 4. Sequence features of the proline-rich regions found in the N-terminus of TvGα402. (A) The N-terminus sequence of TvGα402, up to the end of the G-1 box (residue 97), is shown with all prolines in bold. PX repeats are boxed (X stands for S, Q/E or A). Two potential acylation sites, Gly2 and Cys3 are indicated by (•) above the sequence (see text). The minimal SH3 binding consensus motifs, PxxP, are shaded in grey. Ser9 (✱) is a potential phosphorylation site (NetPhos score = 0.82 [56]). (B) Segments containing PRR from two mammalian Gα splice variants sGi2α [57] and XLαs [48,49] are shown with PS, PE and PA motifs and potential SH3 binding site (motifs PxxP), respectively, boxed and shaded as in (A) for comparison.

divergent compared to known Gα sequences and is the most remarkable feature of this Gα protein (Figs. 2 and 4). However, its size is well within the range of known Gα N-terminal extensions [39,48]. The N-terminus is characterised by an unusually high proportion of proline residues comprising 26 of the 77 N-terminal residues (Fig. 4). The prolines form three distinct clusters of proline-rich repeats (PRR) based on different PX motifs (with X being S, Q/E or A) (Fig. 4). Blast searches with residues 1–77 against the GenBank non-redundant protein database failed to recover any similar repeats indicating that they are specific to *T. vaginalis*. In particular, the TvGα402 PRRs do not resemble the PRR found in the two known mammalian splice variants of Gα [49] (Fig. 4). As often found in PRR there is a potential phosphorylation site (for a putative casein kinase I) [50] (Fig. 4). This posttranslational modification is thought to change the function of studied Gα proteins allowing modulation of signalling pathways [3]. There are also seven potential SH3 binding sites conforming to the minimal consensus motif PxxP among the PRRs (Fig. 4) that could be involved in protein–protein binding [50].

An important feature of the majority of Gα proteins, and key to modulating their binding to membranes and proteins, is acylation of the N-terminus [51]. Acylation can take place at residue Gly2, by a generally irreversible co-translational myristoylation. In addition, Cys3, or other cysteines at the N-terminus, can be reversibly post-translationally palmitoylated or modified with arachidonate. Although the N-terminus extension of TvGα402 is highly divergent, it has the starting sequence MGCSAS, which conforms to the consensus sequence for modification by myristoylation (GXXXS/T/A/G/C/N, with serine favoured in position 5—PROSITE entry PS00008) (Fig. 4), making Gly2 a good candidate for myristoylation. A Cys3 is also present and represents a good candidate for post-translational acylation (Fig. 4).

The presence of the PRR in the N-terminus means that it is not possible to build the entire αN helix on the template Gtα [26]. There is the possibility that the TvGα402 N-terminal region could form a partial α-helix and that the PRR could fold into a structure allowing similar binding properties to the N-termini of mammalian Gα. The αN helix of Gtα is typically involved in binding to the membrane, to Gβγ and to GPCR. Since PRRs are also often involved in protein–protein binding and can form hydrophobic surfaces [50], the TvGα402 PRR might play similar roles, and thus replace the need for a typical helix at the N-terminus (Fig. 4). Any PRR-dependent binding could be modulated by phosphorylation at the extreme N-terminus (Fig. 4). The PRR segments of the N-terminus could also contribute to the targeting of the protein to specific membrane(s) as has been shown for the different PRR of the mammalian XLαs and sGiα splice variants [49].

3.3. Expression and cellular localisation of TvGα402

When the His- and S-tag were proteolytically removed from the cloned fusion protein, the recombinant protein migrated with an apparent molecular mass of 51 kDa. A single protein with the same mass of 51 kDa was also detected in total protein extracts from *T. vaginalis* using the M006 antisera (Fig. 5). The results from the Northern blot (Fig. 1) and features of the cloned gene are also consistent with the hypothesis that the two proteins are the same. However, the observed apparent molecular mass of 51 kDa is greater than the 45 kDa predicted from the sequence. We suggest that this discrepancy may be due to the presence of the N-terminal PRR, since such repeats are known to affect protein mobility in denaturing gels [52].

Using a GTPγS binding assay optimised for mammalian Gα, we were able to show that the purified recombinant TvGα402 protein can bind GTP, albeit with a reduced binding capacity (10%) compared to mammalian Gαi1 (data not shown). The lower binding capacity could be an artefact of the purification procedure (denaturing conditions were used) or due to the recombinant protein structural organisation—it has additional His- and S-tags. However, we note that different mammalian Gαs are also reported to bind GDP/GTP with varying efficiencies [43].

To analyse the cellular distribution of TvGα402, proteins from total membrane and cytosolic fractions were investigated using Western blotting and the specific antisera (Fig. 5B). The TvGα402 protein was enriched in the membrane fraction and a substantial fraction of the total amount was also found in the cytosol (Fig. 5B), two cellular compartments where Gα proteins are found in other systems [51]. However, the protein from the membrane fraction was

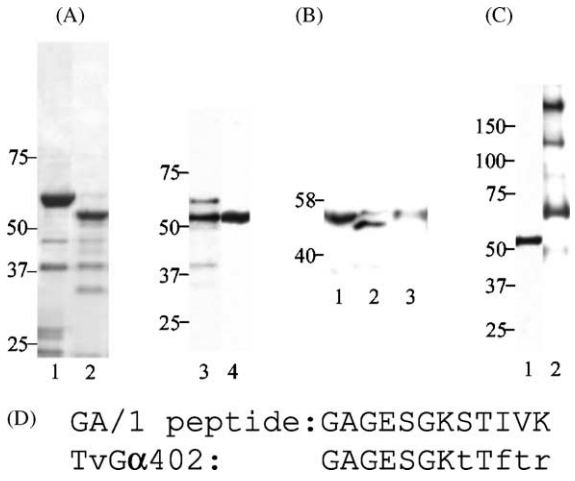

Fig. 5. Analysis of Gα protein expression in *T. vaginalis*. (A) Comparison of apparent molecular mass of the purified TvGα402 recombinant protein (as an His- and S-tagged fusion protein) (lanes 1–3) with native protein from *T. vaginalis* cells (lane 4, 5×10^5 cells). Coomassie staining of SDS–PAGE gel with purified fusion protein (lane 1, 55 kDa protein species) compared with enterokinase digestion that cleaves off the His- and S-tags (lane 2, 51 kDa protein species). Enterokinase digested recombinant protein (lane 3) was electrophoresed in parallel with a total protein extract from *T. vaginalis* (lane 4) and processed for Western blot analysis with M006 antisera. The enterokinase digestion was partial to allow the detection of both the full-length fusion protein and digested material. (B) A total protein extract from *T. vaginalis* (lane 1, as in (A, lane 4)) was processed in parallel with 30 μg protein of a post-nuclear total membrane fraction (lane 2, 0.88% proteins of the fraction) or the corresponding cytosolic fraction (lane 3, 0.97% proteins of the fraction, 1.1× the volume of the equivalent membrane fraction) for Western blot analysis with M006 antisera. (C) *T. vaginalis* total cell extracts were processed for Western blot analysis (as in (A, lane 4)) prior to immunodetection with either M006 (lane 1, 1/20,000 dilution) or GA/1 (lane 2, 1/1000 dilution). (D) Sequence comparison between the peptide (from the G-1 domain) used for generating the GA/1 antisera [31] and the corresponding peptide in the TvGα402 sequence.

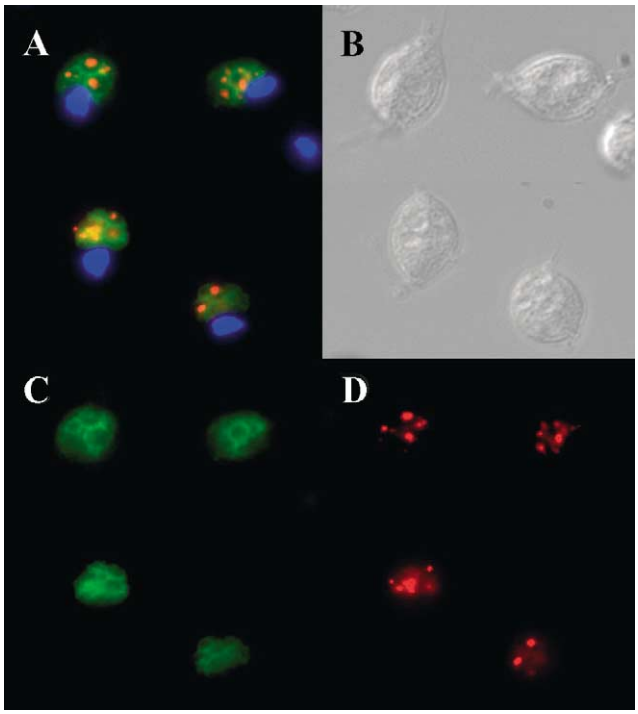

Fig. 6. Cellular localisation of TvGα402 detected by immunocytochemistry with light microscopy. *T. vaginalis* cells were washed and processed for IFA using affinity-purified M006 and goat anti-rabbit antisera Alexa Fluor 488 (green). Nuclei were stained with DAPI (blue). Dextran Texas red (red) was used to label endosomes prior to fixation and processing for TvGα402 immunolocalisation. Specimens were observed with an epifluorescent microscope using the Metamorph software. All three labels were merged (A) and compared with the phase image view of the same field (B). A clear peri-nuclear labelling could be seen for M006 on the ventral side of the cell (C). No labelling could be detected on the plasma membrane. (D) Endosomes were labelled with dextran Texas red for 15 min (red).

degraded as indicated by a reduction in the apparent molecular mass (Fig. 5B). We also observed a high sensitivity of TvGα402 to proteolysis in total cell extracts, particularly when higher cell concentrations were used, indicating a susceptibility of this protein to proteolysis (data not shown).

We used indirect immunofluorescence analysis (IFA) with affinity purified M006 antisera to demonstrate a consistent and specific peri-nuclear labelling of the posterior side of the nucleus (Fig. 6). Since TvGα402 is enriched in the total membrane fraction (Fig. 5B), these labelled structures are likely to represent internal membrane-bound structures. The polar morphology of the *T. vaginalis* cell [53], allows us to speculate on the identity of these structures. They are unlikely to correspond to the Golgi complex, which is located in the anterior side of the nucleus [53], or to endosomes—since we found no co-localisation with dextran Texas Red-labelled structures (Fig. 6A). They could correspond to the RER, a known cellular localisation for Gα proteins in other organisms [7,8], or to another yet to be defined compartment. The observed membrane pool is consistent with TvGα402 being part of a Gαβγ complex since both Gα acylation and binding to Gβγ are needed for a stable membrane association in other systems [54].

We also used the GA/1 antisera, raised against a 12-mer peptide conserved among mammalian Gα proteins [31], to look for additional Gα proteins in *T. vaginalis* (Fig. 5C). A distinct population of proteins was detected in total cell extracts with apparent molecular masses of 48, 61, 120 and >150 kDa (Fig. 5C). The GA/1 antisera did not cross-react with the TvGα402 recombinant protein (data not shown), probably due to the four differences between the 12-mer peptide used to raise GA/1 and the corresponding sequence in TvGα402 (Fig. 5D). These results suggest that, as in *Dictyostelium* and mammals [3,5], there is more than one Gα protein encoded by *T. vaginalis* genome. The protein species with the highest apparent molecular mass (120 and >150 kDa) could represent extra large Gα proteins like the ones found in plants (120 kDa) and mammals (94 kDa) [39,48]. Alternatively, the 120 kDa protein species could represent cross-linked dimers (homo- or hetero-complexes) of the 61 kDa protein as was observed for the polymeric

immunoglobulin receptor previously analysed using identical experimental procedures [55].

3.4. Purification of heterotrimeric G-proteins

To further investigate the presence of heterotrimeric G-protein complexes (Gαβγ) and Gα protein diversity in *Trichomonas*, we adapted a protocol established for extracting heterotrimeric G-proteins from bovine brain. Post-nuclear total membrane fractions contained 95% of the GTPγS binding activity ([S^{35}]GTPγS binding per mg protein) and were used as the starting material for Gαβγ purification. Fractions corresponding to the largest single peak of GTPγS binding activity in the first ion exchange column were pooled and size fractionated (Fig. 7A). Two GTPγS binding peaks segregated from the main protein peak (Fig. 7A), as typically observed for similar preparations from bovine brain [33,34]. Fractions exhibiting the highest GTPγS binding capacity (1st peak—fractions 6–10, protein size range 65–100 kDa) and corresponding to proteins of size range of Gαβγ complexes (ca. 85 kDa) or Gα monomers, were further separated based on hydrophobic interactions (Fig. 7B). Fractions were pooled and tested for both GTPγS binding activity (Fig. 7B) and immunoreactivity to antisera GA/1 (Fig. 7C) and M006 (data not shown). Proteins with an apparent molecular mass of 61 and 45 kDa were detected using GA/1 (Fig. 7B), in accordance with the earlier Western blot analysis of total proteins from *T. vaginalis* (Fig. 5C, 48 and 61 kDa). There was no evidence from Western blot analyses for the 51 kDa TvGα402 protein in these fractions (data not shown). Two factors may help to explain this. Firstly, the TvGα402 protein appears to be particularly susceptible to proteolysis. For example, more than 50% of the M0006 cross-reacting material in the membrane pool is apparently degraded (Fig. 5B), and further proteolysis of TvGα402 was observed in the first column (data not shown). Secondly, the potentially lower binding capacity of the protein for GTPγS could also contribute to a failure to recover TvGα402 during the procedure.

The 61 kDa protein peaked in pooled fractions 1–8 and 25–32 (Fig. 7C), whereas the 45 kDa protein was found in fractions 33–56. These fractions are characterised by enrichment in GTPγS binding per mg protein when compared to the starting total membrane fraction: 165 times for fractions 1–8, 60 times for fractions 24–32 and 90 times for fractions 33–40. To discriminate the two putative Gα proteins from the TvGα402 (51 kDa), we named them TvGα61k and TvGα45k based on their apparent molecular mass. As for mammalian Gαs and Gαi purifications [34], TvGα61k and TvGα45k were found among a complex mix of proteins after the three successive chromatographies, as demonstrated by Coomasie staining (data not shown). An attempt to sequence the TvGα61k protein, further purified on a MonoQ ion exchange column, was not successful. The TvGα61k protein segregates into two major peaks (fractions 1–8 and 25–32) and TvGα45k is found in one major peak (fractions

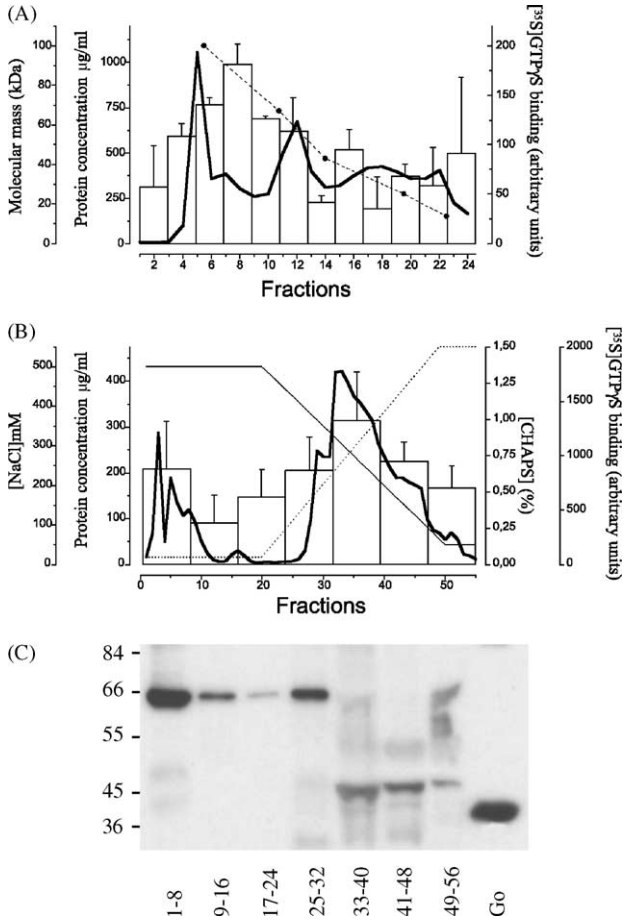

Fig. 7. Purification of heterotrimeric G-proteins from *T. vaginalis* total membrane fractions using column chromatography. A [S^{35}]GTPγS binding assay was used to follow the presence of hetrotrimeric G-proteins in the fractions. (A) Following fractionation of a total membrane extract on a Q-sepharose ion-exchange column, the major peak of GTPγS binding activity was loaded on a G-75 Superdex gel filtration column (shown). Protein concentration (OD_{280}) is indicated by the solid line. The fractions (6–10) corresponding to the major peak of GTPγS binding (bars) were pooled for further purification with hydrophobic interaction chromatography (shown in B). Protein size calibration of the column (dotted line) with a set of five reference proteins (circles) is also shown. (B) Phenyl-sepharose column purification. Protein concentration (solid line), NaCl (thin line) and CHAPS concentration (dashed line) are also indicated with GTPγS binding activity (bars). (C) Aliquots of the same pooled fractions used for GTP binding assays shown in (B) were processed and analysed by Western blot with GA/1 antisera (as in Fig. 5). As a positive control we used purified mammalian brain Gαo protein that has an apparent molecular mass of 39 kDa (lane labelled Go).

33–48). Based on the published biochemical properties of heterotrimeric G-proteins [34] early fractions are potentially Gα monomers, and later fractions Gαβγ heterotrimers, since heterotrimers are more hydrophobic than Gα monomers [34]. Taken together (i) the observed enrichment in GTPγS binding per mg protein during the successive chromatographies, (ii) GA/1 immunoreactive proteins in these enriched fractions, (ii) the apparent molecular mass of immunoreactive proteins (45 and 61 kDa), (iii) the elution profile of these two proteins in the phenyl-sepharose column and

the non-immunoreactivity of TvGα402 to GA/1, strongly suggest that *T. vaginalis* has more than one Gα protein.

4. Conclusions

We have characterised the first Gα protein-encoding gene from *T. vaginalis* and its translation product, TvGα402. Based on sequence comparisons of the protein, its capacity to bind GTPγS and its cellular localisation, we hypothesise that TvGα402 is likely a component of a classic heterotrimeric G-protein functional complex. We have also obtained biochemical data strongly suggesting that the *T. vaginalis* genome has additional genes encoding distinct Gα proteins. These will require further molecular, biochemical and cellular characterization.

Based on its observed peri-nuclear membrane association, we speculate that TvGα402 could be involved in regulating membrane trafficking [7,9] or in signal transduction in a novel way, since it is not localised at the plasma membrane [3]. The possible involvement of one or more heterotimeric G-protein(s) in the cellular transformation from a trophozoite to an amoebal stage upon contact to host tissue [15,18] is only one of the interesting hypotheses that can be tested in future.

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