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Dramatic reorganisation of Trichomonas endomembranes during amoebal transformation: a possible role for G-proteins

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Abbreviations

BSA, bovine serum albumin; ConA, Concanavalin A; DAPI, 4’,6-Diamidino-2-phenylindole dihydrochloride; DIC, differential interference contrast; ECM, extracellular matrix; ER, Endoplasmic Reticulum; IFA, Indirect immunofluorescence analysis; TvGα402, Trichomonas G-protein alpha subunit.

Note: Supplementary data are associated with this article

Keywords

Trichomonas vaginalis, amoeba transformation, G-protein, membrane trafficking, intracellular localisation, multivesicular bodies, secretion.
T. vaginalis is the most common non-viral sexually transmitted pathogen and is thought to represent a significant risk factor for HIV infection [1, 2]. *Trichomonas* transformation from a free-swimming trophozoite to an adherent amoeba is crucial to parasite establishment in the host vagina and subsequent pathogenesis [1-4]. Amoebal transformation takes place upon binding to vaginal epithelial cells or to extracellular matrix (ECM) proteins and can be induced *in vitro* upon binding to ECM components laminin and fibronectin [3]. This process is believed to involve specific cell signalling events [4]. Membrane trafficking is also key to *Trichomonas* pathogenesis; for example perforin secretion leads to host cell lysis [5]. Despite the potential importance of membrane trafficking and signalling to these clinical aspects of *Trichomonas*, little is known of the molecular machinery orchestrating these processes. Heterotrimeric G-proteins (G-proteins) are key to membrane trafficking and cell signalling in most organisms [6] and so we chose to investigate the roles of a *Trichomonas* G-protein. We have previously shown that the *T. vaginalis* G-protein alpha subunit TvGα402 locates at endomembranes in trophozoites [7].

Indirect immunofluorescence analysis (IFA) labelling of TvGα402 together with a nuclear stain (DAPI) shows TvGα402 to localise to posterior perinuclear vesicular structures (up to 2µm in diameter) in *Trichomonas* trophozoite cells (Fig 1A-D) [7]. There are few *Trichomonas* intracellular markers characterised but the mannose-specific lectin Concanavalin A (ConA) preferentially binds glycoproteins at the ER, Golgi and endosome/lysosomes [8] and we show ConA to partially co-localise with TvGα402 (Fig. 1E-H). ConA is likely to represent a highly specific probe for *Trichomonas* glycoproteins as only two bands were detected when a *Trichomonas* total cell extract was probed with ConA by Western blot (Supplementary Fig. S1). ER tracker labels the ER in many cell types [9, 10] and in *Trichomonas* cells appears to highlight several structures including a large oval structure similar to the nuclear membrane in size and location with respect to TvGα402. This is consistent with ER labelling. However, TvGα402 localisation is clearly fully distinct to ER tracker
indicating an absence of TvGα402 at the ER (Fig. 1I-L). A partial co-localisation with ConA (Fig. 1E) suggests that Tvgα402 locates at either the ER, Golgi or lysosomes; the former compartment can be eliminated due to a lack of overlap with ER tracker. Further, Tvgα402 organisation appears distinct from Golgi complex morphology as in *Trichomonas* the Golgi complex is a single copy organelle located anterior to the nucleus [11], whilst Tvgα402 labels structures posterior in the cell (Fig. 1A) [7]. This leads us to suggest that Tvgα402 locates at endosome/lysosomes. Additionally, Tvgα402 labelled vesicles share morphological similarities with *Trichomonas* endosome/lysosomes, which can grow very large (up to ~2 µm in diameter) and are preferentially positioned posterior within the cell [12]. Immunoelectron microscopy shows *Trichomonas* to have multivesicular body-like compartments, i.e. vesicles containing luminal vesicles. Tvgα402 localisation includes the cytoplasmic face of the outer limiting membrane of multivesicular bodies-like compartments and also at unstructured membranes (Fig. 1U and V).

To further investigate the relationship of Tvgα402 to endocytosis we tracked endocytic material using the probes dextran or ConA. The endocytic marker fluorescent dextran (Mwt 10,000 daltons) is taken into many cell types by a fluid-phase route - e.g. [13]. Dextran-labelled endocytic vesicles, less than 1 µm in diameter, frequently appear within Tvgα402 stained structures (Figs. 1M-P, S2 and movie 1) [7]. Thus a subset of Tvgα402 positive vesicles are linked to endocytosis. This staining pattern is consistent with dextran labelling luminal vesicles of multivesicular bodies-like compartments whilst Tvgα402 labels the outer membranes, as suggested from the immunoelectron microscopy data (Fig. 1U and V).

Alternatively, ConA binds glycoproteins on the surface of live *Trichomonas* cells and allows monitoring of endocytosis of ConA-labelled glycoproteins. In contrast to dextran uptake, ConA-labelled endosomes do not appear to be associated with Tvgα402 (Fig 1Q-T). Also, the kinetics of
dextran and ConA uptake are different (Supplementary Fig. S2). Thus, dextran and ConA appear to label distinct populations of vesicles. Also, not all endocytic cargo taken into the cell is passively transported within TvGα402 labelled vesicles. Thus the potential complexity of *Trichomonas* endocytosis is also demonstrated here with the active sorting of the contents of multivesiculur bodies-like compartements. Further, the recent discovery of an extremely large Rab GTPase gene family, encoding proteins key to membrane trafficking in model systems, also strongly suggests an exceedingly complex endomembrane system in *Trichomonas* [14].

Binding to ECM proteins fibronectin and laminin induces differentiation of *Trichomonas* trophozoites into an amoebae form [3]. After 20 minutes of contact with ECM proteins the cellular morphology of *T. vaginalis* clearly reflects the differentiation process as cells in transition show an irregular outline under differential interference contrast (DIC) that is distinct from the trophozoite (compare Fig. 2B and 2D), indicating remodelling of the cell surface and overall cell shape. During this process TvGα402 labelled structures differentiate into well-defined vesicles which proliferate in number (from ~3 to >10 per cell) and dramatically occupy most of the cell volume (Fig. 2C and movie 2). The TvGα402 labelled vesicles appear with a similar morphology in terms of shape and size as in the trophozoite, suggesting the labelling of endosomal/lysosomal compartments into early differentiation. Strikingly, TvGα402 labelling appears brighter and more distinct at vesicles during the differentiation to the amoeba (comparing Fig. 2A and 2C and movies 1 and 2). Semi-quantitative RT-PCR analyses shows a dramatic increase in TvGα402 mRNA in amoebae bound to ECM proteins compared to trophozoites (Fig. S3). It is also possible that the fraction of TvGα402 in the trophozoite that is observed as diffuse staining by immunofluorescence (Fig. 2A) and likely at cytoplasmic membranes by electron microscopy (Fig. 1U and V), may relocate to vesicle membranes during the amoebal transformation together with membrane reorganisation (Fig. 2C). Therefore the increased TvGα402 signal from trophozoite to amoeba detected by immunofluorescence is likely
due to the combined effect of a burst in TvGα402 protein synthesis and the possible relocation of the original pool of protein together with endomembrane reorganisation. After 30 minutes of contact with ECM proteins, in *Trichomonas* “late” amoebae, TvGα402 labelled vesicles often relocate to a single focus towards the cell periphery, often at pseudopodia (Fig. 2E and 2F).

The present data demonstrates dramatic changes occurring in the *Trichomonas* endomembrane system that accompany amoeba transformation following contact with ECM proteins. Significantly these studies also highlight at least two developmental phases in the amoeboid transformation, an early phase involving an increase in TvGα402-labelled vesicle numbers and a later phase where these are reduced. At present the significance of the realignment of TvGα402-labelled membranes is not clear but the relocation of vesicles towards the cell surface suggests a role in secretion. In better characterised systems G-proteins have a well established role at multivesiculuar bodies in the secretion of exosomes [15, 16]. As *Trichomonas* stores HIV particles at multivesicular bodies-like compartements [17] reminiscent of TvGα402-positive compartements, it is possible that TvGα402 plays an important role in the release of harboured HIV upon contact to host tissue and contributes to the well established increased risk of HIV infection for *Trichomonas* infected people [1, 17, 18]. Further study of the functions of TvGα402 and its associated organelles is clearly of importance.

**Acknowledgments**

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References


Legends to Figures

Figure 1. Cellular localization of \( \text{TvG} \alpha 402 \) compared to different markers. \( \text{TvG} \alpha 402 \) at posterior vesicles with partial co-localisation with ConA binding but distinct to ER tracker. \( \text{TvG} \alpha 402 \) vesicles are frequently linked to endocytosed dextran but not ConA. Ultrastructure shows heterogeneous location of \( \text{TvG} \alpha 402 \) including the cytoplasmic face of multivesicular body-like compartments. \textit{Trichomonas} cells were fixed with ethanol or parformaldehyde and permeabilised with Triton X-100 and labelled with anti-\( \text{TvG} \alpha 402 \) (1/5000) and DAPI (A-D) or ConA (E-H). Anti-\( \text{TvG} \alpha 402 \) antibody (M006) was detected with anti-rabbit Alexa 488. Live \textit{Trichomonas} cells were incubated with ER tracker for 30 mins and processed for IFA (I-L). \textit{Trichomonas} endocytosis was monitored after incubation with dextran-texas red for 12 mins (M-P) or ConA-biotin (Q-T) for 2 mins and chased in buffer for 10 mins and processed for IFA. ConA-biotin was detected with streptavidin texas red. Images A-D and I-L were captured with an epifluorescent microscope whilst the remainder were single section confocal images. Images in each row represent one cell and \( \text{TvG} \alpha 402 \) staining is shown in green in the first and second columns. Various co-stains indicated to the left of each row are shown in blue or red in the third column. The overlays of \( \text{TvG} \alpha 402 \) labelling with individual co-stains are shown in the first column. Panel A shows \( \text{TvG} \alpha 402 \) at posterior perinuclear vesicular structures. \( \text{TvG} \alpha 402 \) and ConA labelling (Panel E) shows a partial co-localisation. In panel I, the overlay image of \( \text{TvG} \alpha 402 \) and ER tracker clearly shows distinct labelling. Dextran labelled vesicles often appear inside \( \text{TvG} \alpha 402 \) (M-P) outlined vesicles whilst ConA labelled vesicles do not (Q-T). The bars represent 2 \( \mu \text{m} \). In panels U and V, immuno electron microscopy images of \textit{Trichomonas} cells labelled with anti-\( \text{TvG} \alpha 402 \)-protein A gold, show \( \text{TvG} \alpha 402 \) at heterogeneous vesicles. Multivesicular bodies-like compartments have membrane
bound lumenal vesicles and TvGα402 location includes the cytoplasmic face of the outer limiting membrane of such compartments. TvGα402 is also found at unstructured membranes in the cytosol. The bars represent 500 nm.

**Figure 2. TvGα402 location is dramatically altered with trophozoite to amoeba transformation.**

The cellular location of TvGα402 is compared in trophozoite cells (Fig. 2A-B) to amoeba after incubation with ECM proteins for 20 or 30 minutes. Within 20 minutes of contact with ECM proteins *Trichomonas* start to transform into early amoeboid forms (Fig. 2C-D). After 30 minutes, late amoebae cells show a reduced size and pseudopodia are often apparent (Fig. 2E-F). Trophozoites were fixed and adhered to slides with poly-l-lysine whilst amoebae adhered via ECM proteins for indicated times and then fixed and processed for IFA. Panels A, C and E show overlay images of TvGα402 (green) and DAPI stained the nucleus (blue). Panels B, D and F show the respective corresponding DIC images. TvGα402 labelled structures change from a perinuclear posterior location in the trophozoite (Fig. 2A), to occupying most of the cell in the early amoeba (Fig. 2C). In the late amoeba TvGα402 is found at the cell periphery in reduced numbers and often in pseudopodia (Fig. 2E and 2F). TvGα402 labelling appears brighter and more condensed at vesicles in the amoebal stages. The bar represents 2µm.
Supplementary material

Supplementary Figure S1. ConA is likely a specific probe in *Trichomonas* cells. Western blot analysis of *Trichomonas* total cellular extract probed with biotinylated ConA and peroxidase conjugated streptavidin. ConA specifically detects two glycoprotein bands with apparent Mw of 56 kDa and 62 kDa.

Supplementary Figure S2. Endocytosis of dextran appears to label a distinct set of vesicles to ConA. Each row of panels represents one *Trichomonas* cell. The top three rows show *Trichomonas* cells after incubation with ConA-biotin for 2 minutes and chased with excess buffer for 0, 2 or 10 minutes. The bottom three rows show *Trichomonas* cells incubated with dextran Mwt 10,000 texas red at 37°C for the time points indicated. All cells were washed and fixed and processed for labelling of Tvgα402 and ConA-biotin labelled cells were additionally detected with streptavidin texas red. Images were single sections captured using a confocal microscope. Panels A, E and I show the overlay images of ConA (red) labelling of Tvgα402 (green) whilst panels M, Q and U displays the overlay image of dextran (red) and APM006 (green) labelling. Dextran-labelled vesicles often appear inside Tvgα402 outlined vesicles whilst ConA labelled vesicles do not. Dextran vesicles appear inside Tvgα402 labelled vesicles within 2 minuites of endocytosis (M) whilst ConA requires 10 minutes to reach deeper structures inside the cell (J). From 10-60 mins dextran labelling appears similar to 7 mins and 12 mins time points whilst endocytosed ConA could not be detected after 12 mins and is possibly degraded. Panels B, F and J show the individual staining patterns of ConA (red), panels N, R and V show individual dextran staining (red) and Tvgα402 staining (green) is shown in
panels in the third column. Corresponding DIC images are presented in the fourth column. The bar represents 2 µm.

**Supplementary Figure S3. TvGα402 mRNA is up-regulated in the amoeba life stage.** RT-PCR of *Trichomonas* trophozoite and amoeba life stages compared mRNA levels of TvGα402, α-actinin and actin using specific primers. The size of the amplicon (in base pairs) is indicated on the right hand side. Actin levels are roughly equivalent suggesting similar total mRNA levels from the two cell types. TvGα402 mRNA is clearly up-regulated in the *Trichomonas* amoeba, as is α-actinin as previously reported [19]. TvGα402 amplicon is not visible in the trophozoite under these conditions, but by increasing the template cDNA or the number of amplification cycles TvGα402 becomes visible.

**Movie 1. Dextran vesicles are typically inside TvGα402 outlined vesicles.** *Trichomonas vaginalis* cells were incubated with dextran texas red for 15 minutes at 37°C. Cells were washed thoroughly, fixed with 70% ethanol at -20°C and processed for labelling of TvGα402. Serial z-section images were captured of one cell using a Zeiss confocal microscope. TvGα402 is shown in green and dextran in red. Dextran vesicles often appear inside TvGα402 outlined vesicles. For example the dextran vesicle in the bottom left hand corner of the cell appears inside a TvGα402 outlined vesicle for several frames and the TvGα402 labelled vesicle remains after the dextran vesicle has disappeared from view.

**Movie 2. TvGα402 labelled vesicles proliferate appearing mainly towards the cell surface in the Trichomonas amoeba.** *Trichomonas* cells were induced into amoeba by incubation with mammalian
extracellular matrix proteins, laminin and fibronectin for 20 minutes. Adherent amoebae were processed for IFA and serial z-section images of TvGα402 shown in green were captured using a Zeiss confocal microscope. The DIC image is shown for comparison. TvGα402 outlined vesicles appear numerous, throughout the cell but mainly at the cell periphery when compared to the DIC image. TvGα402 labelling appears brighter at vesicles compared to the trophozoite in movie 1.
Figure 1

Overlay  TvGo402  DIC

DAPI

ConA

ER tracker

Endocytosed dextran

Endocytosed ConA

A  B  C  D

E  F  G  H

I  J  K  L

M  N  O  P

Q  R  S  T

U  V
Figure 2

Free swimming trophozoites

Early amoebae (ECM bound + 20 min)

Late amoebae (ECM bound + 30 min)
Figure S2

Con A
2 mins

Con A
7 mins

Con A
12 mins

Dextran
2 mins

Dextran
7 mins

Dextran
12 mins
Figure S3

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