Flagellar membrane localization via association with lipid rafts

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Summary

The eukaryotic flagellar membrane has a distinct composition from other domains of the plasmalemma. Our work shows that the specialized composition of the trypanosome flagellar membrane reflects increased concentrations of sterols and saturated fatty acids, correlating with direct observation of high liquid order by laurdan fluorescence microscopy. These findings indicate that the trypanosome flagellar membrane possesses high concentrations of lipid rafts: discrete regions of lateral heterogeneity in plasma membranes that serve to sequester and organize specialized protein complexes. Consistent with this, a dually acylated Ca^{2+} sensor that is concentrated in the flagellum is found in detergent-resistant membranes and mislocalizes if the lipid rafts are disrupted. Detergent-extracted cells have discrete membrane patches localized on the surface of the flagellar axoneme, suggestive of intraflagellar transport particles. Together, these results provide biophysical and biochemical evidence to indicate that lipid rafts are enriched in the trypanosome flagellar membrane, providing a unique mechanism for flagellar protein localization and illustrating a novel means by which specialized cellular functions may be partitioned to discrete membrane domains.

Key words: Flagellum, Membrane microdomains, Membrane fluidity, Acylation, Trypanosoma

Introduction

The fluid mosaic model posits that the plasma membrane exists in a liquid disordered state in which there is random diffusion of untethered membrane molecules through contiguous membrane domains (Singer and Nicolson, 1972). The liquid ordered state has been proposed as an exception to the fluid mosaic model (Bagatolli et al., 2001; Brown and London, 1998; Simons and van Meer, 1988), in which sphingolipids and sterols in close proximity form regions of reduced fluidity, so-called membrane microdomains or 'lipid rafts' (Anderson and Jacobson, 2002; Brown and London, 2000; Jacobson and Dietrich, 1999; Yang et al., 2002). Proteins modified by the addition of two or more lipid moieties can be incorporated selectively into lipid rafts (Melkonian et al., 1999), promoting physical association of proteins involved in concerted functions such as cell signaling (Simons and Toomre, 2000). Because of their small size, lipid rafts are difficult to visualize directly; however, recently it has been possible to discriminate areas of membrane heterogeneity where liquid ordered state may be particularly concentrated, using the amphiphilic fluor laurdan (Gaus et al., 2003). In the kinetoplastid *Leishmania*, roles for sphingolipids and, by extension, lipid rafts have recently been demonstrated in cellular differentiation and membrane trafficking, but the basis for these observations is not yet understood (Denny et al., 2004; Zhang et al., 2003).

Ciliary and flagellar membranes form specialized domains of the plasma membrane with peculiar biophysical properties and composition. Flagellar membranes are rich in sterols (Kaneshiro, 1990) and glycolipids (Bloodgood et al., 1995), and may have increased concentrations of sphingolipids (Kaneshiro, 1990; Kaya et al., 1984). Ciliary membranes are often marked by the presence of specific proteins; some of these, such as transducin and arrestin

of the mammalian photoreceptor, appear to associate with lipid rafts in a manner sensitive to external stimuli (Nair et al., 2002). The African trypanosome *Trypanosoma brucei* causes sleeping sickness, a fatal disease epidemic in much of sub-Saharan Africa (Welburn and Odiit, 2002). This organism is an extracellular parasite with a complex digenetic life cycle during which it is able to proliferate both in the midgut of its tsetse fly vector as a procyclic form and in the blood of its mammalian hosts as a bloodstream form. In the nutrient-rich environment of mammalian blood, *T. brucei* is able to scavenge enough cholesterol to meet its sterol requirements. In the tsetse midgut, however, the procyclic form synthesizes ergosterol as its major sterol (Coppens and Courtoy, 2000). Trypanosomes are single-celled, flagellated, eukaryotic organisms, the surface membranes of which are partitioned into flagellar, flagellar pocket and pellicular (cell body) domains. The cytoskeletal architecture of the trypanosome is well defined, with the plasma-membrane being underpinned by a subpellicular array of cortical microtubules (Gull, 1999). The flagellar axoneme has the canonical 9+2 arrangement of microtubules when viewed in cross-section and is closely associated with a paracrystalline structure: the paraflagellar (paraxial) rod. The flagellum is joined to and abuts the cell body for much of the length of the cell and this region of attachment is known as the flagellar attachment zone (Kohl and Bastin, 2005). It was observed early on that sterols could concentrate in the flagellar membrane (Souto-Padron and de Souza, 1983; Tetley, 1986). More recent studies of flagellum-specific proteins have provided further evidence supporting the delineation of plasma membrane domains in the trypanosome cell (Bastin et al., 2000; Bloodgood, 2000; de Souza, 1995; Fridberg et al., 2007; Godsel and Engman, 1999; Landfear and Ignatushchenko, 2001), which has led to increased

interest in how such demarcation can be achieved in a contiguous surface. In this report, we address this issue, documenting the presence of increased concentrations of lipid raft molecules in the trypanosome flagellum and functionally linking these rafts to the specific localization of flagellar membrane proteins.

Results

Calflagin Tb24 is associated with detergent-resistant membranes

The flagellum-specific membrane localization of a flagellar Ca^{2+}-binding protein (FCaBP) of the American trypanosome *T. cruzi* is dependent on modification by both myristate and palmitate (Godsel and Engman, 1999). The requirement of dual acylation for flagellar localization was also recently found to be true of a family of small acylated proteins in *Leishmania* (Tull et al., 2004). Calflagin Tb24, a *T. brucei* homologue of *T. cruzi* FCaBP (Wu et al., 1992), is likewise myristoylated and palmitoylated (Emmer et al., 2008). Dual acylation suggested a potential association with lipid rafts. To test whether this protein is indeed raft associated, we employed two biochemical assays: resistance to solubilization by Triton X-100 at 4°C and buoyancy on sucrose density gradients (Fig. 1). Western blot analysis of Triton X-100 fractions of *T. brucei* procyclic cells using an antiserum we raised to recombinant calflagin Tb24 showed that the protein associated with the detergent-insoluble pellet when fractions were prepared at 4°C but not when they were prepared at 37°C. Furthermore, calflagin Tb24 floated to the top of a sucrose gradient. Thus, this flagellar membrane protein displays the biochemical characteristics of a raft-associated protein. This is in contrast to plasma membrane proteins distributed across the surface (EP procyclin, GP63), to proteins found in the cytoplasm (heat shock protein 70; Hsp70) and to proteins found in the flagellar cytoskeleton (PFR).

Lipid raft components are enriched in the flagellar membrane

Although acylation explains raft association, it does not explain flagellum specificity. We considered the environment that the flagellar membranes represented and their composition. Several reports exist, showing that some membrane species are

overrepresented in the flagellar membranes of ciliates. Taken together, the relative enrichment of flagellar membranes in sterols (Kaneshiro, 1990; Souto-Padron and de Souza, 1983; Tetley, 1986), glycolipids (Bloodgood et al., 1995) and sphingolipids (Kaneshiro et al., 1984; Kaya et al., 1984) already reported suggested that a specialized lipid environment in the flagellar membrane might be the basis for the flagellar localization of some dually acylated proteins in the trypanosome.

In seeking to confirm these reports, we began our investigation at the level of the single cell by fluorescence microscopy of species-specific probes (Fig. 2A). Filipin, a polyene antibiotic that binds specifically to 3-β-hydroxysterols, was used as a fluorescent probe of sterol distribution in *T. brucei*. Staining of procyclic forms, which possess ergosterol as the major 3-β-hydroxysterol, revealed an increased flagellar concentration of filipin, consistent with results of freeze-fracture microscopy (Souto-Padron and de Souza, 1983). There is no fluorescent probe specific for sphingolipids; however, it was possible to determine the likely distribution of galactocerebroside and ganglioside GM1 (Dietrich et al., 2001b) by fluorescence microscopy after staining with galactocerebroside-specific antiserum (Girard et al., 2000) and fluorescinated cholera toxin, respectively. Like the 3-β-hydroxysterols, galactocerebroside and GM1 appeared most concentrated in the flagellum, although some cells showed more diffuse distribution of GM1. Calflagin Tb24 is highly enriched in the flagellum, as expected, whereas the GPI-linked proteins EP procyclin and GP63, which are present on the outer leaflet of the plasma membrane, are distributed throughout the cell surface.

Partitioning of microtubules and microtubule-associated proteins between the cell body and flagellum of the trypanosome has been well studied and has yielded markers that are highly specific to the flagellum and cell body compartments (Gull, 1999). α-Tubulin is present in the subpellicular array of microtubules that underlie the plasmalemma of the cell body and in the microtubules of the flagellar axoneme (Fig. 2B). By contrast, the microtubule associated protein 'whole cell body' (WCB) is associated only with the subpellicular microtubules (Woods et al., 1992), whereas the paraflagellar rod proteins constitute the major paracrystalline structure of flagellar cytoskeleton.

To quantify the flagellar enrichment in lipid raft components, we isolated membranated flagella from *T. brucei* procyclic forms and assessed the abundance of a number of markers in this flagellar preparation relative to their abundance in a whole cell lysate. The purified flagella were evaluated by fluorescence microscopy for purity using cytoskeletal (α tubulin) and surface (EP procyclin) constituents, both common to the flagellum and cell body. There was no microscopic evidence of pellicular contamination (Fig. 2C). Western blot analysis of serial dilutions of flagellar and whole cell lysates was performed for several proteins and relative flagellar enrichment was determined by the ratio of signal in the two preparations (Table 1). These ratios were then normalized to that for EP procyclin, which was given a nominal enrichment value of 1.0. A similar preparation obtained from procyclic forms treated with dehydroergosterol, a fluorescent analog of cholesterol believed to behave similarly in a lipid bilayer (Mukherjee et al., 1998), was assessed spectrophotometrically for accumulation in the flagellum relative to EP procyclin (Table 1). The analysis showed that flagellum-specific molecules of both the membrane (calflagin Tb24, dehydroergosterol) and cytoskeleton [paraflagellar rod (PFR), α tubulin (modest enrichment reflecting the presence of the flagellar axoneme)] were enriched in the flagellar preparation, while pellicular constituents such as the whole cell body antigen were undetectable.
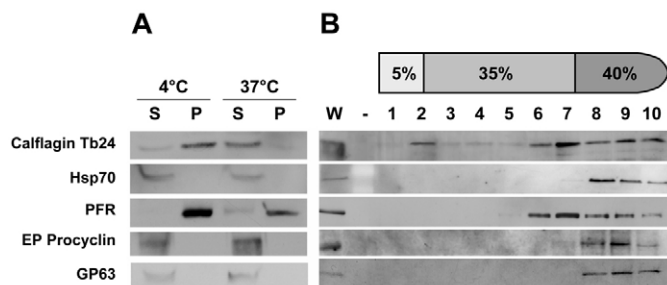

Fig. 1. Calflagin Tb24 is associated with detergent-resistant membranes. (A) To test temperature-dependent resistance to detergent, 10^8 *T. brucei* procyclic forms were extracted in 1 ml of 1% Triton X-100 in PBS at 4°C or 37°C for 10 minute and centrifuged supernatants and pellets were analyzed by SDS-PAGE and western blotting with calflagin Tb24-specific antiserum. Of the proteins tested, only calflagin Tb24 was present in the pellet (P) at 4°C but the supernatant (S) at 37°C. (B) To test buoyancy, cells were extracted in 1% Triton X-100 in TBS at 4°C and floated through an OptiPrep (40%, 30%, 5%) step gradient. Sequential fractions (1-10) were collected and equal volumes (25 μl) were subjected to western blot analysis with the same antibodies used in A. Only calflagin Tb24 floated to the top of the step gradient, with the other proteins remaining close to the loading zone. In both assays, partitioning of Hsp70, PFR, EP procyclin and GP63 were assessed to control for cytosolic, flagellar cytoskeleton and diffuse surface distributions. W, whole-cell lysate.
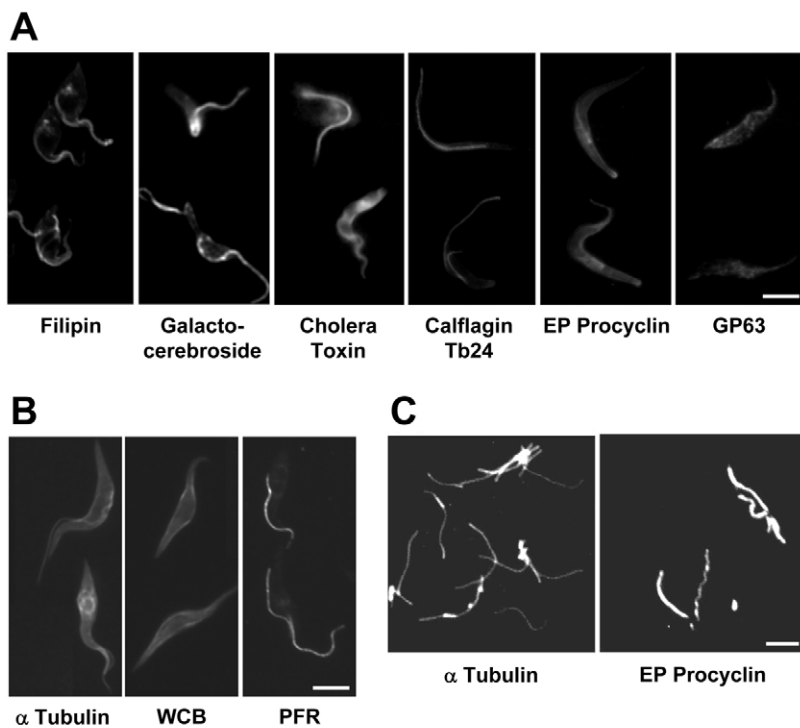
A

Filipin   Galacto-cerebroside   Cholera Toxin   Calflagin Tb24   EP Procyclin   GP63

B

α Tubulin   WCB   PFR

C

α Tubulin   EP Procyclin

Fig. 2. The flagellum of *T. brucei* is enriched in molecules associated with lipid membrane rafts. (A) Paraformaldehyde-fixed procyclic forms were analyzed by fluorescence microscopy for the distribution of raft components. Ergosterol was visualized by staining with filipin, galactocerebroside was visualized by immunofluorescence microscopy, GM1 ganglioside was visualized by staining with FITC-conjugated cholera toxin, the dually acylated calflagin Tb24 was visualized by immunofluorescence microscopy and two GPI-anchored proteins (EP procyclin and GP63) were visualized by immunofluorescence microscopy. (B) Cellular distribution of cytoskeletal controls proteins used in fractionation. α-Tubulin is distributed in both flagellar and pellicular cytoskeleton, WCB is restricted to the pellicular cytoskeleton and PFR is confined to the flagellar cytoskeleton. (C) Purified flagella were evaluated by fluorescence microscopy for purity using cytoskeletal (α tubulin) and surface (EP procyclin) constituents, both of which are common to the flagellum and cell body. The preparation consisted of flagella only; there was no microscopic evidence of cell bodies or pellicular fragments, and kinetoplasts/basal bodies fractionated separately (data not shown). Bar, 5 μm.

Membrane liquid order is highest in the flagellar membrane

The previous experiments demonstrated that raft-associated molecules are enriched in the flagellar membrane. We next sought direct evidence of increased liquid order in the flagellar membrane through the use of the amphiphilic fluor laurdan, which intercalates into the lipid bilayer close to the interfacial region and fluoresces according to the molecular freedom of water in its vicinity (Gaus et al., 2003). The correlation between laurdan emission and membrane order is strong because water dynamics in the lipid interface are closely linked to membrane phospholipid packing and sphingolipid head groups are better aligned and more tightly packed in lipid rafts, conferring increased order to the bilayer. In living *T. brucei* procyclic cells stained with laurdan, a clear difference in generalized polarization (GP) was evident when flagellar and pellicular membranes were compared (Fig. 3). The

GP images shown are ratiometric depictions of fluorescent intensities at 499 nm and 446 nm. The GP value is consequently independent of probe concentration, reflecting variation in the orderliness of the membrane. Areas appearing green are less ordered and those appearing blue are more ordered. A cell with a single flagellum (top) and a dividing cell having two flagella (bottom) are shown. The total fluorescence from laurdan provided an image of the cells with the fluorescence intensity showing regionalized probe concentration and scaled so that yellow corresponds to high intensity and red to low intensity. Blue regions were restricted to the periphery of the cell, and particularly to the single flagellum of the top cell and the two flagella of the dividing cell below, indicating that the flagellum possesses a relatively high degree of liquid order. Consistent with this, laurdan staining intensity in the flagellar membrane was generally less intense than that in the cell body. Although the apparatus does not permit the simultaneous acquisition of phase-contrast images, it was possible to discern the line of the flagella from the unscaled laurdan intensity images. A mask was created from the intensity images at each wavelength and a line drawing was constructed which traced the perceived line of the flagellum and is provided to give orientation.

Flagellar membrane localization is dependent on lipid rafts

If calflagin Tb24 localizes to the flagellar membrane because of its affinity for the lipid rafts resident there, then disruption of those rafts should abolish the flagellar localization of the protein. The major 3-β-hydroxysterol of the bloodstream form of *T. brucei* is not ergosterol, but cholesterol, which is scavenged from the blood. Filipin staining of bloodstream forms revealed a flagellar concentration of cholesterol (Fig. 4A). Calflagin Tb24 is also expressed in bloodstream forms and is associated with the flagellar membrane in a manner resistant to extraction with cold Triton X-100. Interestingly, the anterior tip of the new flagellum shows particularly intense calflagin Tb24 staining where it contacts the old flagellum. This point is coincident with that described as the flagellum connector (Moreira-Leite et al.,
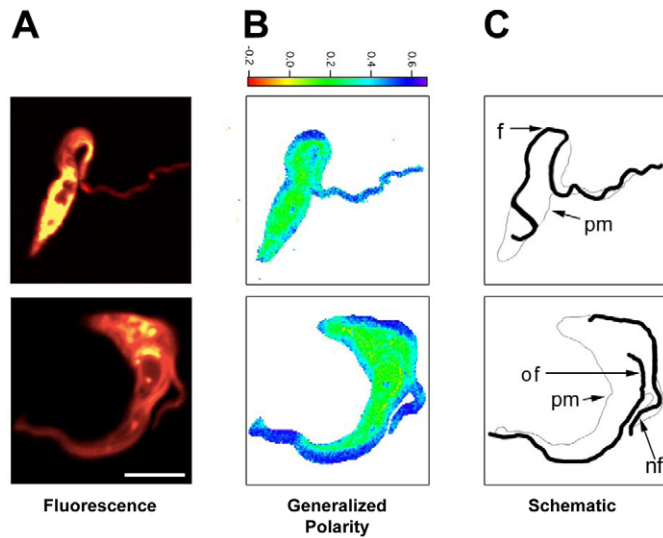
Table 1. Components of lipid rafts are enriched in the trypanosome flagellum

Molecule	Flagellar enrichment
EP Procyclin	1
GP63	0.9
PFR	64
Calflagin Tb24	48
α-Tubulin	8.8
Whole cell body	Not detected
Dehydroergosterol	22

Flagellar and whole cell preparations were analyzed by western blotting (proteins) or by spectrophotometry (dehydroergosterol). The volume of flagellar preparation necessary to give signal equivalent to 1×10^6 whole cells was first calculated from standard curves and tabulated as the 'raw score'. Values for relative flagellar enrichment were tabulated by normalization to EP procyclin. PFR, calflagin Tb24, dehydroergosterol and tubulin were all found to be enriched in the flagellar fraction relative to procyclin, whereas GP63 was not. WCB, an antigen absent from the flagellar cytoskeleton was not detected in the purified flagella.

Fig. 3. The trypanosome flagellar membrane possesses increased liquid order. *T. brucei* procyclic forms were examined by two-photon microscopy after staining with the amphiphilic fluor laurdan. Both non-dividing (top) and dividing (bottom) cells are shown. Procyclic trypanosomes were washed and resuspended to 10^8 cells per ml in PBS containing 1 μM laurdan, then settled on poly-L-lysine-coated slides for 10 minute at room temperature. (A) Laurdan fluorescence intensity relates to probe concentration and is scaled so that yellow corresponds to high intensity and red to low intensity. (B) The generalized polarity (GP) is a ratiometric depiction of fluorescent intensities from two wavelengths. The GP value is probe concentration independent, reflecting variation in the orderliness of a membrane. Areas appearing green are less ordered and those appearing blue are more ordered. (C) Line drawings are shown for orientation of the trypanosome cell: f, flagellum; pm, pellicular membrane; nf, new flagellum; of, old flagellum. Bar, 5 μm.

2001), a structure in procyclic cells that is believed to be involved in regulating the timing of cytokinesis. Although this point in bloodstream form cells does not have the same cytoskeletal composition as that in procyclic organisms, there is electron-dense material present (Moreira-Leite et al., 2001) that apparently contains calflagin, among other molecules. When bloodstream form trypanosome cells were preincubated with 20 mM methyl beta cyclodextrin (MBCD) to specifically adsorb cholesterol and thus disrupt lipid raft stability (Ilangumaran and Hoessli, 1998), the detergent-resistant flagellar membrane localization of calflagin, including enrichment at the flagellum connector, was lost. This coincided with a significant reduction in the flotation of calflagin on a sucrose gradient (Fig. 4B).

Although the ergosterol in procyclic cells is not susceptible to MBCD depletion, it was possible to test the role of the membrane environment on calflagin localization using chemical modulation of membrane fluidity (Fig. 4C). Lipid rafts form when sphingolipids and 3-β-hydroxysterols coalesce in a manner that is dependent on the concentrations of both of these constituents. Depletion of either constituent disrupts the rafts. DMSO stabilizes the liquid ordered phase, whereas diethyl ether disrupts it (Bigi et al., 1975; Regev et al., 1999). Treatment of living cells with 10% DMSO for 1 minute increased the flagellar membrane specificity of calflagin Tb24 without affecting cell viability. The calflagin Tb24 staining pattern in these cells appeared 'straw-like'. As calflagins are sensor proteins that ordinarily exist in a dynamic equilibrium between the flagelloplasm and flagellar membrane, this appearance suggests the loss of soluble calflagin and the stabilization of the interaction between the protein and the flagellar membrane as the liquid order in that
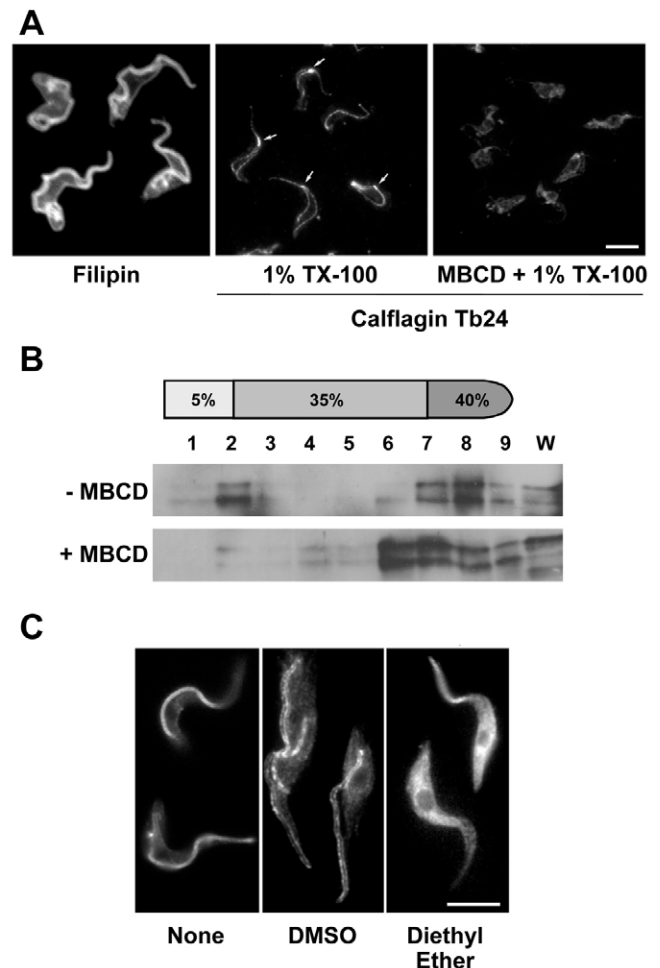

Fig. 4. Calflagin Tb24 distribution reflects membrane raft stability. (A) The flagellum of bloodstream form *T. brucei* is enriched in cholesterol and calflagin Tb24. Filipin staining of untreated cells shows enrichment in the flagellum. The flagellar localization of calflagin Tb24 is resistant to 1% Triton X-100 at 4°C. The region of the flagellum connector (arrows, center panel) is particularly rich in staining for this DRM marker. If cholesterol is depleted by preincubation in 20 mM MBCD for 30 minutes, flagellar concentration of calflagins Tb24 is lost. (B) MBCD treatment leads to loss of buoyancy of calflagin Tb24. Bloodstream form *T. brucei* treated with MBCD and untreated control cells were analyzed by Optiprep gradient centrifugation and western blotting with calflagin Tb24-specific antiserum as described in Fig. 1. W, whole-cell lysate. (C) Chemical modulation of membrane fluidity. Washed procyclic form trypanosomes were treated with 10% DMSO for 1 minute to stabilize the liquid ordered phase or 1% diethyl ether for 30 seconds to fluidize the liquid ordered phase. Cells were then fixed and processed for immunofluorescence microscopy using anti-calflagin Tb24. Bar, 5 μm.

membrane stabilizes. By contrast, all flagellum specificity was lost in cells treated with 1% diethyl ether for 30 seconds when liquid order was disrupted. Collectively, the results of MBCD treatment of bloodstream forms and chemical modulation of membrane fluidity in procyclic cells are consistent with a functional role for lipid rafts in creating an environment favorable for concentrating acyl proteins in the flagellar membrane of trypanosomes.

DRM patches are associated with the flagellar axoneme
The localization of dually acylated protein, sterol and glycosphingolipid to the detergent-resistant 'remnants' of the flagellar membrane suggested that these remnants might be

visualized by SEM. Similarly prepared detergent-extracted bloodstream forms and bloodstream forms that had been preincubated with MBCD were analyzed alongside procyclic cells. In general, the patches were larger in ergosterol-rich procyclic forms (Fig. 5A,B,D) than in cholesterol-rich bloodstream forms (Fig. 5E). In bloodstream forms preincubated with MBCD, patches were not observed (Fig. 5F), suggesting that their formation is sterol dependent. The mean diameter of the DRM patches attached to the axonemes in bloodstream forms was significantly ($P<0.001$) smaller at 245 nm ($n=100$, s.e.m.$=2.7$) compared with 322 nm ($n=100$, s.e.m.$=4.2$) for the procyclic DRM patches. Analysis of detergent-extracted procyclic cells revealed the presence of DRM patches, which assumed a regular linear distribution along much of the flagellar axoneme (Fig. 5B), but were never seen associated with the paraflagellar rod (Fig. 5A,B,D-F). These patches were more abundant in the distal flagellum (Fig. 5A) than in the proximal/mid flagellum (Fig. 5B).

Discussion

Formation of lipid rafts is dependent on the concentrations of just two components, sterols and sphingolipids (Brown and London, 2000). Both of these were found in high concentration in the trypanosome flagellar membrane, suggesting that this membrane would display increased liquid order. Although gangliosides and galactocerebroside have not been well studied in *T. brucei*, studies thus far suggest that they are present, with the gangliosides showing subtle differences from those of mammals (Girard et al., 2000; Sutterwala et al., 2008; Uemura et al., 2004). There are many papers describing the involvement of lipid rafts in a number of important cellular processes (reviewed in Hanzal-Bayer and Hancock, 2007). Despite this, there have been substantial criticisms of the raft hypothesis, largely based on the notion that the use of detergents in the preparation of cell extracts for analysis can cause artifactual association with or dissociation from DRM fractions (Munro, 2003). To test the raft hypothesis directly, without the use of detergents, we used the variable emission properties of the fluor laurdan in

two-photon microscopy of living trypanosomes. This technique has been used extensively for investigating model membranes, and recently has been applied to neutrophil lamellipodia (Kindzelskii et al., 2004) and macrophage filopodia (Gaus et al., 2003). This analysis permitted the direct visualization of increased liquid order in a discrete, stable membrane domain of the trypanosome cell. Although the resolution of the fluorescence microscope is not sufficient for visualization of discrete structures, when considered with corroborating biochemical data, laurdan microscopy provides strong support for the existence of an endogenous liquid ordered phase in the trypanosome flagellum.

Disruption of lipid rafts, by depletion of sterol or by fluidization of the membrane, abolished localization of the acyl protein to the flagellar membrane. This provides proof of principle for lipid raft association as a mechanism by which specialized membrane domain composition and hence function can be achieved, and may be applicable to several putative adhesion and signaling molecules that have been ascribed flagellar membrane localizations in trypanosomes (Bastin et al., 2000; Bloodgood, 2000; Fridberg et al., 2007; Landfear and Ignatushchenko, 2001). It also provides a possible mechanism by which the membrane components of signaling relays may be concentrated and colocalized within the flagellar membrane. This parallels the proposed role of rafts in signaling by metazoan cells, implying evolutionary conservation of the use of rafts for signaling.

In other cells, including the related protozoan *Leishmania*, GPI-linked proteins are typically associated with lipid rafts (Denny et al., 2001; Denny et al., 2004; Zhang et al., 2003). Some have hypothesized that these proteins may be sorted based on their association with sphingolipid microdomains (Muniz and Riezman, 2000). This is apparently not the case in *T. brucei*, which has GPI anchors that are structurally distinct. The variant surface glycoprotein protein (VSG) of bloodstream form *T. brucei* is excluded from DRMs, a finding that was predicted because the saturated acyl chains of the GPI moiety conjugated to the alkyl glycerol group consist of myristate. Even the dimerization of VSG,
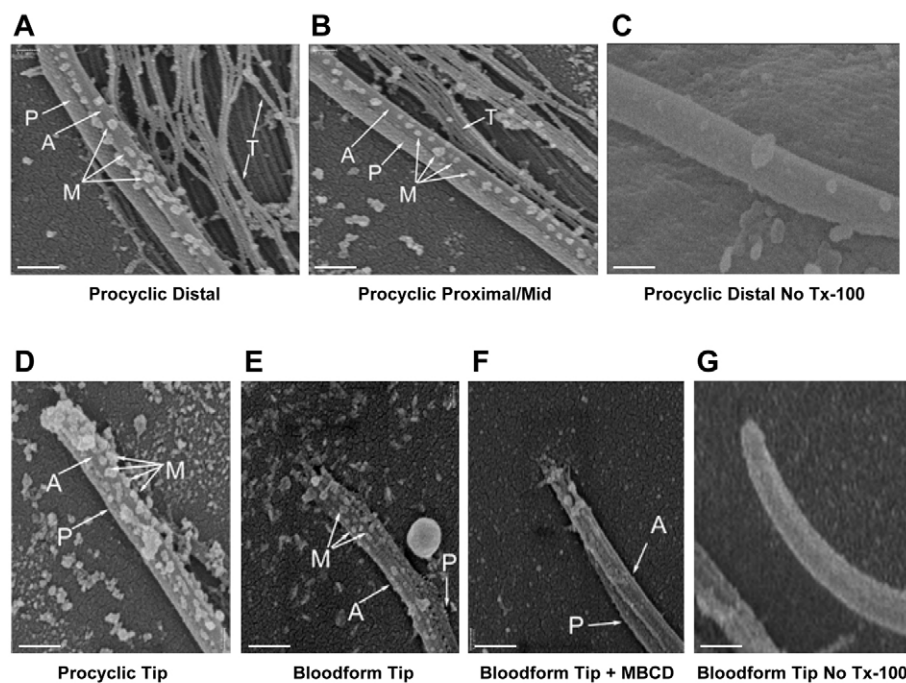

Fig. 5. DRM patches localize to the flagellar axoneme. Cells washed with 1% Triton X-100 at 4°C retain patches of detergent-resistant membrane material along their flagellar axonemes, as visualized by scanning electron microscopy. (A) A more distal section and (B) a more proximal section of the single flagellum of a procyclic form parasite are shown. The patches of membrane (M) revealed are restricted to the flagellar axoneme (A) and absent from the paraflagellar rod (P). Some particles are also seen associated with subpellicular microtubules (T), but these are at much lower density than those distributed along the flagellar axoneme. (C) Micrograph of unextracted procyclic flagellum (no Triton X-100). Patches are most abundant at the flagellar tip and are larger in the ergosterol-containing procyclic form (D) than in the cholesterol-containing bloodstream form (E). Patches are absent from the flagellum and flagellar tip of bloodstream forms pretreated with 20 mM MBCD to deplete cholesterol (F). (G) Micrograph of unextracted bloodform flagellum (no Triton X-100). Bars, 200 nm.

Procyclic Distal   Procyclic Proximal/Mid   Procyclic Distal No Tx-100

Procyclic Tip   Bloodform Tip   Bloodform Tip + MBCD   Bloodform Tip No Tx-100

which effectively doubles the fatty acid complement of this protein, is insufficient to promote the incorporation of this dominant surface protein of the bloodstream forms into lipid rafts under standard conditions (Benting et al., 1999; Denny et al., 2001). The acyl chains associated with procyclin GPI anchors are more interesting, and the data provided here on procyclin may cast light on the structural requirement for acyl chain configuration in order to enter lipid rafts. Structurally, the procyclin GPI anchor contains is a mixture of 2-O-acyl-myo-inositol-1-HPO$_4$-(sn-1-stearoyl-2-lyso-glycerol) and 2-O-acyl-myo-inositol-1-HPO$_4$-(sn-1-octadecyl-2-lyso-glycerol), where the acyl chain substituting the inositol ring shows considerable heterogeneity (Treumann et al., 1997). Although the typical procyclin anchor contains acyl chains of sufficient length to associate with lipid rafts, the conjugation of these acyl chains at the different positions on the inositol ring is likely to introduce strain on the anchor, effectively reducing the lengths of acyl chains available for membrane interaction.

It should be noted that not all myristoylated, palmitoylated proteins in the trypanosome are flagellar. CAP5.5 (Hertz-Fowler et al., 2001) associates with the pellicular cytoskeleton and p41 (Schneider et al., 1988) also associates with the cytoskeleton, although whether this is flagellar or pellicular has not been determined. The dually acylated HASPB protein of *Leishmania* is an extracellular protein (Denny et al., 2000; Stegmayer et al., 2005), whereas the phosphoinositide-specific phospholipase C of *T. cruzi* (TcPI-PLC), a protein that is associated with amastigogenesis in *T. cruzi* and thus with change in flagellar length, has been localized to the plasma membrane and cytoplasm of amastigotes and epimastigotes, though no enrichment in the flagellum was reported (Furuya et al., 2000; Okura et al., 2005). Thus, there appear to be additional factors, such as association with the cytoskeleton, that serve to determine the final destination of dually acylated proteins in these protozoans. The association of calflagin Tb24 with detergent-extracted flagella (Fig. 4A), which contain axoneme-associated membrane patches (Fig. 5), supports this notion. It is also possible that lipid rafts are organized differently in outer and inner leaflets of various plasma membrane domains.

Interestingly, the major 3-β-hydroxysterol of procyclic trypanosomes is ergosterol, not cholesterol (Coppens and Courtoy, 2000). Ergosterol is more efficient than cholesterol at forming rafts in model membranes (Xu et al., 2001), so the use of ergosterol by procyclic forms might be expected to lead to formation of larger rafts than form in bloodstream cells. Indeed, the sizes of the DRM patches visualized by SEM were larger in procyclics. The membrane patches were restricted to the flagellar axoneme and excluded from the paraflagellar rod, indicating a specific connection to the microtubular cytoskeleton. The sizes and distribution of the DRM patches observed are highly reminiscent of intraflagellar transport particles, which are likewise restricted in trypanosomes (Absalon et al., 2008). If this is the case, one would expect this phenomenon to be universal among ciliated cells. In support of this contention, the well-defined DRM protein caveolin was shown to be enriched in the flagellar membrane of mammalian sperm, a membrane that is also cholesterol rich (Travis et al., 2001). Furthermore, it has been demonstrated that the DRM protein prominin accumulates in protrusions from the plasmalemma like microvilli and, indeed, in the evaginations of the outer segment of the rod photoreceptor, a ciliary structure (Corbeil et al., 2001). In addition, recoverin, the prototypical calcium-myristoyl switch protein (Ames et al., 1996; Zozulya and Stryer, 1992) is also found in DRM rafts in retinal rod outer segment (Senin et al., 2004). Lipid raft-associated small

myristoylated proteins have been localized to the flagellum of *Leishmania* (Tull et al., 2004), and raft-associated light-orienting proteins are found in the flagellum of *Chlamydomonas* (Iomini et al., 2006). Importantly, laurdan microscopy was used to demonstrate that the primary cilium of MDCK cells also show a relatively high degree of liquid order compared with the rest of the plasmalemma (Vieira et al., 2006). Considered together, our results and those of others suggest that the surface membranes of many eukaryotic cilia and flagella are chemically distinct from that of the cell body and specifically enriched in components of lipid rafts. Whether this is a universal phenomenon awaits further investigation.

Intraflagellar transport (IFT) involves the trafficking of particles beneath the flagellar membrane to which they are attached (Rosenbaum and Witman, 2002). These IFT particles traffic along microtubules and consequently should be restricted to the flagellar axoneme and absent from the paraflagellar rod in trypanosomes. The size, regular distribution and restriction of the DRM patches to the flagellar axoneme (Fig. 5A,B) are suggestive that the patches observed may overlie IFT particles. The manner by which these assemblies interact specifically with the flagellar membrane and their relationship to the increased order we observe in the flagellum are yet to be determined but our data do suggest that ergosterol rafts are larger, or cluster more effectively, than do cholesterol rafts (Fig. 5D,E), consistent with the observation that ergosterol forms rafts in vitro more effectively than cholesterol (Xu et al., 2001). It should be mentioned that the existence of flagellar membrane remnants physically connected to outer doublet microtubules of detergent-extracted flagella was demonstrated long ago in *Tetrahymena* (Dentler et al., 1980) and some of the components of this linkage have been identified (Kozminski et al., 1995).

We have found that a region akin to the flagellum connector, which links the old flagellum to the growing end of the new flagellum, is specifically enriched in the DRM marker calflagin Tb24. This observation suggests a role for lipid rafts in trypanosome cell division and flagellar biogenesis, in addition to the previous observations that trypanosome flagellar membrane is involved in control of motility, adhesion, cell division and differentiation (Bastin et al., 2000; Bloodgood, 2000; Fridberg et al., 2007; Landfear and Ignatushchenko, 2001). These observations are reinforced by two studies published earlier this year. In the first, *Leishmania* adaptor protein 1 was implicated in a variety of lipid raft-associated activities, including flagellar biogenesis, with sphingolipid and sterol inhibitors exacerbating flagellar biogenesis defects observed in defective mutants (Vince et al., 2008). In the second, depletion of sphingolipids in *T. brucei* bloodstream forms disrupted lipid rafts and abolished the buoyancy of calflagin on a sucrose gradient (Fridberg et al., 2008). Although our results pertain primarily to the trypanosome flagellum and its unique biochemistry, the adjacent flagellar pocket has a unique biochemistry of its own, is sterol rich, contains uniquely localizing glycolipids and is functionally disrupted by ablation of sphingolipids (Fridberg et al., 2008; Tetley, 1986). Although the flagellum arises from the flagellar pocket, membranes of the two surface domains are physically partitioned by a unique structure, a collar that may be a physical barrier to diffusion of some membrane macromolecules (Tetley, 1986). Taken together with the potential for molecules to be actively transported into the flagellum by specific association with IFT partner proteins by the directed movement of the IFT particles themselves, this could provide a mechanism for promoting differential raft composition in different plasma membrane macrodomains of trypanosomes. Purification and identification of the components present in the flagellar membrane rafts is under way

and should provide more direct insights into the molecular mechanisms and pathways localized to, and controlled from, the trypanosome flagellum.

Materials and Methods

Cells lines and culture
The procyclic form used was Treu 927. The bloodstream form used was Lister 427. Procyclic forms were cultured and harvested as previously described (Tyler et al., 1997). Briefly, cells were grown in SDM-79 medium at 27°C and harvested during logarithmic growth ~48 hours after passage at a concentration of between 10^6 and 10^7 trypanosomes per ml. Bloodstream forms were grown in HMI-9 supplemented with 10% fetal calf serum at 37°C, 4% CO_2.

Antibodies and western blotting
Antisera specific for calflagin, Tb24 and GP63 were generated as previously described for the Hsp70 antiserum (Olson et al., 1994). Partial coding sequences of calflagin Tb24 and GP63 were amplified from genomic DNA, expressed in pET23b (Novagen; Madison, WI) with hexahistidine tags and purified over nickel columns to provide the inoculum. On western blots, antiserum to Tb24 reacts with calflagin paralogs Tb24, Tb44 and Tb17 (>95%), which are almost identical in sequence (data not shown). In the procyclic form, Tb24 is the dominant paralog and appears as a singlet; in the bloodstream form, Tb17 is expressed at roughly similar levels to Tb24 and the two proteins appear as a doublet (Fridberg et al., 2008). Antiserum specific for galactocerebroside was purchased from Sigma-Aldrich (St Louis, MO) and EP procyclin (Cedar Lane Laboratories, Ontario, Canada). PFR-specific (L13D6) (Kohl et al., 1999) and WCB210-specific (WCB-1) (Woods et al., 1992) monoclonal antibodies were kind gifts from K. Gull. SDS-PAGE, western blotting and detection using enhance chemiluminescence (Amersham) was performed as previously described (Tyler et al., 1997). For immunofluorescence microscopy, monoclonal antibody supernatants were used undiluted; EP procyclin and galactocerebroside antibodies were used at 1:500; Tb24 and GP63 antisera were used at 1:250; and Hsp70 antiserum was used at 1:1000. For western blotting, monoclonal antibody supernatants were used at 1:100; EP procyclin at 1:5000; Tb24 and GP63 at 1:2000; and Hsp70 at 1:10,000. Densitometry of scanned bands was measured using Kodak 1D 3.5.4 software to generate standard curves of specific protein signals in whole cell and flagellar lysates for each of the markers analyzed. These densities were then compared and normalized to procyclin (assigned a value of 1.0) to generate the ratios used presented in the flagellar enrichment table.

Purification of flagella
Flagella were purified essentially as previously described (da Cunha e Silva et al., 1989). Briefly, 10^{10} trypanosomes were washed and resuspended in buffer A (25 mM tricine, 0.2 mM EDTA, 5 mM $MgCl_2$, 12 mM β-mercaptoethanol, pH 7.0). The cells were then sonicated with two pulses for 5 seconds each at 20 kHz. Cell bodies were then removed by a series of three centrifugations at 420 ***g***. The resulting supernatant was spun at 6900 ***g*** and the pellet was resuspended in 0.32 M sucrose in buffer A. This solution was then layered onto 0.61 M sucrose in buffer A, and spun at 1200 ***g***. The 0.32 M sucrose fraction was removed and the preceding step was repeated for a total of three 1200 ***g*** spins. The final 0.32 M fraction was centrifuged at 10,000 ***g*** and the resulting pellet containing crude flagella was resuspended in 0.32 M sucrose. This flagellar solution was layered onto a 1.11 M, 1.52 M, 1.90 M step gradient of sucrose in buffer A and centrifuged at 130,000 ***g*** for 4 hours. Fractions were collected and the purified flagellar fraction was examined by immunofluorescence microscopy and western blotting compared with whole cell controls. Western blots were loaded as dilution series based on absolute numbers of parasites and flagella.

Microscopy
For fluorescence microscopy, washed trypanosomes were settled onto poly-L-lysine-coated slides and fixed with 2.5% freshly prepared paraformaldehyde for 5 minutes at room temperature. A saturated solution of filipin (emission wavelength of sterol complexed filipin is 480 nm) in PBS was applied to the fixed cells for 5 minutes and the slides were washed three times with PBS. For immunofluorescence microscopy, samples were fixed with 4% paraformaldehyde for 5 minutes. For direct fluorescence microscopy of GM1 by FITC-conjugated cholera toxin and for indirect immunofluorescence microscopy of galactocerebroside, conjugate or antibody was applied directly to unpermeabilized cells. Indirect immunofluorescence microscopy using antiserum specific to the internal flagellar membrane protein calflagin Tb24 required permeabilization with 0.1% saponin, and saponin was added to the diluted antibody and to the PBS for all washing steps. Mounting and visualization of the slides was performed as described (Tyler and Engman, 2000). For two-photon microscopy, procyclic trypanosomes were washed and resuspended to 10^8 cells per ml in PBS containing 1 μM laurdan, then settled onto poly-L-lysine coated slides for 10 minutes at room temperature. Images were captured at 446 nm and 499 nm and GP ratios derived as previously described (Dietrich et al., 2001a). Four fields of cells were captured and imaged ratiometrically. From these, 10 trypanosomes were imaged individually and two representative cells were selected, one with a single flagellum and one with two flagella. Cells were selected for presentation according to which

gave the best resolution of attached and free flagella for the higher resolution fluorescence intensity images. For scanning electron microscopy (SEM), samples were first immobilized on poly-l-lysine coated coverslips, washed five times with pre-chilled (4°C) Triton X-100 in PBS at 4°C. Samples were then processed for SEM using standard methods. Briefly, the coverslips bearing extracted cells were critical point-dried, placed onto specimen mounts using carbon tape and coated with gold in a Bal-Tec MED 020 with single 'cool' sputter device. All samples were examined and photographed in a Hitachi S4500-II cold-field emission scanning electron microscope.

Detergent-resistant membrane assays
To test temperature-dependent resistance to detergent, 10^8 *T. brucei* procyclic forms were washed three time in PBS, and extracted in 1 ml of 1% Triton X-100 in PBS at 4°C or 37°C for 10 minutes and centrifuged. To test buoyancy, 10^8 cells were similarly washed and then treated essentially as previously described (Bagnat et al., 2000). Briefly, cells were extracted in 1% Triton X-100 in TBS at 4°C, which was then adjusted to 40% OptiPrep by mixing with a 60% Optiprep stock. Extracts were then floated through an OptiPrep (40%, 30%, 0%) step gradient under centrifugation for 5 hours at 35,000 rpm and then for 12 hours at 25,000 rpm using a Beckman SW55Ti rotor. Sequential fractions were collected and 25 μl of each fraction was analyzed by western blotting with the same antibodies used in both assays.

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