De novo sphingolipid synthesis is required for the exit of glycosylphosphatidylinositol (GPI)-anchored membrane proteins from the endoplasmic reticulum in yeast. Using a pharmacological approach, we test the generality of this phenomenon by analyzing the transport of GPI-anchored cargo in widely divergent eukaryotic systems represented by African trypanosomes and HeLa cells. Myriocin, which blocks the first step of sphingolipid synthesis (serine + palmitate → 3-ketodihydrosphingosine), inhibited the growth of cultured bloodstream parasites, and growth was rescued with exogenous 3-ketodihydrosphingosine. Myriocin also blocked metabolic incorporation of [3H]serine into base-resistant sphingolipids. Biochemical analyses indicate that the radiolabeled lipids are not sphingomyelin or inositol phosphoceramide, suggesting that bloodstream trypanosomes synthesize novel sphingolipids. Inhibition of de novo sphingolipid synthesis with myriocin had no adverse effect on either general secretory trafficking or GPI-dependent trafficking in trypanosomes, and similar results were obtained with HeLa cells. A mild effect on endocytosis was seen for bloodstream trypanosomes after prolonged incubation with myriocin. These results indicate that de novo synthesis of sphingolipids is not a general requirement for secretory trafficking in eukaryotic cells. However, in contrast to the closely related kinetoplastid Leishmania major, de novo sphingolipid synthesis is essential for the viability of bloodstream-stage African trypanosomes.
Furthermore, temperature-sensitive yeast mutants, defective in ceramide and sphingolipid synthesis, also show defects in the trafficking of GPI-anchored molecules, as well as in endocytosis and stress responses (50, 59, 65). These findings have led to the general assumption that ongoing sphingolipid synthesis is required for efficient exit of GPI-anchored proteins from the ER, although this phenomenon has not been validated for eukaryotic cells other than yeast.

The first and rate-limiting step in sphingolipid biosynthesis (Fig. 1A), occurring in the ER, is the condensation of L-serine and palmitoyl coenzyme A (palmitoyl-CoA) into 3-ketodihydrosphingosine (3-KDS), catalyzed by the enzyme serine palmitoyltransferase (SPT). 3-KDS undergoes further modification and is N acylated to generate ceramide, which, following transport to the Golgi apparatus, is converted into higher-order sphingolipids by addition of various polar head groups (29). Plants typically synthesize neutral glycosphingolipids, while mammalian cells produce sphingomyelin, as well as neutral and acidic glycosphingolipids (43). Among the lower eukaryotes Plasmodium spp. also make sphingomyelin (28), while many others, including fungi (38), Toxoplasma gondii (58), and the kinetoplastids Leishmania (35) and Trypanosoma cruzi (54), synthesize inositol phosphorylceramide (IPC). Surprisingly, in Leishmania major, synthesis of sphingolipids is not necessary for viability or virulence (17, 68), provided the growth medium is supplemented with ethanolamine (67). Due to their close phylogenetic relationship, it has been casually assumed that all kinetoplastids, including African trypanosomes, synthesize IPC (18, 19, 32), and this has recently been confirmed for procyclic-stage Trypanosoma brucei (27).

In this study, we investigate these assumptions by using myriocin to block sphingoid base synthesis in bloodstream-stage trypanosomes and mammalian cells. This strategy allows us to directly investigate whether de novo sphingolipid synthesis is required for trypanosome viability and for VSG trafficking. In agreement with previous studies on yeast and mammalian cells (30, 51), and in contrast to Leishmania, we find that de novo synthesis of sphingoid base is essential for the viability of African trypanosomes. However, disruption of sphingolipid catabolism has no effect on the trafficking of GPI-anchored proteins in trypanosomes or in human HeLa cells. Indeed, other than ultimate lethality, the only significant effect we observe in bloodstream trypanosomes is a general depression of endocytosis following prolonged inhibition of sphingoid base synthesis. Finally, metabolic radiolabeling studies with [3H]serine indicate that bloodstream trypanosomes synthesize a novel sphingolipid that is neither IPC, as in Leishmania, nor sphingomyelin, as in mammalian cells. Thus, African trypano-
somes differ significantly from related ketoplastids in the biosynthesis and utilization of sphingolipids.

**MATERIALS AND METHODS**

**Compounds and reagents.** Myriocin (Sigma, St. Louis, MO) was prepared as a 1 mg/ml stock solution in methanol. Ethanolamine in liquid form, tunicamycin, brevetoxin A (BFA), bovine liver phosphatidylinositol (PI), and bovine brain sphingomyelin were also obtained from Sigma. Both d18:1/8:0 ceramide from Avanti (Alabaster, AL) and 3-KDS from Matreya (Pleasant Gap, PA) were prepared as 10 mM stock solutions in chloroform-methanol (1:1, vol/vol). Glucosylceramide was obtained from Avanti. Staphylococcus aureus sphingomyelinase was from Sigma, and S. aureus PI-specific phospholipase C (PI-PLC) was a generous gift from Vicky Stevens (Emory University).

**Metabolic labeling and analysis of radiolabeled lipids.** Cultured procyclic and bloodstream-stage Trypanosoma brucei brucei strain 427 parasites were grown and maintained as described in references 2 and 5. Monolayer cultures of HeLa cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Bloodstream-stage trypanosomes were harvested at late-log phase (0.5 × 10⁷ to 1 × 10⁸/ml) and resuspended (5 × 10⁶ cells/ml) in TMB medium (48 mM NaHCO₃, 48 mM Na₂CO₃, pH 8.2) and incubated at 37°C. Alkaline phosphatase activity was measured in the presence of 50 µM Myriocin (Sigma, St. Louis, MO) at 50 µg/ml in methanol, and 0.1 N KOH in methanol (pH 7.4)–10 mM MgCl₂–0.05% (wt/vol) Triton X-100 with or without 1 U/ft h of Pseudomonas fluorescens phosphatase C (PI-PLC) was a generous gift from Vicky Stevens (Emory University).

**Flow cytometry and immunofluorescence microscopy.** For flow cytometry following uptake of FITC-TL, cells were fixed with PBS containing 2% formaldehyde for 15 to 30 min on ice, centrifuged, and resuspended in 1 ml PBS–5% FBS. Labeled cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA) using CellQuest software. For immunofluorescence microscopy, trypanosomes were smeared on glass slides, fixed, permeablized, and stained as previously described (2). Specific staining was determined by capturing Alexa 488- and Alexa 633-conjugated secondary antibodies (Molecular Probes, Seattle, WA), and 500 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) was included to visualize nuclear and kinetoplast DNA. Cells were analyzed on a motorized Zeiss Axioplan II with a rear-mounted excitation filter wheel, a triple-pass (DAPI/FITC/Texas Red) emission cube, and differential interference contrast (DIC) optics. Serial 0.2-µm image Z-stacks at a magnification of ×100 (PlanApo; numerical aperture, 1.4) were collected with a Zeiss AxioCam black-and-white coupled device camera. Fluorescence images were deconvolved using a constrained iterative algorithm, pseudocolored, and merged using OpenLabs (version 4.1) software (Improvement, Inc., Lexington, MA).

**RESULTS**

Sphingoid base synthesis is essential for the growth of trypanosomes. Studies of both yeast and mammalian cells have shown that ongoing sphingolipid synthesis is essential for cell proliferation (14). However, recent work with the kinetoplastid parasite Leishmania demonstrate that log-phase promastigote-
FIG. 2. Myriocin inhibition of de novo sphingolipid synthesis in trypanosomes. (A) Bloodstream-stage trypanosomes grown for 4 h in the presence (bottom) or absence (top) of 200 nM myriocin were pulse-radiolabeled with 50 µCi/ml of [3H]serine for 4 h. Base-resistant lipids were extracted as described in Materials and Methods and analyzed by TLC. The mobilities of sphingomyelin (Rf, 0.12) and ceramide (Rf, 0.89) standards were determined by iodine staining (arrowheads, bottom panel only). (B) Saponified [3H]serine-labeled lipids were subjected to mock treatment (top) or acid methanolyis (bottom) (2 h) as described in Materials and Methods and then analyzed by TLC. Ceramide was added as an internal marker/substrate prior to hydrolysis. The mobilities of unhydrolyzed ceramide (open arrowhead) (Rf, 0.25), and the sphingamine product (solid arrowhead) (Rf, 0.15) are indicated. (C) Base-resistant [3H]serine-labeled lipids from untreated bloodstream trypanosomes were either mock treated (not shown) or digested with either sphingomyelinase (S-Mase) (top) or bacterial PI-PLC (bottom) with internal sphingomyelin and PI controls, respectively. Samples were prepared and analyzed as for panel A. Solid arrowheads indicate the respective ceramide (top) (Rf, 0.9) and diacylglycerol (bottom) (Rf, 0.8) products. Note that the solvent systems used for panels A and C are different from that for panel B.

Stage mutants lacking SPT activity fail to synthesize ceramides and sphingolipids yet show no marked growth defect compared to wild-type cells (17, 68), provided that they are supplemented with exogenous ethanolamine (67). In light of this key difference between Leishmania and other eukaryotes, we wanted to investigate the requirements for de novo sphingolipid synthesis for the growth of the closely related kinetoplastid T. brucei. Treatment of bloodstream-stage trypanosomes with myriocin, an inhibitor of sphingolipid synthesis (44), resulted in a dose-dependent inhibition of growth with a 50% inhibitory concentration of ~100 to 150 nM (Fig. 1B). Proyclic-stage trypanosomes showed a similar myriocin-induced growth inhibition, albeit with a higher 50% inhibitory concentration (~800 nM) (data not shown).

We wanted to determine if myriocin-induced growth inhibition was due to a block at a specific cell cycle checkpoint. Most trypanosomes in an asynchronously growing population are in interphase and contain a single kinetoplast (mitochondrial genome) and nucleus (1k/1n). During cell division, the kinetoplast divides, first generating intermediates with two kinetoplasts and a single nucleus (2k/1n); this is followed by nuclear division to produce cells with two kinetoplasts and two nuclei (2k/2n). Finally, cytokinesis results in two interphase daughter cells (26). Microscopic examination of myriocin-treated bloodstream cells stained for DNA with DAPI showed a decrease in the proportion of interphase cells (1k/1n) with a concomitant increase in the products of aberrant cell division (1k/0n, 1k/2n, and >2k/>2n) compared to untreated control cells (Fig. 1C). These results indicate that myriocin treatment of bloodstream trypanosomes leads to arrest of cell growth at all stages of the cell cycle.

The first step in sphingolipid biosynthesis, catalyzed by SPT, produces the sphingoid base 3-KDS, which is converted to sphinganine (dihydrosphingosine) and then N acylated and desaturated to generate ceramide, the immediate precursor for all higher-order sphingolipids (Fig. 1A). Addition of 3-KDS completely rescued the myriocin-induced growth inhibition (Fig. 1D), indicating that the defect is not due to general toxicity but rather is specifically due to inhibition of ongoing sphingoid base synthesis by SPT. In contrast, addition of ceramide failed to rescue cell growth, likely due to inefficient uptake and/or delivery of exogenous ceramide to the Golgi apparatus, consistent with previous observations of mammalian cells and Leishmania (30, 68). Growth and differentiation defects observed in Leishmania promastigote mutants lacking either SPT or sphingosine-1-phosphate lyase (SPL) activity can be rescued by exogenous ethanolamine (67). This issue is discussed in detail below, but briefly, the basis for the phenomenon is believed to be acquisition of essential ethanolamine via SPL activity (Fig. 1A). Loss of sphingoid base synthesis leads to ethanolamine auxotrophy by depletion of sphingosine-1-phosphate. However, African trypanosomes appear to differ from Leishmania in this respect; addition of ethanolamine was unable to rescue growth for either procyclic (data not shown) or bloodstream (Fig. 1D) cells.

De novo sphingolipid synthesis is inhibited by myriocin. In order to confirm that myriocin-induced growth inhibition is due to a block in de novo sphingoid base synthesis, we metabolically radiolabeled bloodstream-form trypanosomes with [3H]serine and extracted base-resistant cellular lipids for analysis by thin layer chromatography (Fig. 2A). Myriocin-treated cells showed a decrease in total protein synthesis (trichloroacetic acid-insoluble incorporation of [3H]serine) of ~25% compared to untreated control cells, indicating that the compound had minimal effect on general metabolic function (data not shown). In control, untreated cells (Fig. 2A, top panel), a single major radiolabeled lipid (designated X) was detected, as well as several variable minor peaks (Fig. 2, compare panels A
and C), and myriocin treatment completely blocked incorporation into all of these species (bottom panel). The combined base resistance and myriocin sensitivity of all the labeled peaks indicate that they are sphingolipids. This was verified by acid methanolysis, which released radiolabeled products that comigrated with sphingosine and sphinganine in TLC analysis (Fig. 2B). Sphingolipid X routinely migrated slightly ahead of the sphingomyelin marker in our standard solvent system (CMW, 2B). Sphingolipid X was sensitive to hydrolysis by sphingomyelinase (Fig. 2C, bottom), indicating that it is not IPC. These results indicate that myriocin effectively blocks de novo synthesis of sphingoid bases, and they further suggest that bloodstream-stage African trypanosomes may synthesize a novel higher-order sphingolipid.

**De novo sphingolipid synthesis is not required for forward transport in trypanosomes.** Ongoing sphingolipid synthesis is required for efficient ER exit of GPI-anchored proteins in yeast (33, 59). We wanted to examine whether there is a similar requirement for sphingolipids in the forward transport of GPI-anchored proteins in bloodstream-form trypanosomes. First, we characterized the forward transport of a non-GPI-anchored protein in order to determine if myriocin treatment had any globally deleterious effects on transport. We have previously characterized the kinetics of protein secretion in bloodstream-form trypanosomes. For example, the time points used in this assay fall in the linear range for BiP transport in bloodstream cells (60). (A) Bloodstream-form trypanosomes constitutively expressing the soluble secretory reporter BiPN were cultured for 14 h in the presence (+) or absence (−) of 200 nM myriocin (myr.). Cells were pulse-radiolabeled (15 min) with [35S]Met-Cys and then chased for the indicated times in the continued presence or absence of the inhibitor. The BiPN reporter was immunoprecipitated from cell and medium fractions and analyzed by SDS-PAGE and phosphorimaging. The mobilities of endogenous BiP and VSG, and of the BiPN reporter, are indicated on the left. All lanes contain 5 × 10^9 cell equivalents. Note that the time points used in this assay fall in the linear range for BiP transport in bloodstream cells (60). (B) Bloodstream cells were cultured for 14 h in the presence or absence of 200 nM myriocin. Cells were then pulse-radiolabeled (3 min) with [35S]Met-Cys and chased in the continued presence (myr) or absence (control [cont]) of the inhibitor. At the indicated times, samples were washed and subjected to hypotonic lysis as described in Materials and Methods, and VSG polyepitopes were immunoprecipitated from the released fractions. VSG was also immunoprecipitated from total-cell fractions at the beginning and end of the chase period (T0 and T30), and all samples were analyzed as for panel A. The recovery of released VSG as a fraction of total VSG (average of T0 and T30) is given for each lane. All lanes contain 2 × 10^9 cell equivalents.

**Sphingolipid synthesis is required for endocytosis in trypanosomes.** Temperature-sensitive SPT mutants in yeast show a defect in endocytosis (65), but the connection between ongoing synthesis of sphingoid bases and endocytosis remains to be fully defined. Consequently, we investigated whether myriocin treatment of bloodstream trypanosomes would affect endocytosis. Since myriocin treatment led to an observable decrease in cell motility and a fattening of the cell shape, we first visualized the localization of two key organelles in the secretory/endocytic pathway, the ER and the lysosome, by immunofluorescence microscopy (Fig. 4A to H). In both control and myriocin-treated cells, staining with anti-BiP (Fig. 4B and F) revealed a fine reticulated network characteristic of the ER, and staining with an antibody to the lysosomal membrane protein p67 (Fig. 4C and G) revealed a vesicular compartment...
located properly between the nucleus and the kinetoplast. However, compared to control cells, the majority of myriocin-treated cells showed a distorted and enlarged p67 compartment (Fig. 4G and H). To more carefully investigate the effects of myriocin on trypanosomal ultrastructure, we performed transmission electron microscopy using Tf-gold as a lysosomal marker. Cells were preloaded with Tf-gold to label the terminal lysosome and were then treated with myriocin. In untreated control cells, lysosomes typically had diameters of 300 nm (Fig. 5A, B, E, and F). In contrast, myriocin-treated cells typically exhibited lysosomes with diameters of >600 nm (Fig. 5C, D, G, and H). No other obvious ultrastructural abnormalities were observed in myriocin-treated cells. These results confirm that lysosomal morphology is altered, and they imply that ongoing sphingolipid synthesis may play a role in membrane trafficking events involved in the formation and/or maintenance of the lysosomal compartment.

We used the uptake of FITC-TL to assess receptor-mediated endocytosis (Fig. 4I to P). Tomato lectin binds poly-N-acetyllactosamine-containing N-glycans on membrane glycoproteins in the flagellar pocket of bloodstream trypanosomes (46). Many of these glycoproteins are internalized into the endocytic pathway, and TL eventually accumulates in the terminal lysosomal compartment (2). Microscopic analysis of TL uptake failed to detect any obvious defects in lysosomal delivery; the majority of internalized TL colocalized completely with the resident lysosomal protein p67 in both untreated and myriocin-treated cells (Fig. 4L and P). The distribution of internalized TL is more diffuse in myriocin-treated cells, presumably due to the enlarged lysosomal compartment. Next, we employed a quantitative assay using flow cytometry to measure the uptake of FITC-TL. Bloodstream cells pretreated for 4 h with myriocin were similar to untreated cells in their ability to take up TL (Fig. 6A). However, after a 14-h myriocin pretreatment, cells showed a significant decrease in the uptake of TL compared to untreated cells (Fig. 6B). Collectively these results suggest that sphingolipids play an important role in endocytosis.

**Sphingolipid synthesis and intracellular transport in mammalian cells.** In contrast to the situation for yeast (50, 59, 65), ER exit and intracellular transport of GPI-anchored proteins in trypanosomes apparently does not require ongoing de novo synthesis of sphingolipids. To further investigate this issue in a broader biological context, we assessed the effects of myriocin
on sphingolipid synthesis and GPI transport in mammalian HeLa cells. First, we determined that myriocin indeed blocks the incorporation of \(^3\)Hserine into sphingomyelin and glucosylceramide (data not shown), confirming its inhibitory activity in HeLa cells. Next, we measured the transport of a GPI-anchored influenza virus hemagglutinin reporter (HA-GPI) by pulse-chase analysis in transiently transfected HeLa cells (Fig. 7). In this assay, the arrival of the reporter at the cell surface is measured by susceptibility to discrete cleavage by exogenously added trypsin to yield a diagnostic HA2 fragment.

FIG. 5. Ultrastructure of myriocin-treated trypanosomes. Cultured bloodstream cells were first pulse-loaded (2 h, 37°C) with colloidal gold (5 nm) coupled to bovine holotransferrin to label the terminal lysosome. Cells were then cultured in the absence (A and B) or presence (C and D) of myriocin (200 nM) for 14 h and processed as described for electron microscopy. (A to D) Representative cell sections. Bar, 1.0 μm. White arrowheads indicate lysosomes containing colloidal gold particles. cont, control; myr, myriocin. (E to H) Corresponding images in the lysosomal region taken at a higher magnification. Bar, 0.2 μm.

FIG. 6. Myriocin affects endocytic transport in trypanosomes. Bloodstream cells were cultured for 4 h (A) or 14 h (B) in the presence or absence of 200 nM myriocin. Cells were then incubated at 4°C or 37°C for 1 h in a medium containing 20 μg/ml FITC-tomato lectin, washed, and analyzed by flow cytometry. Uptake is presented as relative fluorescent intensity, in arbitrary units. Values are means ± standard deviations from three independent experiments.

FIG. 7. Transport of HA-GPI to the plasma membranes of HeLa cells is unaffected by myriocin treatment. (A) Schematic representation of HA-GPI. The protein is synthesized as a GPI-anchored polypeptide (HA0), corresponding to the ectodomain of influenza virus hemagglutinin protein, containing a disulfide bond as shown. Upon trypsin treatment, HA0 yields two disulfide-linked fragments, HA2 and HA1. (B) HeLa cells expressing HA-GPI were preincubated for 30 min with myriocin (125 μM) or BFA (5 μg/ml) as indicated and were then pulse-labeled with 200 μCi/ml \(^{35}\)S]methionine-cysteine for 20 min. The cells were chased for 2 h in the presence of drugs before being trypsinized to convert cell surface HA-GPI molecules into HA2 and HA1. The extent of conversion of radiolabeled protein to HA2 and HA1 was determined after immunoprecipitation and SDS-PAGE. A scan of a typical fluorography is presented. No HA2 is seen in the BFA-treated cells, whereas radiolabeled HA2 is readily observed in both control (−) and myriocin-treated cells.
At the end of the chase period, the reporter is as sensitive to trypsin in the presence of myriocin (Fig. 7B, lane 3) as it is in control cells (lane 1), indicating that transport is unimpeded when de novo sphingolipid synthesis is blocked. In control cells treated with brefeldin A, an inhibitor of vesicular secretory trafficking (21), HA-GPI cleavage is completely blocked (Fig. 7B, lane 2), confirming the validity of the assay. Finally, we measured the effect of myriocin treatment on the trafficking of \([^{3}H]\)mannose-labeled free GPI, a process that we have previously demonstrated to be dependent on vesicular trafficking (9). In this assay, the arrival of free GPI of a known structure (H8) (Fig. 8A) at the cell surface is determined by susceptibility to oxidation by exogenously added metaperiodate, which is impermeant to intact biological membranes. Following an extended chase period, periodate treatment converts a substantial portion of \([^{3}H]\)mannose-labeled GPI H8 to the oxidized H8* form (Fig. 8B). More-precise kinetic analysis indicates that myriocin treatment has no effect on the susceptibility of H8 to oxidation throughout the linear range for transport to the cell surface, while brefeldin A treatment completely abrogates periodate sensitivity (Fig. 8C). Collectively these data indicate that in mammalian cells, as in bloodstream trypanosomes, de novo synthesis of sphingolipids is not necessary for the transport of GPI structures at any stage of the secretory pathway.

**DISCUSSION**

The most current models of GPI-dependent protein sorting are based on the lateral association of GPI-anchored proteins (and free GPI) with ceramide or sphingolipid/sterol-rich microdomains (lipid rafts), which are proposed to act as selective platforms for vesicle budding at different sites along the secretory and endocytic pathways (56, 57). Consistent with these models, ceramide synthesis is required for both efficient membrane association and ER exit of GPI-anchored proteins in yeast (3, 59). We have investigated the universality of these concepts for the trafficking of GPI-anchored proteins and free GPI in two systems representing the broad sweep of eukaryotic evolution, African trypanosomes and nonpolarized human HeLa cells. In contrast to the prevailing view on the role of sphingolipids in secretory trafficking, we find for both systems that ER exit and transport to the cell surface of GPI-anchored proteins occur in the absence of ongoing sphingolipid synthesis. In addition, we have found a similar lack of effect on secretory trafficking in procyclic insect-stage trypanosomes (data not shown). We cannot rule out the possibility that ongoing transport is supported by the pool of ceramide and sphingolipid synthesized prior to treatment with myriocin, but nevertheless, de novo synthesis of sphingoid bases is clearly not required.

It should be noted that similar studies using both pharmacological and genetic strategies to block sphingolipid synthesis have been performed on *Leishmania*. Myriocin had no effect on overall ER-to-plasma membrane transport or on the incorporation into surface DRMs of free GPI or the major surface GPI-anchored protein GP63 (52). Similarly, genetic ablation of a subunit of SPT, which prevents sphingoid base synthesis, did not perturb the overall transport of GP63 or its association with plasma membrane DRMs (17, 68). Denny and coworkers performed a more detailed kinetic assay that revealed a decrease in the rate of association of newly synthesized GP63 with DRMs, suggesting a role for lipid rafts in ER exit (17); however, this finding could not be reproduced by others (66).
Thus, the results for *Leishmania* are contradictory. So is the evidence for association of GPI-anchored proteins with DRMs in African trypanosomes. Nolan et al. reported that VSG does not significantly associate with DRMs, while Denny et al. found such association by using a lower detergent concentration (16, 47). Consequently, the existence of DRMs in trypanosomes must be more systemically investigated before an assessment of the role of lipid rafts in VSG and GPI trafficking in trypanosomes can be made. Whatever the status of DRMs in *T. brucei*, or the situation in *Leishmania*, our results for African trypanosomes and HeLa cells clearly establish that ongoing sphingolipid synthesis is not a universal requirement for forward transport of GPI-anchored proteins in eukaryotic cells.

On the other hand, we do find a general defect in endocytosis for African trypanosomes when sphingolipid synthesis is blocked. Typically, newly synthesized sphingolipids traffic as ceramide from the ER to the Golgi apparatus, where higher-order modifications occur, and then on to the plasma membrane (64). Consequently, it seems unlikely that inhibition of sphingoid base synthesis would lead to rapid depletion of sphingolipids at the plasma membrane. Consistent with this notion, pulse-chase analysis indicates only minor turnover of [3H]serine-labeled sphingolipid X over a 14-h period in both control and myriocin-treated cells (~10% [data not shown]). Thus, our results argue that a prolonged myriocin treatment is required before defects in endocytosis are detected. This is in contrast to the situation for yeast, where a sphingoid base-mediated signaling pathway rapidly regulates endocytosis through protein phosphorylation (25, 65). Although we cannot rule out secondary effects due to prolonged inhibition, the time frame of our results for trypanosomes is more consistent with a general role for sphingolipids in cellular membranes.

One surprising implication of our studies is that bloodstream-stage African trypanosomes may synthesize unusual sphingolipids. After the production of ceramide in the ER, eukaryotes go on to synthesize a diverse array of higher-order sphingolipids. Many of the lower eukaryotes, including fungi (38) and the kinetoplastids *Leishmania* (35) and *Trypanosoma cruzi* (54), synthesize IPC, and it has been generally assumed that African trypanosomes likewise synthesize IPC (18, 19, 32). In fact, earlier compositional analyses of African trypanosomes found no evidence for IPC; rather, sphingomyelin was detected at fairly high levels in bloodstream-stage parasites (49). Although the presence of IPC in procyclic trypanosomes has recently been confirmed (27), our results suggest that neither sphingomyelin nor IPC is made by bloodstream-form *T. brucei* in our biosynthetic assays, and they raise the possibility of developmental regulation of sphingolipid synthesis. The nature of the major sphingolipid detected in our biosynthetic assays remains uncertain. The close but imprecise migration of sphingolipid X with authentic sphingomyelin in TLC analyses could account for the previous identification of sphingomyelin in compositional analyses. Alternatively, sphingolipid X may be related to one of the recently identified neutral glycosphingolipids in *T. brucei* (61). Although further structural work is needed to identify this lipid, our analyses suggest that African trypanosomes synthesize novel sphingolipids relative to their kinetoplastid brethren.

African trypanosomes also differ from *Leishmania* in two additional ways: growth dependence on sphingoid base synthesis and rescue of sphingoid base deficiency with exogenous ethanolamine. *SPT*-null *Leishmania* parasites replicate well as log-phase promastigotes but differentiate poorly to infectious stationary-phase metacyclic promastigotes (17, 68). Thus, de novo synthesis of sphingoid bases is apparently not necessary for replicative growth in vitro but is necessary for differentiation. However, Zhang and coworkers have recently found that *SPT* is absolutely essential for growth under conditions that are strictly limiting for exogenous phospholipids (67). Interestingly, the defect in differentiation can be complemented by exogenous ethanolamine, and furthermore, the same phenotype, including rescue by ethanolamine, can be duplicated in an *SPL*-null mutant. The likely explanation for these related phenotypes is that cleavage of sphingosine-1-phosphate, a downstream product of SPT activity, generates essential ethanolamine phosphate (EtN-P) for anabolic purposes such as phosphatidyethanolamine synthesis via the Kennedy pathway (reaction 1, EtN-P + CTP → CDP:Etn + PPi; reaction 2, CDP:Etn + DAG → PE + CMP [36, 37]). A major implication of this finding is that *Leishmania* is not dependent on de novo synthesis of higher-order sphingolipids, e.g., IPC, for growth, viability and virulence.

Unlike *Leishmania*, however, African trypanosomes are critically dependent on de novo synthesis of sphingolipids. The trivial explanation for our major finding, that myriocin toxicity is due to nonspecific side effects, is ruled out because the immediate product of SPT activity, 3-KDS, completely rescues cell viability. Although *T. brucei* has an orthologue (Tb927.6.3630) of the *Leishmania* SPL gene, our data suggest that the deleterious effect of blocking sphingoid base synthesis is not due to ethanolamine deficiency either, since exogenous ethanolamine is unable to complement growth. Nor can this be due to a deficiency in uptake, since exogenous ethanolamine is readily transported and incorporated into GPI and phospholipids (42, 53). The failure of exogenous ethanolamine to rescue myriocin toxicity does not rule out the possibility that trypanosomes also acquire ethanolamine via the SPL pathway, but clearly sphingoid base synthesis is required for some other purpose. The obvious candidates are ceramide and higher-order sphingolipids, presumably as important structural components of cell membranes. Precisely what these higher-order sphingolipids are in African trypanosomes, and how they relate to the lipids detected in our biosynthetic assays, will require careful structural characterization of steady-state lipids in both life cycle stages of these unusual parasites.

ACKNOWLEDGMENTS

We are indebted to Steve Beverley and Kai Zhang for thoughtful discussions and sharing of unpublished data. We thank Debbie Brown (SUNY, Stony Brook, NY) for suggesting the HA-GPI trafficking assay, Saulius Vainauskas for the HA-GPI construct (originally obtained from Judy White, University of Virginia), Niki Baumann for input on the initial experiments with GPI trafficking in HeLa cells, Henna Ohvo-Rekila for assistance with sphingolipid analysis, Dawn Ransom for the initial myriocin experiment on trypanosomes, and Kevin Schwartz for technical assistance with parasites. We also thank the staff at the UW Medical School Electron Microscopy Facility, particularly Ben August, for technical assistance and helpful suggestions in performing electron microscopy.

This work was supported by National Institutes of Health grants AI35739 (to J.D.B.) and GM55427 (to A.K.M.) and the Cornell/
Rockefeller/Sloan-Kettering Tri-Institutional Training Program in Chemical Biology (to S.S.).

REFERENCES

1. Agosti, R. A., S. C. Coito, O. Campetella, A. C. C. Frasch, and R. M. de Lederkremer. 1998. Structure of the glycosylphosphatidylinositol-anchor of the trans-sialidase from Trypanosoma cruzi metacyclic trypomastigote forms. Mol. Biochem. Parasitol. 97:123–131.

2. Alexander, D. L., K. J. Schwartz, A. E. Balber, and J. D. Bangs. 2002. Developmentally regulated trafficking of the boomsomal membrane protein p67 in Trypanosoma brucei. J. Cell Sci. 115:3235–3263.

3. Bagnat, M., S. Kera¨nen, A. Shevchenko, A. Shevchenko, and K. Simons. 2003. Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. Proc. Natl. Acad. Sci. USA 98:3254–3259.

4. Bangs, J. D., N. Andrews, G. W. Hart, and P. T. Engh. 1986. Posttranslational modification and intracellular transport of a trypanosome variant surface glycoprotein. J. Cell Biol. 103:255–263.

5. Bangs, J. D., L. Uyetake, D. A. Ransom, and J. L. Roggy. 1996. A soluble secretory reporter system in Trypanosoma brucei: studies on endoplasmic reticulum targeting. J. Biol. Chem. 271:18367–18393.

6. Bangs, J. D., D. Herald, J. L. Krakow, G. W. Hart, and P. T. Engh. 1985. Rapid processing of gp67 into boomsomal membranes is a regulator of intracellular levels of surface glycoprotein. Proc. Natl. Acad. Sci. USA 82:3207–3211.

7. Bangs, J. D., D. M. Ransom, M. A. McDowell, and E. M. Brouch. 1997. Expression of bloodstream variant surface glycoproteins in procyclic stage Trypanosoma brucei: role of GPI anchors in secretion. EMBO J. 16:4285–4294.

8. Bangs, J. D., L. Uyetake, M. J. Brickman, A. E. Balber, and J. C. Boothroyd. 1993. Molecular cloning and cellular localization of a BIP homologue in Trypanosoma brucei: differential regulation of ER retention signals in a lower eukaryote. J. Cell Sci. 105:1011–1113.

9. Baumann, N. A., J. Vidugiriene, C. A. Machamer, and A. K. Menon. 2000. Cell surface display and intracellular trafficking of free glycosylphosphatidylinositol in mammalian cells. J. Biol. Chem. 275:5778–5783.

10. Bertiello, L., M. F. Goncalvez, W. Colli, and R. M. de Lederkremer. 1995. Structural analysis of inositol phospholipids from Trypanosoma cruzi epimastigote forms. Biochem. J. 308:253–261.

11. Bertiello, L. E., M. M. Alves, W. Colli, and R. M. de Lederkremer. 2000. Evidence for phospholipases from Trypanosoma cruzi active on phosphatidylinositol and inositolphosphoceramides. Biochem. J. 345:77–84.

12. Brown, D. A., B. Crise, and J. K. Rose. 1989. Mechanism of membrane anchoring affects polarized expression of two proteins in MDCK cells. Science 245:1499–1501.

13. Brown, D. A., and E. London. 1998. Functions of lipid rafts in biological membranes. Annu. Rev. Cell Dev. Biol. 14:111–136.

14. Cerbon, J., A. Falcon, C. Hernandez-Luna, and D. Segura-Cabos. 2005. Inositol phospholipid turnover in human lung fibroblasts. Mol. Microbiol. 53:725–733.

15. Derenbourg, M., G. D. Finazzi, and R. D. Klausner. 2001. Synthesis of cytidine diphosphate choline, cytidine diphosphate ethanolamine, and related compounds. J. Biol. Chem. 222:193–214.

16. Lester, R. L., and C. Dickson. 1993. Sphingolipids with inositol phosphate-containing head groups. Adv. Lipid Res. 26:253–274.

17. Lim, S. I., M. P. Caris, M. A. Davitz, and E. Rodriguez-Boulan. 1989. A glycosylphosphatidylinositolanchored membrane protein acts as an apical targeting signal in polarized epithelial cells. J. Cell Biol. 109:2145–2156.

18. Mayo, S., and H. Riezman. 2004. Sorting GPI-anchored proteins. Nat. Rev. Mol. Cell Biol. 5:110–120.

19. McDonald, M. A., D. A. Ransom, and D. J. Bangs. 1998. Glycosyl phosphatidylinositol-dependent secretory transport in Trypanosoma brucei. Biochem. J. 335:681–689.

20. Menon, A. K., S. Mayor, M. A. J. Ferguson, Duszenko, and G. A. M. Cross. 1988. Candidate glycosylphosphatidyl inositol precursor for the glycosylphosphatidylinositol membrane anchor of Trypanosoma brucei variant surface glycoprotein. J. Biol. Chem. 263:1970–1977.

21. Merrill, A. H., Jr., and K. Sandhoff. 2002. Sphingolipid: metabolism and cell signalling. p. 373–407. In D. E. Vance and J. E. Vance (ed.), Biochemistry of Lipids, Lipoproteins and Membranes, 4th ed. Elsevier, Boston, MA.

22. Miyake, T. Y., Y. Kozutsumi, S. Nakamura, T. Fujita, and T. Kawasaki. 1995. Ceramide synthesis and other stress responses. Biochim. Biophys. Acta 1294–1303.

23. Nolan, D. P., G. Genskens, and E. Pays. 1999. N-linked glycans containing linear poly-N-acetyllactosamine as sorting signals in endo-lysosomes. Biochem. J. 342:1169–1172.

24. Nolan, D. P., D. J. Jackson, M. J. Biggs, E. D. Brahazon, A. Pays, F. Van Laethem, F. Patisiaux-Hanoog, J. Elliott, H. P. Voorhees, and E. Pays. 2000. Characterization of a novel aluminrich protein associated with lysosomes in Trypanosoma brucei. J. Biol. Chem. 275:4072–4080.

25. Overath, P., Z. Czichos, and C. Hass. 1986. The effect of citrate/ctc-acetate on oxidative metabolism during transformation of Trypanosoma brucei. Eur. J. Biochem. 167:75–118.

26. Patnaik, P. K., M. C. Field, A. K. Menon, G. A. M. Cross, M. C. Yee, and P. Butkofiker. 1993. Molecular species analysis of phospholipids from Trypanosoma brucei bloodstream and procyclic forms. Mol. Biochem. Parasitol. 58:147–160.

27. Perry, D. K. 2002. Serine palmitoyltransferase: role in apoptotic de novo ceramide synthesis and other stress responses. Biochem. Biophys. Acta 1585:146–152.

28. Pilot, W. J., B. Brinivasan, S. Shepherd, A. Schmidt, R. C. Dickson, and R. L. Lester. 1992. Sphingolipid long-chain-base auxotrophs of Saccharomyces cerevisiae: genetics, physiology, and a method for their selection. J. Bacteriol. 174:2563–2574.

29. Ralston, J. K., A. A. Mullin, and M. J. McCouville. 2002. Intracellular trafficking of glycosylphosphatidylinositol (GPI)-anchored proteins and free GPIs in Leishmania mexicana. Biochem. J. 363:365–375.

30. Rifkin, M. R., C. A. Strobos, and A. H. Fairlamb. 1995. Specificity of etha-
nolamine transport and its further metabolism in *Trypanosoma brucei*. J. Biol. Chem. 270:16160–16166.

54. Salto, M. L., L. E. Bertello, M. Vieira, R. Docampo, S. N. J. Moreno, and R. M. de Lederkremer. 2003. Formation and remodeling of inositolphospho-ceramide during differentiation of *Trypanosoma cruzi* from trypomastigote to amastigote. Eukaryot. Cell 2:756–768.

55. Schuck, S., and K. Simons. 2006. Controversy fuels trafficking of GPI-anchored proteins. J. Cell Biol. 172:963–965.

56. Schuck, S., and K. Simons. 2004. Polarized sorting in epithelial cells: raft clustering and the biogenesis of the apical membrane. J. Cell Sci. 117:5955–5964.

57. Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. Nature 387:569–572.

58. Sonda, S., G. Sala, R. Ghidoni, A. Hemphill, and J. Pieters. 2005. Inhibitory effect of aureobasidin A on *Toxoplasma gondii*. Antimicrob. Agents Chemother. 49:1794–1801.

59. Sutterlin, C., T. Doering, F. Schimmoller, S. Schroder, and H. Riezman. 1997. Specific requirements for the ER to Golgi transport of GPI-anchored proteins in yeast. J. Cell Sci. 110:2703–2714.

60. Triggs, V. P., and J. D. Bangs. 2003. Glycosylphosphatidylinositol-dependent protein trafficking in bloodstream stage *Trypanosoma brucei*. Eukaryot. Cell 2:76–83.

61. Uemura, A., S. Watarai, Y. Kushi, T. Kasma, Y. Ohnishi, and H. Kodama. 2006. Analysis of neutral glycosphingolipids from *Trypanosoma brucei*. Vet. Parasitol. 140:264–272.

62. Vainauskas, S., Y. Maeda, H. Kurniawan, T. Kinoshita, and A. K. Menon. 2002. Structural requirements for the recruitment of Gaa1 into a functional glycosylphosphatidylinositol transamidase complex. J. Biol. Chem. 277:30535–30542.

63. Vainauskas, S., and A. K. Menon. 2004. A conserved proline in the last transmembrane segment of Gaa1 is required for glycosylphosphatidylinositol (GPI) recognition by GPI transamidase. J. Biol. Chem. 279:6540–6545.

64. van Meer, G., and Q. Lisman. 2002. Sphingolipid transport: rafts and trans-locators. J. Biol. Chem. 277:25855–25858.

65. Zanolari, B., S. Friant, K. Funato, C. Sutterlin, B. J. Stevenson, and H. J. Riezman. 2000. Sphingoid base synthesis requirement for endocytosis in Saccharomyces cerevisiae. EMBO J. 19:2824–2833.

66. Zhang, K., F. F. Hsu, D. A. Scott, R. Docampo, J. Turk, and S. M. Beverley. 2005. *Leishmania* salvage and remodelling of host sphingolipids in amasti-gote survival and acidocalcisome biogenesis. Mol. Microbiol. 55:1566–1578.

67. Zhang, K., J. M. Pompey, F.-F. Hsu, J. Turk, and S. M. Beverley. 2007. Redirection of sphingolipid metabolism towards de novo synthesis of ethanolamine in *Leishmania*. EMBO J. doi:10.1038/sj.emboj.7601565.

68. Zhang, K., M. Showalter, J. Revollo, F.-F. Hsu, J. Turk, and S. M. Beverley. 2003. Sphingolipids are essential for differentiation but not growth in *Leish-mania*. EMBO J. 22:6016–6026.