Requirement for acetyl-CoA carboxylase in Trypanosoma brucei is dependent upon the growth environment

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Summary

Trypanosoma brucei, the causative agent of human African trypanosomiasis, possesses two fatty acid synthesis pathways: a major de novo synthesis pathway in the ER and a mitochondrial pathway. The 2-carbon donor for both pathways is malonyl-CoA, which is synthesized from acetyl-CoA by Acetyl-CoA carboxylase (ACC). Here, we show that T. brucei ACC shares the same enzyme architecture and moderate ~30% identity with yeast and human ACCs. ACC is cytoplasmic and appears to be distributed throughout the cell in numerous puncta distinct from glycosomes and other organelles. ACC is active in both bloodstream and procyclic forms. Reduction of ACC activity by RNA interference (RNAi) resulted in a stage-specific phenotype. In procyclic forms, ACC RNAi resulted in 50–75% reduction in fatty acid elongation and a 64% reduction in growth in low-lipid media. In bloodstream forms, ACC RNAi resulted in a minor 15% decrease in fatty acid elongation and no growth defect in culture, even in low-lipid media. However, ACC RNAi did attenuate virulence in a mouse model of infection. Thus the requirement for ACC in T. brucei is dependent upon the growth environment in two different life cycle stages.

Introduction

The deadly protozoan parasite Trypanosoma brucei, the causative agent of African sleeping sickness in humans and Nagana in livestock, is vectored by the bloodsucking tsetse fly and infects the blood and cerebrospinal fluid of its human and animal hosts. As it transits through its life cycle, the parasite encounters a number of different host microenvironments that differ in their availability of key nutrients such as proteins and lipids, including fatty acids. For example, there is a ~300× lower concentration of lipids in the cerebrospinal fluid compared with blood (Lentner, 1981). One important function of fatty acids in T. brucei is to anchor cell surface glycoproteins as part of their glycosylphosphatidylinositol (GPI) anchors. These cell surface glycoproteins play key roles in the parasite’s ability to evade host defences. For example, switching of the GPI-anchored Variant Surface Glycoprotein (VSG) surface coat via antigenic variation protects T. brucei against immune attack in the mammalian bloodstream (reviewed in Mansfield and Paulnock, 2005; Morrison et al., 2009). Similarly, the GPI-anchored procyclin proteins may protect T. brucei against proteolytic attack in the tsetse midgut (Acosta-Serrano et al., 2001). The parasite has two ways to supply itself with fatty acids: acquire fatty acids from the host or synthesize its own fatty acids de novo (Smith and Bütikofer, 2010). There is a significant difference between the energy required for fatty acid uptake and synthesis: uptake of a 16-carbon fatty acid by passive diffusion would require one ATP for activation to its CoA derivative, while synthesis of the same 16-carbon fatty acyl CoA would require six ATPs and 12 reducing units (Lee et al., 2006). Thus, fatty acid uptake is likely preferred over the more energy intensive fatty acid synthesis pathway. However, when the host fatty acid supply is insufficient, the parasite must then synthesize its own fatty acids to meet its needs.

Trypanosoma brucei has two fatty acid synthesis pathways: the fatty acid elongase pathway of the endoplasmic reticulum that serves as the major pathway for synthesis (Lee et al., 2006), and a minor pathway in the mitochondrion that catalyses the synthesis of mitochondrial fatty acids (Stephens et al., 2007; Guler et al., 2008). T. brucei fatty acid elongation consists of a conserved cycle of reactions that starts with the condensation of the 2-carbon donor, malonyl-CoA, with an acyl-CoA primer (4–16 carbons long) followed by reduction, dehydration and reduction steps to yield a fatty acyl chain that is two carbons longer. Malonyl-CoA is synthesized from acetyl-CoA by acetyl-CoA carboxylase (ACC), a member of the biotin-dependent carboxylase family of enzymes (Jitrapakdee and Wallace, 2003). The ACC reaction is catalysed in two steps: first, the ATP-dependent carboxylation of the biotin prosthetic group, followed by transfer of the carboxyl group from biotin to the acceptor acetyl-CoA. Because the synthesis of malonyl-CoA requires the hydrolysis of ATP, the ACC reaction is considered the first
committed step in fatty acid synthesis and is a well-documented control point for the regulation of this pathway in mammals and yeast (reviewed in Tehlivets et al., 2007; Saggerson, 2008).

As T. brucei can acquire fatty acids from the host as well as synthesize them, the parasite likely has a mechanism to modulate its fatty acid synthesis pathway(s) in response to the environmental supply. Two published observations support this idea. First, bloodstream form T. brucei labelled with [3H]myristate (C14:0) in whole blood showed no elongation, but cells labelled in medium with only 5% serum lipids showed efficient elongation of [3H]myristate into longer fatty acids (Doering et al., 1993). Second, T. brucei midgut procyclic forms grown in lipid-depleted medium had upregulated the entire fatty acid elongase pathway compared with cells grown in normal medium (Lee et al., 2006). We propose that this ability to control fatty acid synthesis in response to the environment is critical to the process of host adaptation, allowing maximal usage of host resources to conserve energy that otherwise would be used for biosynthesis. To begin elucidating the mechanism(s) by which T. brucei fatty acid synthesis is regulated in response to the environment, we focused on ACC because it catalyses the first committed step of fatty acid synthesis, is known to be highly regulated by multiple mechanisms in other systems, and could theoretically control flux through the fatty acid synthesis pathway via the availability of its key substrate, malonyl-CoA.

Here, we performed an initial characterization of T. brucei ACC. We show that ACC has a punctate cytoplasmic localization and that ACC is required by procyclic forms for growth in culture under lipid-limited conditions and by bloodstream forms for full virulence in mice.

Results

ACC is expressed in both bloodstream and procyclic forms

The TriTrypDB indicates that the T. brucei genome encodes a single predicted ACC isoform (Tb927.8.7100) (Aslett et al., 2009), which was confirmed by Southern blotting (data not shown). T. brucei ACC possesses the same overall enzyme architecture as other eukaryotic ACCs (Fig. S1), shares a moderate ~30% identity to yeast and human ACC (Fig. S2), and shares a higher ~60% identity to other trypanosomatid ACCs (Fig. S3). Northern analysis of total mRNA revealed that the ACC mRNA is ~8.8 Kb and is expressed in both bloodstream and procyclic form life cycle stages (Fig. 1A). ACC (and other biotinylated proteins) can be detected on Western blots using streptavidin conjugated to horseradish peroxidase (SA-HRP), which recognizes the biotin prosthetic group (Nikolau et al., 1985; Haneji and Koide, 1989). In addition to ACC, the T. brucei genome contains one other biotinylated protein: the 74 kDa alpha subunit of 3-methylcrotonyl-CoA carboxylase (Tb927.8.6970), which is a mitochondrial enzyme involved in amino acid degradation. SA-HRP blotting of bloodstream and procyclic form lysates revealed a predominant >200 kDa band, roughly consistent with the predicted size of ACC (243 kDa) (Figs 1B and 2B) given the resolving power of the gel in this size range. The 74 kDa alpha subunit of the 3-methylcrotonyl-CoA carboxylase was not readily detected in total lysates, but could be detected in partially purified mitochondrial fractions (data not shown). Although additional cross-reacting bands become evident upon longer exposures, we show that the >200 kDa band is specifically depleted upon ACC RNA interference (RNAi), as discussed below (Fig. 4B). Finally, ACC enzyme activity was detected in both bloodstream and procyclic form lysates and was dependent upon the addition of ATP and acetyl-CoA (Fig. 1C). Taken together, these data show that ACC is expressed and active in both life cycle stages.

ACC is cytoplasmic and localized to numerous puncta

Multiple prediction algorithms (WoLF PSORT, TargetP/SignalP and PredoTar) found no known targeting motifs in the ACC protein, predicting ACC to be cytosolic (Small et al., 2004; Emanuelsson et al., 2007; Horton et al., 2007). To experimentally assess the localization of ACC, we used an epitope-tagging strategy to create a procyclic-form cell line (PF ACC-myc) with a c-myc tag fused to the C-terminus of ACC. To minimize the possibility of mislocalizing the tagged protein due to overexpression of an ectopic copy, we tagged the genomic locus of only one ACC allele. Using immunoprecipitation with anti-c-myc antibody covalently linked to beads, we found that ACC-myc immunoprecipitates possessed ACC activity, while control immunoprecipitates from untagged cells were inactive (Fig. S4). This result indicates that the myc-tagged ACC allele encoded a functional enzyme.

First, we subjected lysates of PF ACC-myc cells to subcellular fractionation by differential centrifugation (see Fig. 2A for scheme) and analysed the fractions by SDS-PAGE and Western blotting (Fig. 2B). ACC-myc showed a fractionation pattern similar to the cytoplasmic marker HSP70, and distinct from the markers for the glycosome and ER. Moreover, the ACC-myc fractionation pattern was the same as that of native ACC in both PF ACC-myc cells (Fig. 2B) and wild-type procyclic cells (data not shown), indicating that the c-myc epitope was not affecting the localization of the tagged ACC.

Next, we examined the subcellular localization of ACC by immunofluorescence microscopy. ACC-myc was not uniformly distributed in the cytoplasm, but instead localized to a multitude of small distinct puncta (Fig. 3B, D, J, N, R and V). Wild-type cells had no visible fluorescent signal at the same exposure time (Fig. 3F and H) and only a faint haze
with an exposure 3.5 times longer (data not shown). A field of cells captured at a lower magnification demonstrated that specific labelling with the anti-c-myc antibody was reflected in the whole-cell population (Fig. 3C, D, G, and H). The ACC puncta did not colocalize with markers for the cytoplasm (cytoplasmic HSP70), the glycosomes (pyruvate phosphate dikinase, aldolase and glyceraldehyde phosphate dehydrogenase), the ER (BiP) or the mitochondrion (lipoamide dehydrogenase) (Fig. 3M–P, I–L, Q–T and U–X respectively). Furthermore, the distribution of ACC-myc is distinct from that of Nile Red-stained lipid droplets (Fig. 3Y and Z), Golgi (Ho et al., 2006; Ramirez et al., 2008) and acidocalcisomes, which are larger and less numerous (Fang et al., 2007; de Jesus et al., 2010).

**RNAi of ACC is efficient in both bloodstream and procyclic forms**

Because our attempts to delete both alleles of ACC were unsuccessful, we chose to assess the functional role of ACC in *T. brucei* using the pZJM RNAi vector to induce knock-down of ACC mRNA in bloodstream and procyclic cells (Wang et al., 2000; Morris et al., 2001). Northern analysis of total RNA showed that induction of RNAi reduced ACC mRNA by 76% in bloodstream forms and 85% in procyclic forms (Fig. 4A). Similar results were obtained with at least four independent clones (data not shown). Like others, we have observed that the ACC RNAi cells can undergo RNAi reversion (Chen et al., 2003; Motyka and Englund, 2004). By day 25, Northern analysis showed that procyclic cells had completely recovered expression of ACC mRNA, even though ACC dsRNA was still being produced (data not shown). SA-HRP blotting revealed the loss of ACC protein over 10 days of RNAi (Fig. 4B). A separate analysis of four independent inductions showed that 4 days of ACC RNAi reduced ACC protein by 91 ± 7% in both bloodstream and procyclic forms. Four days of ACC RNAi also significantly reduced ACC activity in lysates (Fig. 4C), with an 87 ± 1% and 90 ± 1% reduction in bloodstream and procyclic forms respectively.
procyclic cells respectively (Fig. S6A and B). Finally, ACC RNAi resulted in no growth inhibition in either bloodstream or procyclic cells (Fig. 4D), with doubling times of 8 h and 15 h respectively (Fig. S6C). Fluorescence microscopy revealed that ACC RNAi resulted in no gross defects in cell morphology, or in the structure of the mitochondrion, ER or nuclear/mitochondrial DNA as revealed by immunostaining with specific antibodies to a mitochondrial marker (lipoamide dehydrogenase), an ER marker (BiP), or by staining with the DNA intercalating dye 4′,6-diamidino-2-phenylindole (DAPI) (data not shown).

Effect of ACC RNAi on overall lipid metabolism

To look for ACC RNAi-induced changes in fatty acid metabolism, ACC RNAi cells were incubated with [3H]laurate (C12:0) or [3H]myristate (C14:0), which can be elongated by the fatty acid elongase pathway and incorporated into lipids. Analysis of the labelled lipids by thin-layer chromatography (TLC) in the absence of RNAi showed labelling of neutral lipids at the top, free fatty acids co-migrating with the free fatty acid marker, myristate (Myr), and various phospholipids co-migrating above and below the phospholipid marker, phosphatidylcholine (PtdC) (Fig. 5A, lanes 1, 3, 5 and 7). Migrating below the phospholipids, we also observed labelling of the bloodstream form-specific lipids, Glycolipids A and C (and their intermediates) (Buxbaum et al., 1994), which are precursors to the VSG GPI anchor (Fig. 5A, lanes 5 and 7). In procyclic forms, ACC RNAi resulted in little change in the overall labelling patterns of [3H]laurate or [3H]myristate, except for an accumulation of free fatty hypotonic lysates

Fig. 2. Subcellular fractionation shows ACC to be cytosolic.
A. Scheme for subcellular fractionation by differential centrifugation of lysates prepared from procyclic-form ACC-myc cells. B. Starting lysate (L) and subcellular pellet (P) and supernatant (S) fractions were probed by Western blotting for ACC-myc (c-myc), total ACC (SA-HRP), cytoplasmic HSP70 (cytoplasmic marker), glycosomes and BiP (ER marker). The anti-glycosome antibody recognizes three glycosomal enzymes: pyruvate phosphate dikinase (PPDK) (~100 kDa), aldolase (~41 kDa) and glyceraldehyde phosphate dehydrogenase (GAPDH) (~39 kDa). The identity of the ~50 kDa band is not known. Example shown is representative of two independent fractionations.

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Fig. 3. Immunofluorescence microscopy shows ACC is localized to cytoplasmic puncta.
A–H. Procyclic-form ACC-myc cells (A–D) and wild-type procyclic cells (E–H) were fixed, permeabilized, and ACC-myc localized by staining with mouse anti-c-myc primary antibody and Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (green). I–X. Procyclic-form ACC-myc cells were colocalized for ACC-myc as above (green) along with rabbit antibodies for various subcellular markers: (I)–(L) glycosome (anti-glycosomal); (M)–(P) cytoplasm (anti-cytoplasmic HSP70); (Q)–(T) ER (anti-BiP); and (U)–(X) mitochondrion [anti-lipoamide dehydrogenase (LipDH)]. Secondary antibody was goat anti-rabbit conjugated to Alexa Fluor 594 (red). Y and Z. Wild-type procyclic cells were stained with Nile red to show lipid droplets. All cells were co-stained with DAPI (blue) to indicate nuclear and mitochondrial DNA. Cells were imaged at 100× (A, B, E, F, I–Z) and 60× (C, D, G, H). Scale bars = 10 μm.

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acids and one of the phospholipids (indicated by asterisk; likely phosphatidylethanolamine based on its migration) (Fig. 5A, lanes 2 and 4). In bloodstream forms, ACC RNAi labelling resulted in no change in the species labelled by [3H]laurate (Fig. 5A, lanes 5 and 6). In contrast, ACC RNAi reduced incorporation of [3H]myristate into phospholipids (Fig. 5A, lanes 7 and 8), while no loss of labelling was observed in the bloodstream-specific Glycolipids A and C.

**ACC is required for elongation of fatty acids**

We next examined the effect of ACC RNAi on the major pathway for fatty acid synthesis in *T. brucei*. Because the cell-free fatty acid elongation assay bypasses the ACC step (Morita *et al.*, 2000a), we examined fatty acid elongation in vivo. Cells labelled with [3H]laurate (C12:0) and [3H]myristate (C14:0), which should be converted to longer fatty acids if the elongase pathway is functioning (Lee *et al.*, 2006), were analysed for elongation products using reverse-phase TLC. In uninduced procyclic forms, both [3H]laurate and [3H]myristate were elongated to products up to 18 carbons (Fig. 5B, lanes 1 and 3). ACC RNAi in procyclics resulted in a 74±H1006 6% and 53±H1006 5% inhibition of [3H]laurate and [3H]myristate elongation respectively (Fig. 5B, lanes 2 and 4). Uninduced bloodstream forms readily elongated [3H]laurate (Fig. 5B, lane 5), but little [3H]myristate elongation occurred (Fig. 5B, lane 7). ACC RNAi in bloodstream forms resulted in a 15±H1006 5%
inhibition of [3H]laurate elongation (Fig. 5B, lane 6), and completely abolished the minor elongation that occurred with [3H]myristate (Fig. 5B, lane 8).

**Effect of ACC RNAi on growth in low-lipid media**

Even though ACC RNAi reduced elongase activity, the cells were still able to grow normally in culture (Fig. 4D). Because *T. brucei* can also readily acquire fatty acids from the medium (Dixon *et al.*, 1971; Voorheis, 1980; Bowes *et al.*, 1993; Coppens *et al.*, 1995; Lee *et al.*, 1999), we assessed the growth of ACC RNAi cells in low-lipid media. ACC RNAi in bloodstream forms still showed no effect on growth in two different formulations of low-lipid medium compared with the uninduced control (Fig. 6A). In contrast, ACC RNAi in procyclic forms reduced growth by 64% in low-lipid medium (Fig. 6B). Furthermore, 68% of this growth defect could be reversed by the addition of 35 μM stearate (C18:0), suggesting that the growth defect arose from a lack of fatty acids rather than some other limiting factor in the medium. Finally, pre-adaptation of bloodstream and procyclic cells by growth in low-lipid media for 10 days prior to induction of ACC RNAi did not enhance the effect of ACC RNAi on growth (Fig. S7).

**ACC is required for full virulence in mice**

To assess the virulence of ACC RNAi cells, NIH Swiss mice (*n* = 10 per group) were either left untreated (uninduced control) or treated with doxycycline [a bioavailable tetracycline (Tet) analogue] in their drinking water to induce ACC RNAi. Mice were then infected intraperitoneally with 1 × 10⁵ freshly thawed bloodstream-form ACC RNAi trypanosomes. The uninduced control infection resulted in a mean time-to-death of 12.7 days by Kaplan–Meier survival analysis (Fig. 7). However, when ACC RNAi was induced in the doxycycline-treated mice, the mean time-to-death was significantly increased to 22.3 days (P = 0.0021, Wilcoxon test).

**Discussion**

Among the protozoa, only the ACCs of the Apicomplexan parasites *Toxoplasma gondii* and *Plasmodium falciparum* have been characterized. These Apicomplexans possess two eukaryotic-type multi-domain ACC isozymes: a plastid ACC1 that functions in plastid de novo fatty acid synthesis and a cytosolic ACC2, with proposed functions in fatty acid elongation, polyketide synthesis and mitochondrial
fatty acid synthesis (Zuther et al., 1999; Jelenska et al., 2001; Gardner et al., 2002; Waller et al., 2003; Mazumdar and Striepen, 2007). Here, we have performed the first characterization of the sole trypanosome ACC isozyme in bloodstream and procyclic forms and explored its role in fatty acid metabolism.

The cytosolic punctate distribution of ACC-myc in *T. brucei* has not been observed previously in other eukaryotes and thus, appears to be novel. What are these puncta? They could represent a fixation artefact from the paraformaldehyde. However, ACC-myc showed the same punctate pattern when cells were fixed in cold methanol, suggesting this is not the case (data not shown). The puncta could also represent non-specific aggregation due to the c-myc epitope tag. Four reasons argue against this: first, ACC-myc showed the same fractionation pattern as native ACC, suggesting that the myc tag has no significant effect upon the subcellular distribution of ACC; second, ACC-myc immunoprecipitates possess ACC activity, suggesting that the myc tag did not affect enzyme function; third, because the myc tag was incorporated into the genomic locus, ACC-myc is likely expressed at endogenous levels rather than at the high levels associated with epitope-tag artefacts; fourth, one previous report of a cytosolic myc-tagged protein showed diffuse staining in *T. brucei* rather than the puncta we observe for ACC (Petersen et al., 1997).

Fig. 6. Growth of ACC RNAi cells in low-lipid conditions. ACC RNAi cells were seeded into normal or low-lipid media, induced for ACC RNAi for 10 days, and the cell densities of induced (+ RNAi) and uninduced control (No RNAi) cultures were recorded every other day. A. Bloodstream-form ACC RNAi cells in normal medium (NM), or two types of low-lipid media: medium made with delipidated FBS (DL) and medium made with only Serum Plus (SP). B. Procyclic-form ACC RNAi in normal medium (NM) or low-lipid medium made with delipidated FBS (DL). C. Procyclic-form ACC RNAi in DL medium or DL medium supplemented with 35 μM stearate (DL + C18). For all panels, average of three replicates is shown. Error bars show SD, but are smaller than the data symbols.

An intriguing alternative is that these puncta might represent polymerization of ACC in *T. brucei*. Mammalian and avian ACCs polymerize into filaments (Kleinschmidt et al., 1969; Mackall et al., 1978), and there is evidence suggesting yeast ACC may also polymerize (Schneiter et al., 1996). In birds and mammals, ACC polymerization is dynamic and the polymer form is the active form (Ashcraft et al., 1980; Beaty and Lane, 1983; 1985; Thampy and Wakil, 1988; Kim et al., 2010). Whether they are non-specific aggregates or polymers, the nature of the ACC puncta must be independently confirmed using an...
ACC RNAi cells show reduced virulence in a mouse model of infection. Kaplan–Meier survival analysis of mice infected with bloodstream-form ACC RNAi trypanosomes. NIH Swiss mice (10 per group) were pre-dosed in their drinking water for 48 h with 1 mg ml⁻¹ doxycycline in 5% sucrose water (+ RNAi) or 5% sucrose water as a control (No RNAi). At 48 h, mice were infected by intraperitoneal injection of 1 × 10⁵ ACC RNAi trypanosomes and monitored for time of death for 30 days. Mice were maintained on the doxycycline/sucrose or sucrose water for the duration. Significance was determined by Wilcoxon test.

Fig. 7. ACC RNAi cells show reduced virulence in a mouse model of infection. Kaplan–Meier survival analysis of mice infected with bloodstream-form ACC RNAi trypanosomes. NIH Swiss mice (10 per group) were pre-dosed in their drinking water for 48 h with 1 mg ml⁻¹ doxycycline in 5% sucrose water (+ RNAi) or 5% sucrose water as a control (No RNAi). At 48 h, mice were infected by intraperitoneal injection of 1 × 10⁵ ACC RNAi trypanosomes and monitored for time of death for 30 days. Mice were maintained on the doxycycline/sucrose or sucrose water for the duration. Significance was determined by Wilcoxon test.

Alternative epitope tag or an antibody to native T. brucei ACC before their function can begin to be explored.

Among unicellular eukaryotes, ACC has been most extensively characterized in the yeasts. In both Saccharomyces cerevisiae and Schizosaccharomyces pombe, deletion of ACC is lethal (Hasslacher et al., 1993; Saitoh et al., 1996), while a reduction in ACC activity leads to growth inhibition and a range of defects in nuclear and vacuolar membrane function (Saitoh et al., 1996; Schneider et al., 1996; 2000). Thus, we predicted that ACC RNAi would reduce overall lipid biosynthesis activity, resulting in growth inhibition in T. brucei. Instead, we found that bloodstream form and procyclic cells differed in the effect of ACC RNAi upon fatty acid elongation and growth in culture. We also found that the effect of ACC RNAi was dependent upon the growth environment.

Based on our results, we propose that procyclic form T. brucei is dependent upon ACC only when environmental lipids are limiting. It is well known that T. brucei can readily take up and use lipids from their environment (Dixon et al., 1971; Voorheis, 1980; Bowes et al., 1993; Coppens et al., 1995; Lee et al., 1999). Thus, in normal medium, procyclic cells primarily rely on fatty acid uptake to satisfy their needs, rather than de novo synthesis. Therefore, reduction of fatty acid elongation upon ACC RNAi had a limited effect on overall lipid metabolism because the cells were already relying upon exogenous lipids. In low-lipid medium, however, the procyclics require ACC and fatty acid elongation to compensate for the fatty acid deficit. Under these conditions, reduction of ACC activity and fatty acid elongation rendered the cells unable to grow efficiently. This growth defect of procyclic ACC RNAi cells in low-lipid conditions is very similar to that seen with RNAi of the enoyl-CoA reductase in the fatty acid elongation pathway (Lee et al., 2006), consistent with the coupling of these enzymes into the same metabolic pathway.

In bloodstream forms, the response to ACC RNAi differed significantly from procyclic forms. Despite efficient knock-down of ACC activity, fatty acid elongation was only moderately reduced, and the cells exhibited no growth defect in either normal or low-lipid media. This suggests that in cultured bloodstream forms, the fatty acid elongation pathway may not be very dependent upon ACC. This result was unexpected for two reasons: first, in all other eukaryotes examined to date, fatty acid synthesis and elongation are dependent upon malonyl-CoA supplied by ACC; second, bloodstream form cells have a high demand for myristate to anchor their VSG surface coat, which is a relatively scarce fatty acid in serum and scarcer still in standard culture medium (Paul et al., 2001). One possible explanation is that the residual ~10% ACC activity supports sufficient fatty acid elongation. If true, this suggests that the level of ACC expression in bloodstream forms is at > 10-fold excess over what is required for growth in culture. Other metabolic enzymes, such as trypanothione reductase (Krieger et al., 2000) and several glycolytic enzymes (Albert et al., 2005; Caceres et al., 2010), have been reported to be present in excess, although a 75–90% knock-down of these enzymes did cause an observable growth defect.

In contrast to procyclics, loss of ACC had little apparent impact upon bloodstream forms in culture, except one notable effect upon the metabolism of [³H]myristate. As previously reported (Doering et al., 1993; Morita et al., 2000a), very little elongation of [³H]myristate occurs in bloodstream forms, likely resulting from the exclusive use of myristate as the fatty acid moiety in the VSG GPI anchors (Ferguson et al., 1988). However, under ACC RNAi conditions, we observed a general loss in the incorporation of [³H]myristate into phospholipids, while incorporation into the VSG GPI anchor precursors, Glycolipids A and C was preserved. Thus, ACC RNAi revealed a partitioning of the myristate pool, where the myristoylation of the GPI anchors takes priority over incorporation into phospholipids. This phenomenon has been observed previously (Doering et al., 1993; Morita et al., 2000a), and highlights the special importance of myristate in bloodstream-form T. brucei. Morita et al. reported that myristate produced by the ELO pathway was preferentially incorporated into the VSG GPI anchors. Here, we show that exogenous myristate is likewise preferentially incorporated into the VSG GPI anchors, perhaps by special delivery from acyl-CoA-binding protein (Milne and Ferguson, 2000), or by one or more acyl-CoA
This work extends our understanding of fatty acid synthesis in *T. brucei* and points to the importance of exogenous sources of fatty acids in the overall lipid metabolism of these parasites. Finally our data raise key questions about how *T. brucei* senses environmental fatty acids and transduces this information into regulatory decisions governing its fatty acid metabolism. Such processes are key to survival, enabling the parasite to adapt its fatty acid metabolism to each host environment to satisfy its lipid needs while minimizing wasteful energy expenditure.

**Experimental procedures**

**Reagents**

All chemicals and reagents were purchased from Thermo Fisher Scientific and Sigma except: Minimum Essential Medium Eagle (MEM), Iscove’s Modified Dulbecco’s Medium (IMDM), DAPI (Invitrogen), Serum Plus (JRJ Biosciences), delipidated fetal bovine serum (FBS) (Cocalico Biologicals), poly-L-lysine solution and normal goat serum (Electron Microscopy Sciences). [U-14C]-dATP was from Perkin-Elmer. [14C]NaHCO3 and [3H]-labelled fatty acids were from American Radiolabelled Chemicals. The mouse monoclonal 9E10 anti-c-myc antibody was from Santa Cruz Biotechnology. The rabbit polyclonal antibodies to BiP and cytoplasmic HSP70 were generously provided by Dr Jay Bangs (University of Wisconsin-Madison) (Bangs et al., 1993; McDowell et al., 1998). The rabbit polyclonal anti-lipoamide dehydrogenase antibody was a kind gift of Dr Luise Krauth-Siegel (University of Heidelberg) (Schoneck et al., 1997). The rabbit polyclonal 2841D anti-glycosome antibody was a generous gift from Dr Marilyn Parsons (Seattle Biomedical Research Institute) (Parker et al., 1995) and recognizes three glycosomal enzymes: Pyruvate Phosphate Dikinase (PPDK) (∼100 kDa), Aldolase (∼41 kDa) and Glyceraldehyde Phosphate Dehydrogenase (GAPDH) (∼39 kDa).

**Trypanosome strains and cell lines**

Wild-type procyclic and bloodstream form *T. brucei* strain 427 were provided by Dr Paul Englund (Johns Hopkins School of Medicine). Procyclic and bloodstream form *T. brucei* transgenic cell lines containing genomically integrated Tet repressor and T7 polymerase [29-13 and 90-13 respectively (Hirumi and Hirumi, 1989; Wirtz et al., 1999)] were generously provided by Dr George Cross (Rockefeller University). Bloodstream form parasites were grown in HMI-9 medium (Hirumi and Hirumi, 1989) containing 10% heat-inactivated FBS/10% Serum Plus and supplemented with 2.5 µg ml−1 G418, 5 µg ml−1 hygromycin and 2.5 µg ml−1 phleomycin, as needed. Procyclic form parasites were grown in SDM-79 medium (Brun and Shonenberger, 1979) containing 10% heat-inactivated FBS and supplemented with 15 µg ml−1 G418, 50 µg ml−1 hygromycin and 2.5 µg ml−1 phleomycin, as needed.

**Preparation of low-lipid media**

The only source of lipids in media comes from the serum additives. According to the manufacturers, both Serum Plus...
and delipidated FBS contain ~20% serum lipids. Two types of low-lipid HMI-9 media were prepared. Delipidated medium (DL) was prepared with 10% Serum Plus and 10% delipidated FBS. Serum Plus only medium (SP) was prepared with 10% Serum Plus only. Thus the DL and SP media contain serum lipids equivalent to 4% and 2% FBS, respectively, compared with 12% for normal HMI-9 medium. For procyclic cells, low-lipid DL medium was prepared with 10% delipidated FBS, and contained serum lipids equivalent to 2% FBS, compared with 10% for normal SDM-79. For fatty acid rescue experiments, a final concentration of 35 μM stearate (C18:0) was added to the medium.

**RNA purification and Northern analysis**

Total RNA was purified and Northern analysis was performed as previously described (Wang et al., 2000), except 1 x 10^7 cell equivalents or 10–15 μg of total RNA was loaded per lane and blots probed with a 32P-labelled DNA probe corresponding to the same ACC sequence used for ACC RNAs (see below).

**Preparation of cell lysates**

Hypotonic lysates were prepared as described (Morita et al., 2000a). We also prepared lysates using an alternative method developed for radioimmunoprecipitation assays (RIPA): 1 x 10^6 cells were washed twice in BBSG (50 mM Bicine-NaCl pH 8, 50 mM NaCl, 5 mM KCl, 70 mM glucose) and the final cell pellet frozen on dry ice. The frozen pellet was overlaid with 100 μl of TBS-RIPA buffer (1 x Tris-Buffered Saline (TBS), 2 mM EDTA, 0.5 mM DTT, 1% (v/v) nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS) supplemented with 0.5 μg ml⁻¹ leupeptin, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM Nα-p-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), 2 μM pepstatin A and allowed to thaw on ice for 10 min. Cells were vortexed every 5 min for 30 min, with 20 s of resting on ice between vortexing. Cell lysates were centrifuged for 30 min at 4°C at 16,000 g to remove cell debris. Supernatant was removed, aliquoted, snap frozen in liquid nitrogen and stored at −80°C.

**Streptavidin blotting**

For streptavidin blotting, lysates were fractionated on 8% SDS-PAGE gels, transferred to nitrocellulose and blocked in Wash Buffer (1% dry milk, 1 x TBS, 0.05% Tween-20). Blots were probed for ACC with SA-HRP (Pierce) diluted 1:200 in Streptavidin Wash Buffer (0.2% dry milk, 1 x TBS, 0.05% Tween-20). Blots were washed 4 x in Streptavidin Wash Buffer, followed by two washes in 1 x TBS/0.05% Tween-20. The blots were developed using the Pico SuperSignalEnhanced Chemiluminescence kit (Pierce), and exposed to HyBlot CL film (Denville). For some experiments the blot was cut, and the top half probed for ACC with SA-HRP as above, while the bottom half was probed for tubulin as follows. Blot was incubated with a mouse anti-tubulin antibody (clone B-5-1-2; Sigma) diluted 1:500 000 in Wash Buffer, washed 4 x in Wash Buffer, and probed with HRP-conjugated goat anti-mouse IgG antibody (Invitrogen) diluted 1:20 000 in Wash Buffer. After four washes in Wash Buffer and two washes in 1 x TBS, 0.05% Tween-20, blots were developed for ECL. Semi-quantitative analysis of blots was performed using densitometry (NIH Image J software) of appropriately exposed films (unsaturated signal within the linear range of the film).

**ACC enzyme activity**

To assay ACC activity, we modified a biotin carboxylase assay described previously (Wurtele and Nikolau, 1990). To remove endogenous CoA substrates, lysates were either dialysed into BC Buffer (50 mM Tris-Cl pH 8, 5 mM MgCl₂, 2 mM DTT) for 4–12 h at 4°C or alternatively, were desalted on a G50-80 sephadex column (Sigma) equilibrated in BC Buffer. Treated lysates were then incubated in a final volume of 100 μl of BC Buffer supplemented with 5 mM ATP, 0.6 mM acetyl-CoA, 1 mg ml⁻¹ fatty acid-free bovine serum albumin (BSA) (Sigma) and 2 mM [14C]NaHCO₃ (14.9 mCi mmol⁻¹) for 30 min at 30°C, mixing every 10 min. Reactions were stopped by transferring tubes to ice for 5 min. Unreacted [14C]NaHCO₃ was released as [14C]CO₂ by the addition of 50 μl of 6 N HCl. Acid-precipitated [14C]malonyl-CoA product was collected on Whatman #1 filter circles, air-dried and quantified by scintillation counting. Linear regressions and Student’s t-test analyses were performed using Microsoft Excel.

**Generation of ACC-myc cell line**

We used an in situ epitope-tagging strategy to generate a procyclic cell line with the C-terminus of one ACC allele fused to the c-myc epitope. We used PCR with bipartite primers and the appropriate plasmid template to generate a 936 bp linear tagging construct (ACC-MYC/Phleo/ACC 3’ UTR) with the following features (in 5’-3’ order): 3’ end of ACC gene fused in-frame with c-myc epitope ending with stop codon, α/β tubulin intergenic region, phleomycin resistance gene, 5’ end of ACC 3’ UTR sequence. To make this construct we used a two-step PCR procedure. First, we used a forward primer comprised of the last 54 bp of the ACC gene (without stop codon) followed by 6 bp of the c-myc epitope (5’-GACGAAAGGATGCCTGGCAGGCGCATGAGCGCTGG AAGTACAACCGGAAAGGCAGGCCTTGGTGGCAGCAA-3’). The reverse primer sequence is comprised of 21 bp of the phleomycin resistance gene followed by the first 44 bp of the ACC 3’ UTR (5’-TAATTCTTATCCTTGGCCTCCAATGTCGCCC GCATCCCCAGCCATGTCAGTCCTGGTGGGCGACC-3’). For template DNA, we used the mycPHLEO plasmid (a generous gift of Dr Meredith Morris, Clemson University), which contains the c-myc sequence, α/β tubulin intergenic region and phleomycin resistance gene. The resulting ampiclon encoding the linear tagging construct was cloned into the pCR 2.1-TOPO vector and sequenced. For the second PCR step, we used shorter forward and reverse primers that flank the linear tagging construct (5’-GACGAAAGGATGCCTGGCCTGGTGGCAGGCCG CCCAGCCATGTCAGTCCTGGTGGGCGACC-3’ and 5’-TAATTCTTATCCTTGGCCTCCAATGTCGCCC GCATCCCCAGCCATGTCAGTCCTGGTGGGCGACC-3’ respectively) to perform a large-scale PCR. The resulting ampiclon (ACC-MYC/Phleo/ACC 3’ UTR) was purified using a MinElute column (Qiagen), and 15 μg of purified targeting construct
was electroporated into $1 \times 10^8$ procyclic-form 427 cells and selected in 2.5 µg ml$^{-1}$ phleomycin. Integration of the tagging construct into the genomic locus via homologous recombination generated an in-frame fusion of the c-myc epitope to the 3’ end of one allele of ACC. Correct integration was confirmed by diagnostic PCR and Western blotting.

**Subcellular fractionation**

Hypotonic lysates of wild-type and ACC-myc-expressing procyclic form cells were subjected to differential centrifugation (Bangs et al., 1993; Roggy and Bangs, 1999). Briefly, lysate and then supernatants were fractionated by three successive centrifugation steps: a 1000 g step yielding P1 (cell fragments, nuclei, mitochondria) and S1 fractions; a 100 000 g step yielding P2 (microsomes) and S2 fractions; and a second 100 000 g step yielding P3 (residual microsomes) and S3 (cytosol) fractions (Fig. 2A). Samples of each subcellular fraction (1.5 $\times 10^6$ cell equivalents) were separated by 10% SDS-PAGE and transferred to nitrocellulose. Membranes were processed for streptavidin blotting as described above. To probe for ACC-myc or subcellular markers, membranes were blocked $\approx 1$ h in 5% milk/1× TBS and probed with primary antibodies diluted in 5% milk/1× TBS/0.5% Tween-20 as follows: anti-c-myc (clone 9E10), 1:250; anti-glycosome, 1:7,500; anti-cytosolic HSP70, 1:100; anti-lipoamide dehydrogenase (glycosome, 1:7,500; anti-cytosolic HSP70, 1:1000; anti-BiP (ER marker); and anti-lipoamide dehydrogenase (glycosome, 1:100). Membranes were washed twice in Cytomix (van den Hoff et al., 1992). For transfection into 29–13 procyclic cells, 100 µg of linearized pZJM.ACC was electroporated into 1 $\times 10^8$ washed cells in a final volume of 0.5 ml. A stable non-clonal procyclic ACC RNAi population was established first, followed by isolation of clonal cell lines by limiting dilution. For transfection into 90-13 bloodstream form cells, five replicate transfections were prepared, each containing 100 µg of linearized pZJM.ACC and 3 $\times 10^7$ washed cells in a final volume of 0.5 ml. After electroporation, the five transfections were pooled and dispensed into 24-well plates, resulting in clonal cell lines.

For growth curves, ACC RNAi cells were diluted into normal or low-lipid media, induced for RNAi by the addition of Tet (1 µg ml$^{-1}$ final) (Wang et al., 2000) and cell density monitored using either a Z1 dual-threshold Coulter Counter (Beckman) or a FACScan flow cytometer (Becton Dickinson). For comparison purposes, the slopes of the growth curves (linear correlation coefficients) were derived from linear regressions performed using Microsoft Excel. Doubling times were calculated from the slopes. Student’s t-test analysis was performed using Microsoft Excel.

**Metabolic labelling and lipid analysis**

Metabolic labelling was performed essentially as described (Paul et al., 2004). Briefly, after 7 days of RNAi induction, $1 \times 10^6$ cells were washed 3× in BBSG and resuspended in 1 ml of either HMI-9 (for bloodstream forms) or SDM-79 (for procyclics). The 1 ml of cell suspension was then added to tubes containing 50 µCi of dried down [11,12-3H]laurate (C12:0; 50–60 mCi mmol$^{-1}$) or [9,10-3H]myristate (C14:0; 50–60 Ci mmol$^{-1}$) and incubated for 2 h in a 37°C (bloodstream forms) or 28°C (procyclic forms) CO$_2$ incubator. Total lipids were extracted using a modified Folch method, equal DPMs were loaded per lane (4000–10 000), and analysed by normal-phase TLC on Kieselgel 60 plates as described previously (Morita et al., 2000a,b; Paul et al., 2004). Labelled lipid species were identified based on known migration was performed using the Nikon NIS Elements software package.

**RNA interference**

To make the ACC RNAi construct, a fragment bracketing the start codon of ACC (–110 to +467 nt) was amplified by PCR (Roche Expand High Fidelity) from wild-type procyclic-form 427 genomic DNA using a forward primer containing a 5′ Xhol site (5′-CCGctcgagTCCGAGCTGCACAAATG-3′) and a reverse primer containing a 5′ HindIII site (5′-CCCagacctGTGCCCCAAAAAGCAATC-3′). This 572 bp amplicon was cloned first into pCR2.1 TOPO prior to subcloning into the Tet-inducible RNAi vector pZJM (Wang et al., 2000). The pZJM.ACC plasmid was confirmed by sequencing (one T-to-C difference from 927 sequence at nt –55).

Bloodstream and procyclic form RNAi cell lines were generated as described previously (Wang et al., 2000; Morris et al., 2001), with modifications suggested by J. Roper and M. Ferguson (pers. comm.). pZJM.ACC plasmid was linearized by NotI digestion and precipitated in ethanol to a final concentration of 10 mg ml$^{-1}$. Prior to transfection, cells were washed twice in Cytomix (van den Hoff et al., 1992). For transfection into 29–13 procyclic cells, 100 µg of linearized pZJM.ACC was electroporated into 1 $\times 10^8$ washed cells in a final volume of 0.5 ml. A stable non-clonal procyclic ACC RNAi population was established first, followed by isolation of clonal cell lines by limiting dilution. For transfection into 90-13 bloodstream form cells, five replicate transfections were prepared, each containing 100 µg of linearized pZJM.ACC and 3 $\times 10^7$ washed cells in a final volume of 0.5 ml. After electroporation, the five transfections were pooled and dispensed into 24-well plates, resulting in clonal cell lines.

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patterns in this TLC system (Doering et al., 1993) or co-migration with the following markers: [1,14C]myristoyl phosphatidylcholine (American Radiolabelled Chemicals) for the various phospholipid species, and the bloodstream-specific VSG anchor precursors, Glycolipids A and C, which were generated using a cell-free GPI anchor biosynthesis reaction (Morita et al., 2000b; Paul et al., 2004). To analyse the fatty acids by chain length, total lipid extracts were converted to fatty acid methyl esters (FAMES), extracted in hexane, equal DPMs were loaded per lane (4000–10 000), and analysed by C18 reverse-phase TLC (Morita et al., 2000a; Paul et al., 2004). For chain length markers, FAMES were prepared in parallel from [9,10-3H]palmitate (C16) and [9,10-3H]stearate (C18). Semi-quantitative analysis of TLCs was performed using densitometry (NIH Image J software) of appropriately exposed autoradiographic films with an unsaturated signal within the linear range of the film. To determine fatty acid elongation, each FAME spot was quantified and then calculated as follows: total C12 elongation = 100 × [(C14 + C16 + C18)/(C12 + C14 + C16 + C18)]; total C14 elongation = 100 × [(C16 + C18)/(C14 + C16 + C18)].

Mouse infections

Analysis of ACC RNAi in mice was performed essentially as described (Lecordier et al., 2005). Twenty female NIH Swiss mice (10–12 weeks) were divided into two groups of 10 and pre-treated via their drinking water for 2 days with either 1 mg ml−1 doxycycline/5% sucrose (+RNAi group) or 5% sucrose alone (no RNAi group). Doxycycline is a bioavailable Tet analogue that will induce RNAi in vivo (Lecordier et al., 2005) and does not itself affect the course of infection (Rothberg et al., 2006; Abdulla et al., 2008). Mice were then infected by intraperitoneal injection with 1 × 107 bloodstream form ACC RNAi cells freshly thawed from frozen stabilates. Mice were maintained on treated water for the duration of the experiment, with fresh changes every 2 days. Course of infection was monitored and time to death was recorded. Parasitaemias were monitored periodically in a randomly selected subset of mice by tail stick and examination of blood smears. Mice were monitored daily for general appearance, behaviour and weight loss. If a mouse reached a humane end-point with fresh changes every 2 days. Course of infection was maintained on treated water for the duration of the experiment, with fresh changes every 2 days. Course of infection was varied with nutritional conditions. We thank Maurizio del Poeta, Jim Morris, Meredith Morris, Kerry Smith, Lesly Temesvari and Marilyn Parsons, and members of our laboratory for their helpful suggestions. We also thank Paul Englund, Jenny Guler, Soo Hee Lee, Yasu Morita, Sunayan Ray and Jamie Wood for critical reading of the manuscript. We are indebted to Paul Englund in whose laboratory this work was initiated.

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