A Novel Protein Kinase Localized to Lipid Droplets Is Required for Droplet Biogenesis in Trypanosomes\textsuperscript{\textdagger}\textasteriskcentered

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Ubiquitous among eukaryotes, lipid droplets are organelles that function to coordinate intracellular lipid homeostasis. Their morphology and abundance is affected by numerous genes, many of which are involved in lipid metabolism. In this report we identify a \textit{Trypanosoma brucei} protein kinase, LDK, and demonstrate its localization to the periphery of lipid droplets. Association with lipid droplets was abrogated when the hydrophobic domain of LDK was deleted, supporting a model in which the hydrophobic domain is associated with or inserted into the membrane monolayer of the organelle. RNA interference knockdown of LDK modestly affected the growth of mammalian bloodstream-stage parasites but did not affect the growth of insect (procyclic)-stage parasites. However, the abundance of lipid droplets dramatically decreased in both cases. This loss was dominant over treatment with myriocin or growth in delipidated serum, both of which induce lipid body biogenesis. Growth in delipidated serum also increased LDK autophosphorylation activity. Thus, LDK is required for the biogenesis or maintenance of lipid droplets and is one of the few protein kinases specifically and predominantly associated with an intracellular organelle.

\textit{Trypanosoma brucei} is a single-celled eukaryotic pathogen responsible for human African trypanosomiasis (also known as African sleeping sickness) and nagana in domestic animals. More than 50,000 cases of human disease occur yearly, with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk. No vaccine exists, and chemotherapy is difficult to administer and prone to pathogenicity with over 70 million people at risk.
bloodstream-form (BF) and insect procyclic-form (PF) stages of the parasite life cycle.

MATERIALS AND METHODS

Cell culture. The single marker BF line of T. brucei, which is derived from the 427 strain (52), was grown in HMI-9 supplemented with 10% fetal calf serum and 2.5 μg of G418/ml. PF T. brucei strain 29-13 genomic DNA by using the primers LDK 5'-CCCTAGGATGCTTACGGGAAAGATAATTGGTG-3' and LDK 3'-CCCAAGCTTGTTCTTCTCCAGCCAACGGAGCAC-3'. The LDK open reading frame (ORF) was cloned into pGEM-T Easy and sequenced. The ORF was then subcloned into the AvrII and HindIII sites in the inducible expression vector pLew79-3V5(PAC), which contains the puromycin resistance marker in lieu of the hygromycin gene of the original plasmid (1) was replaced by the puromycin resistance gene. The plasmid contains cloning sites between AvrII and LDK 3' opposing T7 promoters under the control of the Tet operator, as well as sequences mediating integration into the ribosomal DNA intergenic spacer region. RNA interference (RNAi) constructs were based on the plasmid p2T7TAblue (PAC), in which the hygromycin gene of the original plasmid (1) was replaced by an equal volume of PBS.

Immunoprecipitation of LDK-V5 from PF cell lysates (107 cells) of T. brucei was performed as previously described (15). Kinase assays were performed as described elsewhere (42) using [γ-32P]ATP and in some cases casein (5 μg/reaction) as substrate. Results were separated by SDS-PAGE, transferred to nitrocellulose, and labeled proteins detected by phosphorimaging. Signals were quantified by using ImageQuant (Molecular Dynamics). The same blots were probed with anti-V5 as described above to allow normalization of activity.

RESULTS

Tb11.01.0670 was identified by searching GeneDB (www.genedb.org) for T. brucei genes encoding proteins possessing a protein kinase catalytic domain, as well as a predicted transmembrane domain(s). The Tb11.01.0670 protein is 554 amino acids (aa) long; the protein kinase domain begins 12 aa from the N terminus (see schematic in Fig. 1A). It contains all of the essential motifs and residues required for protein kinase activity (see Fig. S1 in the supplemental material) (26). The kinase domain is followed by a 267-aa extension, which contains a single predicted transmembrane domain of 24 aa, suggesting potential membrane localization. This extension shows little homology with any known predicted proteins except with the orthologous kinases of trypanosomatids (see Fig. S1 in the supplemental material for alignment and systematic names). The orthologues in the related genus Leishmania are highly similar within the kinase domain, and also have a similarly placed predicted transmembrane domain within the C-terminal extension, but the extension is longer and shows reduced sequence similarity (see Fig. S1 in the supplemental material). These trypanosomatid kinases belong to the calmodulin-dependent kinase (CAMK) group of protein kinases and appear to be most closely related to the CAMKL (for CAMK-like) family (44). However, there is no obvious orthologous relationship to any specific eukaryotic protein kinase outside of the trypanosomatids. Our previous genome-wide microarray anal-
FIG. 1. Lipid droplet kinase structure and kinase activity. (A) Schematic of LDK, showing location of protein kinase (PK domain) and hydrophobic region (Hyd), which lies in the C-terminal extension. Between the kinase domain and the hydrophobic region, the protein is enriched for acidic aa (−), whereas distal to the hydrophobic region it is enriched for basic aa (+). The region included in the RNAi construct is marked. The annotated sequence is shown in Fig. S1 in the supplemental material. (B) Immunoblot. Lysates were prepared from PF induced (+Tet) or uninduced (−Tet) for LDK-V5 expression. After SDS-PAGE, samples were transferred to nitrocellulose. The blot was incubated with anti-V5 and goat anti-mouse immunoglobulin (anti-V5), Western analysis. Asterisks mark the heavy and light chain of myeloma protein. (C) Kinase assay. The gel containing the samples was blotted and subjected to nase activity in the presence of casein as a candidate exogenous substrate. The gel containing the samples was blotted and subjected to nase activity in the presence of casein as a candidate exogenous substrate (data not shown). We observed modest protein kinase activity directed toward a small synthetic peptide that is a favored substrate for other kinases in the CAMKL kinase family and another level of phosphorylation on myelin basic protein, another exogenous substrate (data not shown).

LDK protein localizes to the periphery of lipid droplets. As shown in Fig. 1C, immunoblotting of the same gel lanes, allowing normalization of activity. The graph depicts the relative level of phosphorylation by LDK K41A immunoprecipitates compared to the wild type.

ysis showed that the corresponding mRNA was expressed to low levels in both BF and PF T. brucei (31). Based on the presence of a protein kinase domain and the data below, we named the protein lipid droplet kinase (LDK).

To assess whether LDK has protein kinase activity, we expressed LDK tagged with three V5 epitopes at its C terminus in PF. Induction of LDK-V5 expression with Tet resulted in expression of an immunoreactive species migrating at ∼75 kDa, slightly larger than the predicted mass of 68 kDa (Fig. 1B). In this experiment and some others, LDK-V5 appeared as a doublet, but this was not consistently observed. We immunoprecipitated LDK-V5 from Tet-induced PF lysates and performed in vitro kinase reactions. As shown in Fig. 1C, immunoprecipitates from cells induced for LDK-V5 expression showed a significant amount of phosphorylation of a species comigrating with LDK-V5, which likely represents autophosphorylation. The LDK-V5 immunoprecipitates also phosphorylated the upper casein band, although some casein phosphorylation from control immunoprecipitates was also observed. The latter may result from casein kinases such as CK1, CK2α, and CK2α′, each of which is ranked among the eight most abundant kinases at the RNA level (31). Both LDK and casein phosphorylation were seen with purified TAP-tagged LDK compared to a mock purification from untransfected cells (not shown). We observed modest protein kinase activity directed toward a small synthetic peptide that is a favored substrate for other kinases in the CAMKL kinase family and another level of phosphorylation on myelin basic protein, another exogenous substrate (data not shown).

With rare exceptions, a lysine in subdomain II is required for activity. Mutation of this residue (K41A, see Fig. S1 in the supplemental material) strongly reduced phosphorylation of LDK, but the phosphorylation of casein was not decreased (Fig. 1D). This finding suggests that LDK possesses kinase activity which autophosphorylates in cis. The activity of the kinase-dead LDK-V5 immunoprecipitates toward casein may result from association with the native kinase or another kinase; hence, casein phosphorylation was not considered further. Autophosphorylation was similar in 50 to 100 mM NaCl and was dependent on Mg2+ (Mn2+ could not substitute). Ca2+ (with or without calmodulin) did not increase activity.
brane of lipid droplets. A similar ringlike staining pattern has been seen for proteins localized to the lipid droplet surface in other eukaryotes (see references 6 and 8 for examples). In the culture conditions used, images of cultured PF showed an average of four lipid droplets, whereas cultured BF typically showed two smaller lipid droplets. Staining of the ER, in addition to lipid droplets, was seen in some cells, most noticeably in those with a higher level of expression (Fig. 2E). This is consistent with the concept that lipid droplets derive from the ER.
Unlike other subcellular organelles, lipid droplets are bounded by a membrane monolayer in which the charged moieties face outwards. The presence of a single hydrophobic, predicted transmembrane domain suggested that this region might be critical for localization of LDK to lipid droplets. We therefore expressed a mutant version of the kinase lacking the 24 aa comprising the hydrophobic region (LDKΔ432-455) in PF. The mutant kinase was found throughout the cell, with no apparent association with lipid droplets (Fig. 2C). We examined the association of LDK-V5 with the lipid droplet using Triton X-114 and carbonate extractions (Fig. 3). In Triton X-114 extractions, LDK-V5 partitioned to the aqueous phase, although a C-terminal degradation fragment predicted to contain the hydrophobic region partitioned equally between the aqueous and detergent phases. EP-procyclin, a heterogeneously glycosylated glycosylphosphatidylinositol-anchored protein, was predominantly in the detergent phase, whereas the cytosolic and glycosomal phosphoglycerate kinase were present in the aqueous phase. When hypotonically lysed cells were extracted with carbonate, pH 11, control proteins fractionated as expected: phosphoglycerate kinases were in the supernatant, whereas the acidocalcisomal H⁺ pyrophosphatase (VP1), an integral membrane protein, was present only in the pellet. Most LDK-V5 remained in the integral membrane protein fraction (pellet), but some was released into the supernatant. The partial resistance of LDK to carbonate extraction indicates that the protein is strongly associated with the lipid droplet membrane.

LDK RNAi parasites show minimal growth defects but are depleted of lipid droplets. To further examine the role of LDK and ascertain its importance for survival of T. brucei, we performed RNAi knockdown analysis. A segment of the LDK ORF was cloned into the p2T7TAblue vector between Tet-inducible T7 promoters. Addition of Tet initiates RNAi knockdown. We isolated several clonal lines of BF transfectants, as well as nonclonal lines of PF transfectants. RNAi knockdown of LDK mRNA in PF was verified by Northern analysis and phosphorimaging, which demonstrated a 8% of the uninduced condition (Tet−) is dramatically reduced upon induction of RNAi (Tet+). The small species in the Tet+ lane corresponds to the double-stranded RNA produced upon induction. At right is the control hybridization with α-tubulin. Migration of molecular mass markers is shown (in kb).

FIG. 3. Extraction of LDK. (A) Triton X-114 extraction. After incubation with Triton X-114 the samples were separated into aqueous (Aq1) and detergent phase by centrifugation extraction, and the detergent phase was re-extracted (Aq2 and Det). Cell equivalents were analyzed by Western blotting with anti-V5, anti-procyclin, and anti-phosphoglycerate kinase (which detects both the 45-kDa cytosolic and the 56-kDa glycosomal matrix isoforms). (B) Carbonate extraction. Cell equivalents of the carbonate supernatant and pellet in SDS-PAGE sample buffer were loaded onto SDS-PAGE gels and blotted. The sample used for detection of the integral membrane protein VP1 was not boiled but heated to 45°C. Blots were probed with anti-V5, anti-phosphoglycerate kinase (anti-PGK), and anti-VP1. TCL, total cell lysate; P, carbonate pellet; S, carbonate supernatant.

FIG. 4. RNAi targeting LDK in PF and BF does not abrogate parasite proliferation. (A) Northern analysis of PF transfectants bearing an RNAi cassette targeting LDK. The abundance of full-length 3.32-kb mRNA seen in the uninduced condition (Tet−) is dramatically reduced upon induction of RNAi (Tet+). The small species in the Tet+ lane corresponds to the double-stranded RNA produced upon induction. At right is the control hybridization with α-tubulin. Migration of molecular mass markers is shown (in kb). (B) Growth curve for PF, induced (Tet+) or uninduced (Tet−) for RNAi. Standard deviations of cell counts are obscured by markers. (C) Growth curve for BF (Tet+) or uninduced (Tet−) for RNAi. Standard deviations of cell counts are obscured by markers.
conditions examined (Fig. 4B). Several independently derived clonal BF lines showed a small change in growth rate upon induction of RNAi; the results obtained with one of these lines are shown in Fig. 4C.

We next analyzed BF and procyclic RNAi transfectants for numbers of lipid droplets, utilizing Nile Red staining and microscopic analysis. Parasites were induced with Tet for RNAi and then examined on day 5 (PF) and day 2 (BF) for the presence of Nile Red staining bodies. The numbers of lipid droplets were enumerated by examining at least 50 randomly selected cells for each condition. In both developmental stages, LDK RNAi knockdown resulted in a 90% reduction in the number of lipid droplets (Fig. 5). In addition, the remaining lipid droplets appeared to be somewhat smaller and stained more faintly than those seen in the control samples. In control experiments with the parental 29-13 PF line and an irrelevant BF RNAi line (CK2/H9251/H11032), Tet treatment gave rise to a small increase in lipid droplet numbers (7 and 16%, respectively), indicating that neither Tet nor the process of RNAi itself are responsible for the reduction in lipid droplets seen upon LDK RNAi. These data show that LDK is required for maintaining normal numbers of lipid droplets in vitro.

Cells respond to an increase in fatty acids or to various stresses in lipid homeostasis by increasing the number of lipid droplets (48). We therefore investigated whether stimuli that normally induce lipid droplet biogenesis were still able to do so when LDK was depleted. Two different conditions were tested, both of which were tolerated by PF, although they were much more toxic to BF. Each condition deprives cells of at least one type of lipid, presumably yielding a compensatory increase in lipid synthesis or uptake and subsequent storage in lipid droplets. Treatment of PF with 1.5 μM myriocin, a potent inhibitor of the first step in sphingolipid biosynthesis, has been reported to increase the number of lipid droplets (20). Reduction of lipids in the medium has been previously shown to induce fatty acid synthesis in T. brucei (34), and we observed that replacing fetal calf serum with delipidized calf serum for 24 h almost doubled the number of lipid droplets in PF (see Table S1 in the supplemental material). Both of these treatments were applied to PF that had been induced for LDK RNAi for 5 days. Although the treated PF remained viable for the treatment period, both treatments increased the proportion of parasites with two, three, or four nuclei as revealed by DAPI staining. We therefore normalized the number of lipid droplets to the number of nuclei in these experiments to more accurately reflect any potential induction of lipid droplets (Fig. 6B; Table S1 in the supplemental material provides detail by number of nuclei per cell). Myriocin increased the number of lipid droplets per nucleus from ∼4 to >10, whereas delipidated serum increased the number to ∼7. Under both inducing conditions, cells depleted of LDK averaged less than one droplet per nucleus (Fig. 6). The reduction was apparent when only “normal” cells (one or two nuclei) were considered (Table S1 in the supplemental material). These findings indicate that LDK plays a critical role in the biogenesis and/or maintenance of lipid droplets.

**Induction of lipid droplets increases LDK autophosphorylation.** Given that LDK appears to be important for the induction or maintenance of lipid droplets, we hypothesized that the kinase might show increased activity after cells were exposed to lipid droplet inducing conditions. Because the studies with the K41A mutant suggested that autophosphorylation was a better reflection of LDK activity, we focused on autophosphorylation. Cell lysates were prepared after growing the cells in delipidated serum for 4 h. Immunoprecipitates were prepared and assayed for kinase activity, and

![FIG. 5. LDK RNAi depletes lipid droplets. Parasites induced for LDK RNAi were stained for lipids using Nile Red after 5 days (PF) or 48 h (BF) and compared to parallel untreated cultures. (A) Representative images of cells stained with Nile Red. Bar, 5 μm. (B) The number of lipid droplets per cell under each condition was enumerated. The means and standard deviations are shown.](image-url)
the same blots were incubated with anti-V5 to quantitate LDK-V5. As shown in Fig. 7, growth in the absence of lipids resulted in significantly increased phosphorylation of LDK in the assay. Because phosphorylation of LDK was shown to require its catalytic lysine (Fig. 1D), the enhanced phosphorylation is likely attributable to increased activation of LDK.

DISCUSSION

We describe here a novel protein kinase, LDK, which localizes to the periphery of lipid droplets in both BF and PF T. brucei. LDK was identified by searching T. brucei predicted proteins for those possessing both a protein kinase catalytic domain and at least one putative transmembrane domain. Although we anticipated that our screen might yield genes encoding proteins localized to the plasma membrane, this kinase was instead localized to an intracellular organelle. Previous work has shown some partial localization of mitogen-activated protein kinases to lipid droplets of leukocytes (53), as well as anonymous kinase activity associated with lipid droplets (33). However, these physical associations have not been tied to droplet function. Recently, a genome-wide RNAi screen of Drosophila cells identified over 200 genes that modulate lipid droplet morphology, affecting their size, subcellular distribution, or number (25). Of those that resulted in a fewer lipid droplets phenotype, two were protein kinases: cdc2 and tlk. These protein kinases are involved in cell cycle regulation and are not localized to lipid droplets. Hence, their modulation of lipid droplet number likely results from pleiotropic effects. Neither of these kinases is closely related to LDK at the sequence level. Thus, LDK is unique in that it is both positioned at the lipid droplet surface and is involved in lipid droplet maintenance.

To our knowledge, LDK is the first protein kinase identified as localized to intracellular organelles of trypanosomatids and the first protein shown to primarily reside on lipid droplets of these organisms under normal conditions. Lipid droplet membranes are unusual since they consist of a monolayer of phospholipids. The internal core of the lipid droplet is composed primarily of neutral lipids, an environment unfavorable to al-
most all proteins. The topology of proteins associated with the lipid droplet membrane is therefore likely to be distinct from transmembrane proteins in lipid bilayers that make up other membranes of the cell. Because deletion of the hydrophobic domain led to diffuse cytosolic staining, we propose that LDK associates with the monolayer membrane via its single hydrophobic domain, leaving both the N-terminal kinase domain and the C-terminal portion extending toward the cytosol. Because LDK-V5 is partially extractable with carbonate and partitions to the aqueous phase upon Triton X-114 extraction (not shown), we suggest it is a tightly associated peripheral membrane protein. However, given the lack of studies on the behavior of proteins in the lipid body monolayer, this topic bears further study. Indeed, a previous publication showed membrane-dipping proteins may be more readily extracted by carbonate than transmembrane proteins (28). Unlike some membrane-dipping domains (13), the LDK hydrophobic domain is not predicted to form an amphipathic alpha helix. However, it is flanked by charged regions, especially basic amino acids. Basic amino acids have been shown to work in concert with the hydrophobic domain of cavelin to mediate its interaction with the lipid body membrane when cells are treated with fatty acids (29).

The location of LDK at the lipid droplet surface suggests it could function as a mediator of the lipodomic status in the cell, reminiscent of the sensing of AMP/ATP ratio by protein kinase AMPK. Indeed, LDK is most similar to members of the CAMKL family, which includes AMPK. We propose that localization to the lipid droplet surface may also be important for modulating activity, similar to what is seen for a key enzyme in the membrane interaction of amphipathic proteins, and as modulators of membrane curvature. CTP:phosphocholine cytidylyltransferase. This enzyme shows a dramatic increase in kcat and decrease in Km when associated with membranes and specifically associates with lipid droplets as they enlarge (21). Changes in membrane curvature are thought to affect the conformation and hence the activity of the molecule (13). The location of LDK at the lipid droplet membrane suggests it, too, could be modulated by the composition or curvature of the membrane, allowing it to transmit signals regarding the lipid status of the cell. We observed an increase in autophosphorylation activity of LDK when cells were incubated in a lipid droplet inducing condition, indicating activation of the kinase. Phosphorylation of natural substrates by the kinase anchored at the droplet surface might provide a more accurate measure of activation. Identification of the substrates would be a step toward understanding the regulation of lipid droplet homeostasis in the parasite.

The observation that LDK RNAi knockdown causes little growth phenotype despite a dramatic reduction in the number of detectable lipid droplets indicates that droplet loss does not inevitably lead to parasite death. It is possible that small lipid droplets, undetectable by the techniques we used, remained in the cells or that survival is facilitated by other lipid storage and mobilizing mechanisms. The higher sensitivity of BF to knockdown of LDK may result from their high requirement for myristic acid (used to anchor the variant surface glycoprotein that covers the parasite surface) or their upregulated exocytic and endocytic transport systems (2, 19). In either case, it appears that LDK is not essential in vitro, but confirmation will require gene knockout approaches. Using conditional knockouts in studies that manipulate the lipids provided to and synthesized by the cells may elucidate conditions in which the protein provides important regulatory information. In addition, such knockouts will provide a means to dissect the structural features of LDK that are essential for lipid droplet biogenesis and maintenance.

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