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***Trypanosoma brucei* AMP-Activated Kinase Subunit Homologs Influence Surface Molecule Expression**

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Abstract

The African trypanosome, *Trypanosoma brucei*, can gauge its environment by sensing nutrient availability. For example, procyclic form (PF) trypanosomes monitor changes in glucose levels to regulate surface molecule expression, which is important for survival in the tsetse fly vector. The molecular connection between glycolysis and surface molecule expression is unknown. Here we partially characterize *T. brucei* homologs of the β and γ subunits of the AMP-activated protein kinase (AMPK), and determine their roles in regulating surface molecule expression. Using flow cytometry and mass spectrometry, we found that TbAMPK β or TbAMPK γ -deficient parasites express both of the major surface molecules, EP- and GPEET-procyclicin, with the latter being a form that is expressed when glucose is low such as in the tsetse fly. Last, we have found that the putative scaffold component of the complex, TbAMPK β , fractionates with organellar components and colocalizes in part with a glycosomal marker as well as the flagellum of PF parasites.

Keywords

Trypanosoma brucei; African trypanosome; nutrient sensing; procyclicins; mass spectrometry

Trypanosoma brucei, the protozoan parasite that causes African sleeping sickness, is a hemoflagellate that is transmitted to its mammalian host by the bite of an infected tsetse fly. In the mammalian infection, trypanosomes proliferate within the blood as long slender forms and can differentiate into short stumpy forms that are pre-adapted for life in the insect. When the insect ingests short stumpy trypanosomes during a blood meal, the parasites differentiate into procyclic form parasites (PF¹) in the fly midgut (Vickerman, 1985). After about 3 weeks, they are found in the salivary glands where they develop into metacyclic forms that are infectious to mammals.

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¹Abbreviations used: AMPK, AMP-activated kinase; Con A, concanavalin A; aq. HF, aqueous hydrofluoric acid; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; PF, procyclic form trypanosome; RNAi, RNA interference; TbAMPK β , *T. brucei* homolog of AMP-dependent kinase β subunit; TbAMPK γ , *T. brucei* homolog of AMP-dependent kinase γ subunit; tet, tetracycline;

The PF parasites are covered by a coat consisting of members of the procyclin family of glycoproteins (Matthews and Gull, 1994, Roditi, et al., 1989, Ziegelbauer, et al., 1990). The GPI-anchored procyclins, EP-procyclins and GPEET-procyclin, have at their C-terminus 22–30 Glu-Pro repeats (EP-procyclin) or five or six Gly-Pro-Glu-Glu-Thr repeats followed by three EP repeats (GPEET-procyclin) (Mowatt and Clayton, 1987, Mowatt, et al., 1989, Richardson, et al., 1988, Roditi, et al., 1987). The different isoforms of procyclins have different features. For example, EP1-1, EP1-2, and EP3 are *N*-glycosylated with a homogeneous Man₅GlcNAc₂ while EP2 is unglycosylated (Acosta-Serrano, et al., 1999, Hwa, et al., 1999, Treumann, et al., 1997). GPEET is unglycosylated but is phosphorylated at the threonine residues (Butikofer, et al., 1999, Mehlert, et al., 1999). Expression of procyclins can be influenced by culture conditions *in vitro*. Glycerol in the culture medium triggers GPEET expression, as can growth in glucose-depleted (~ 0.03 mM) medium (Butikofer, et al., 1997, Morris, et al., 2002, Treumann, et al., 1997). Early in the *in vitro* differentiation of bloodstream forms to PFs, both types of procyclins are expressed (Vassella, et al., 2001). GPEET expression increases in the first 24 hours, to be replaced by glycosylated EP-procyclins later in development (Vassella, et al., 2001). A similar program of expression has been observed in parasites isolated from tsetse fly infections, though insufficient parasites were available for analysis prior to day 3 of the infection (Acosta-Serrano, et al., 2001, Vassella, et al., 2000). Although the function of the different procyclin isoforms remains to be determined (Vassella, et al., 2009), it is suggested that its polyionic nature may help the parasite to resist attack by tsetse midgut proteases (Acosta-Serrano, et al., 2001).

Previously, we screened an RNAi-based genomic library for cells resistant to the lectin concanavalin A (Con A), which binds the *N*-glycan on EP-procyclins and induces cell death (Hwa, et al., 1999, Pearson, et al., 2000, Welburn, et al., 1996). We found that glycolysis (or the rate of glycolytic flux) was a regulator of procyclin expression. Under conditions in which the rate of glycolysis was reduced, EP-procyclin expression was repressed, while GPEET-procyclin expression was upregulated.

Complicating the connection between glucose metabolism and surface molecule expression is the compartmentalization of a majority of glycolysis in peroxisome-like organelles, the glycosomes. This compartmentalization suggests that some extra-glycosomal metabolic sensor may be involved in the transmission of information about glycolysis to the nucleus. To resolve the components that connect glucose metabolism and surface molecule expression, we have used reverse genetics to characterize genes that were predicted to be involved in glucose sensing based on observations from other systems. In other organisms, AMP-activated kinase (AMPK), a heterotrimeric enzyme complex consisting of α , β , and γ subunits, is a key regulator in nutrient sensing (reviewed in (Carling, 2004)). The α subunit contains a kinase domain as well as a regulatory domain that inhibits the enzyme in the absence of AMP (Crute, et al., 1998). The β subunit acts as a scaffold for the other components, while the γ subunit is thought to be involved in AMP binding (Cheung, et al., 2000).

AMPK is a member of the AMPK/SNF1 family, which has been described in many eukaryotes (Hardie, et al., 1998, Hardie and Hawley, 2001, Kemp, et al., 1999) and acts in nutrient sensing pathways in yeast and mammals. Under conditions that reduce glycolysis, the yeast SNF1 complex (a homolog of AMPK (Carlson, 1999)) is activated. This kinase modulates the cellular response to decreased glycolysis by phosphorylating (and activating) metabolic enzymes, including glycolytic enzymes such as hexokinase. Similarly, low glucose conditions activate mammalian AMPK (Salt, et al., 1998), which triggers changes in gene expression, including increase in expression of metabolic genes involved in glycolysis (reviewed in (Hardie and Hawley, 2001)).

While the α subunit remains elusive, we have identified homologous genes for the β and γ subunits (TbAMPK β and TbAMPK γ , respectively) of the heterotrimeric components (α , β , and γ) of AMPK in *T. brucei* and have explored the role of these genes in the regulation of surface molecule expression in response to glucose levels. Silencing these genes triggers changes in surface molecule expression, while localization of the scaffold (β) subunit suggests positioning in the cell consistent with a role as an intermediary between surface molecule expression and glycolysis.

MATERIALS AND METHODS

Trypanosome growth and RNAi

PF 29-13 *T. brucei*, a 427 strain that expresses T7 RNA polymerase and the tetracycline repressor, were grown in SDM-79 as described (Wang, et al., 2000, Wirtz, et al., 1999). Low glucose SDM-79 was prepared with glucose-free RPMI 1640 replacing the liquid MEM. Also, additional glucose and glucosamine (normally present in SDM-79 at 1 mg/ml and 50 ng/ml, respectively) were omitted. This mixture was supplemented with normal FBS (10%), resulting in a final glucose concentration of ~ 0.5 mM.

RNAi constructs were generated by ligation of *TbAMPK β* (276 bp, from nt 436 to 711 of the open reading frame) or *TbAMPK γ* (461 bp, from nt 895 to 1356) PCR products amplified from *T. brucei* strain 427 genomic DNA into *XhoI/HindIII* cut pZJM (Wang, et al., 2000). Parasites (1×10^8) were transformed with 10 μ g *NotI* linearized pZJM(AMPK β) or pZJM(AMPK γ) and stable integrants selected with phleomycin (2.5 μ g/ml) as described (Wang, et al., 2000). RNAi was induced by the addition of tetracycline (tet, 1 μ g/ml) to cultures.

Flow cytometry and surface glycoprotein labeling

Parasites were labeled with fluorescein-conjugated Con A as described (Morris, et al., 2002). Briefly, cells (1×10^6) were resuspended in 1 ml cytomix supplemented with 1 mM MnCl₂ (cytoM (van den Hoff, et al., 1992)) containing 10 μ g/ml FITC-labeled Con A (Sigma, St. Louis, MO). After 15 min at room temperature, trypanosomes were applied to a FACScan flow cytometer (Becton Dickinson Biosciences, Franklin Lakes, NJ) and 10,000 cells analyzed per sample. Antibody labeling of surface molecules was performed using 5×10^6 parasites that were washed in PBS, fixed in 4% paraformaldehyde/0.1% glutaraldehyde (12 hr, 4°C), and washed in 1% BSA in PBS (blocking solution). Antibodies to EP-procycalin (monoclonal antibody TBRP1/247, Cedarlane Laboratories, Ontario Canada (Richardson, et al., 1988, Richardson, et al., 1986)) or GPEET-procycalin (monoclonal 5H3 (Butikofer, et al., 1999)), which was a generous gift from Dr. Terry Pearson (University of British Columbia) were diluted (1:100) and incubated with cells (1hr, 4°C) in blocking solution. Parasites were washed, incubated in FITC-conjugated goat anti-mouse antibody (1 hr, 4°C in blocking solution), and then resuspended in sheath fluid for flow cytometry.

MALDI-TOF-MS analysis of procyclins

For mass-spectrometry analysis, procyclins were purified from freeze-dried parasites (1×10^8) by sequential extraction with organic solvents (Acosta-Serrano, et al., 1999). To remove GPI anchors from polypeptides, dry butanolic extracts were dephosphorylated with 25 μ l of 48% aqueous hydrofluoric acid (aq. HF) for 20 h at 0 °C. After treatment, samples were dried in a Speed-Vac and resuspended in 20 μ l of 0.1% TFA. An aliquot (~ 0.5 μ l; ~ 5×10^6 parasite equivalents) was co-crystallized with 0.5 μ l 10 mg/ml sinapinnic acid in 70 % acetonitrile, 0.1 % TFA and analyzed by positive-ion mode. Data collection was in linear mode on a PerSeptive Biosystems Voyager-DE mass spectrometer (located at the Sir Henry Wellcome Functional Genomics Facility, Glasgow University). The accelerating voltage was 2500 V and the grid voltage was set at 91 % with an extraction time delay of 100 nsec. Data were collected manually

at 200 shots per spectrum, with laser intensity set at 2800. To confirm assignments, HF-treated samples ($\sim 5 \times 10^7$ parasite equivalents) were submitted to mild acid hydrolysis with 40 mM TFA for 20 min at 100 °C, and an aliquot analyzed by MALDI-TOF-MS as described above (Acosta-Serrano, et al., 1999).

Digitonin fractionation and Hexokinase assays

Digitonin fractionation was performed using a method adapted from (Lorenz, et al., 1998). Parasites (1×10^7) were washed in cold PBS, followed by a wash in STE buffer (250 mM sucrose, 25 mM Tris, pH 7.4, 1 mM EDTA). Cells were resuspended in STE+N (150 mM NaCl) with 0.1 M PMSF, pelleted, and resuspended in STE+N with digitonin (0.2 mg/ml). The mixture was vortexed for 5 min and then incubated at 25°C for 4 min. Lysates were centrifuged (2 min, $15,000 \times g$, 4°C) and pellets resuspended in 0.1 M TEA, pH 7.5 (5×10^5 cell equivalents/ml).

Hexokinase assays were performed in a coupled assay as previously described on equal volumes of the resulting supernatant and pellet from the digitonin fractionation (Misset and Opperdoes, 1984, Morris, et al., 2002). Assays were performed in triplicate in 96-well microtiter plate format using a GENios spectrophotometer (Phenix Research Products, Hayward, CA).

Localization of TbAMPK β subunit

To localize TbAMPK β , PF parasites expressing eYFP fused to the *T. brucei* aldolase peroxisomal targeting sequence 2 (PTS2, which targets proteins to glycosomes) were harvested by centrifugation at $800 \times g$, washed with Voorheis's modified PBS (vPBS; 137 mM NaCl, 3 mM KCl, 16 mM Na₂HPO₄, 3 mM KH₂PO₄, 46 mM sucrose, 10 mM glucose, pH 7.6) and fixed in an equal volume of 6% paraformaldehyde and vPBS for 1 hr on ice. Cells were washed with vPBS and then allowed to settle on poly-lysine coated slides prior to permeabilization with 0.1% Triton X-100 in PBS (10 min, RT) and then washed in excess PBS three times before the addition of block (1% BSA and 0.25% Tween in PBS, 1 hr, RT). Primary affinity purified rabbit polyclonal antibodies for TbAMPK β , which was purified using an antigen coupled AminoLink Coupling Resin (Thermo Scientific, Rockford IL) following the manufacture's instructions, and a mouse monoclonal against GFP (3E6, Molecular Probes, Eugene, OR) were applied at a dilution of 1:10 and 1:100, respectively, for 1 hour. The slides were washed 3 times in excess PBS before the addition of the secondary antibodies (TexasRed-conjugated goat anti-rabbit and FITC-conjugated goat anti-mouse; Rockland, Gilbertsville, PA, both at 1:100). Slides were then washed 3 times in excess PBS and prepared for visualization by addition of Vectashield mounting media with DAPI. Images were taken on a Zeiss Axiovert 200M using Axiovision software version 4.6.3 for image analysis.

To express TbAMPK β with a C-terminal fusion to GFP in live parasites the TbAMPK β open reading frame was cloned into pLew111(2T7)GFP β (the generous gift of Drs. Shawn Motyka and Paul Englund, Johns Hopkins School of Medicine). PF 29-13 strain trypanosomes were transformed with linearized DNA and selected as described above. Recombinant TbAMPK β GFP expression was induced for 4 days by addition of tet (1 μ g/ml) and GFP expression monitored by microscopy.

RESULTS

Identification and silencing of AMPK subunit homologs in *T. brucei*

We have identified candidate single copy homologous genes for the β and γ subunits components of the heterotrimeric enzyme AMPK in the *T. brucei* genome database. The putative *T. brucei* AMPK β subunit (TbAMPK β systematic name Tb927.8.2450) is similar to

the rat AMPK β 1 protein (E value = 2.8×10^{-10}), sharing 33% amino acid identity. TbAMPK β is predicted to be a 34.4 kDa protein that contains a 5' AMP-activated protein kinase β -subunit complex interacting region (α/γ interaction domain, residues 195–303) found in the β subunit of mammalian AMPK and yeast homologs of AMPK (Thornton, et al., 1998) (Fig. 1A). In other systems, this domain allows interaction with the kinase (α) subunit and is characteristic of β subunits. TbAMPK β also shares with the rat AMPK β 1 protein a conserved putative N-myristoylation signal (Fig. 1A) and two (of four) conserved Ser (at residue 119 and 126) (Carling, 2004).

The putative *T. brucei* AMPK γ subunit (TbAMPK γ , systematic name Tb10.70.3670) is predicted to be a 55.2 kDa protein that is most similar to human AMPK γ 3 (E value = 1.1×10^{-11} , 27% identical at the amino acid level). The trypanosome protein is predicted to have 4 cystathionine- β -synthase (CBS) domains (residues 80–134, 187–244, 361–414, 430–484), which have been found in other γ subunits (but are not limited to AMPK γ subunits) (Fig. 1A). The CBS domains have been implicated to function in AMP binding (Cheung, et al., 2000, Daniel and Carling, 2002).

To initiate analysis of the function of TbAMPK subunits, we silenced the putative subunits using the pZJM RNAi vector. The impact of RNAi of either subunit on cell viability was minimal, causing ~2-fold increase in doubling time (not shown). Induction of RNAi for 40 hours led to a dramatic decrease in *TbAMPK β* and *TbAMPK γ* transcript abundance (Fig. 1B) (Wang, et al., 2000). Due to low transcript abundance, the northern blots of TbAMPK β were particularly difficult to assess. Since RNAi ultimately leads to protein depletion, we used western blotting to confirm the *TbAMPK β* knock down using polyclonal antibodies to recombinant TbAMPK β protein after RNAi (Fig. 1C). The ~34 kDa polypeptide was detectable by western blot from 1×10^6 parental 29–13 cell equivalents, while silencing *TbAMPK β* led to a ~5-fold reduction in detectable protein with 5×10^6 cell equivalents (Fig. 1C, lanes 3 and 4).

Bioinformatics-based approaches suggested there were 14 active members of the CAMK group of kinases in the *T. brucei* genome, which include the AMPK α subunit (Parsons, et al., 2005). Of these potential α subunits, a candidate *T. brucei* gene that clustered closely with yeast SNF1 and human AMPK α 1, Tb10.70.1760. RNAi of Tb10.70.1760 failed to yield a discernable Con A binding phenotype (see below), suggesting that we had either identified and tested the incorrect homolog, or that functional redundancy was obscuring the resolution of the α subunit. For these reasons, we have focused on the single-copy subunits for the remainder of this manuscript.

RNAi of *TbAMPK β* or *TbAMPK γ* impacts surface molecule expression

Previously, we found through an RNAi-based genomic library screen that glycolysis modulated surface glycoprotein expression in PF parasites (Morris, et al., 2002). Therefore, we reasoned that knockdown of proteins that play a role in the connection of glycolysis to surface molecule expression may yield cells with similar dis-regulation of surface molecule expression.

To determine if TbAMPK subunit silencing leads to this phenotype, we first stained TbAMPK β and TbAMPK γ -deficient cells with the lectin Con A, which predominantly binds to glycosylated EP-procycylins but not to GPEET on PF trypanosomes (Pearson, et al., 2000) (Fig. 2). We have used this approach to previously characterize the impact of silencing glycolytic enzymes on surface molecule expression (Morris, et al., 2002). The impact of silencing TbAMPK β and TbAMPK γ on Con A binding was assessed in cells grown in standard SDM-79 medium or in reduced glucose (~0.5 mM) medium for 7 days. While growth in reduced glucose medium did not alter parental PF 29-13 cell Con A-FITC binding (Fig. 2A, light gray line compared to dark grey line, and (Morris, et al., 2002)), culturing of

TbAMPK β or TbAMPK γ -deficient cells in normal medium caused a subtle reduction in Con A binding while growth in low glucose medium for 7 days caused a dramatic reduction in Con A binding (Fig. 2B and 2C, black line compared to grey line).

To further characterize the nature of the surface molecule change, we used antibodies to the major surface glycoproteins to assess expression by flow cytometry. We found that cells deficient in either TbAMPK β or TbAMPK γ have a similar glucose-dependent RNAi phenotype that is not observed in the parental cell line. Parental 29-13 PF trypanosomes predominantly expressed EP-procycalin (Fig. 3A, left panel) with little detectable GPEET-procycalin (Fig. 3A, right panel). These findings are in agreement with the procycalin repertoire that has been described from PF 29-13 parasites (Acosta-Serrano, et al., 1999, Morris, et al., 2002).

Like parental PF 29-13 cells, TbAMPK β -deficient parasites expressed EP-procycalin (Fig. 3B, left panel) and expressed very little GPEET-procycalin (Fig. 3B, right panel). However, if parasites deficient in TbAMPK β were grown under reduced glucose (~ 0.5 mM) conditions, a greater proportion expressed GPEET-procycalin (Fig. 3B, right panel, +tet, -glc). These cells continued to express EP-procycalin (Fig. 3B, left panel).

Cells with *TbAMPK γ* silenced also expressed EP-procycalin (Fig. 3C, left panel) and demonstrated a subtle increase in the surface expression of GPEET-procycalin (Fig. 3C, right panel). Under reduced glucose (~ 0.5 mM) conditions, a greater proportion expressed GPEET-procycalin (Fig. 3C, right panel, +tet, -glc). Again, these cells continued to express EP-procycalin (Fig. 3C, left panel), with both procyclins expressed for the duration of these experiments (~14 days). Addition of glucose to these cells blocked the increased expression of GPEET-procycalin (Fig. 3C, right panel, +tet - glc/+glc).

MALDI-TOF mass spectrometry confirms that normally glycosylated EP-procycalin, in addition to GPEET-procycalin, is expressed after RNAi of *TbAMPK β* or *TbAMPK γ*

Silencing either *TbAMPK β* or *TbAMPK γ* subunits triggered a loss of Con A binding, while antibody staining and flow cytometry analysis of these cells indicated that EP-procycalin was expressed on the surface. These observations seem to conflict, as Con A binds to the *N*-glycan of EP-procycalin. How is there a reduction in Con A binding while the protein to which Con A binds is still expressed? These data suggested that GPEET-procycalin could be masking the *N*-glycan of EP-procycalin. Alternatively, EP-procyclins may not be properly *N*-glycosylated in the TbAMPK β or TbAMPK γ -deficient cells, or EP-2 (a naturally occurring unglycosylated EP-procycalin) could be the dominant EP-procycalin in these cells. To resolve these possibilities, we performed MALDI-TOF mass spectrometry on partially purified procyclins from parental PF 29-13, TbAMPK β -deficient, or TbAMPK γ -deficient trypanosomes grown in low glucose medium (Fig. 4).

Expression of GPEET was confirmed by positive-ion MALDI-TOF-MS on HF-treated procycalin polypeptides. Parental PF 29-13 expressed only glycosylated EP-procycalin species (*i.e.* EP1-1, EP1-2 and EP3) (Fig. 4A). Ions representing GPEET (*m/z* 6141) or EP2-procycalin (*m/z* 8345; the only naturally unglycosylated procycalin) were not detected, consistent with the Con A and antibody binding observed by flow cytometry. In contrast, silencing of either TbAMPK β or TbAMPK γ yielded a reduction (but not complete ablation) of glycosylated EP-procyclins with a concurrent increase in expression of GPEET-procycalin. GPEET-procycalin expression is characterized by expression of full length polypeptides (*m/z* 6,141) and the presence of a truncated form lacking the N-terminus sequence VIVK (marked as "GPEET-4", Fig 4B and 4C) (Acosta-Serrano, et al., 2000). Assignments of the procyclins species present in all samples were confirmed by negative ion MALDI-TOF-MS analyses of the C-terminus after mild acid hydrolysis (not shown). Since the EP-species have the expected masses of polypeptides bearing the parental oligomannose *N*-glycan, the reduction in Con A binding

observed in TbAMPK β or TbAMPK γ -deficient cells is not likely due to alteration in *N*-glycosylation but rather is possibly due to masking of the glycan by GPEET-procycalin.

Localization of the TbAMPK β subunit homolog

In mammals, the AMPK β -1 subunit localizes to extranuclear particulate structures that are neither mitochondria nor endoplasmic reticulum (Warden, et al., 2001). The localization and activity of the AMPK complex are altered if the AMPK β -1 subunit N-myristoylation site is mutated, suggesting that this component of the complex directs the subcellular localization of the complex. To explore the localization of TbAMPK β , affinity-purified antibodies were generated against recombinant TbAMPK β . These antibodies recognized a single polypeptide (that does not react with pre-immune sera) in western blots of whole trypanosomal lysates (Fig. 5A and data not shown).

Digitonin fractionation of trypanosomes was used to separate soluble cytoplasmic proteins from organellar components. When this fraction was analyzed by western blot, TbAMPK β was detected exclusively in the particulate fraction (Fig. 5B, P). As a control, the hexokinase activity of each of the fractions was determined and ~90% of the hexokinase activity was found associated with the particulate fraction (which should include intact glycosomes). Not all proteins fractionated with the particulate matter, as a putative cyclophilin-like protein (Tbcyp22) was found with near-equal distribution in both fractions (Fig. 5B).

Immunofluorescence of parasites expressing yellow fluorescent protein (YFP) targeted to glycosomes (as a result of fusion with an N-terminal PTS2) was used to further assess the cellular localization of TbAMPK β . These parasites had a TbAMPK β distribution that overlapped with the glycosomal YFP marker but was not exclusively glycosomal (Fig. 6A). For example, signal was also detected in or near the flagellum. Similarly, live parasites expressing a fusion of TbAMPK β with GFP yielded punctate fluorescence consistent with glycosomes, as well as faint flagellar signal (Fig. 6B). YFP bearing an N-terminal PTS2 (for glycosome targeting, Fig. 6B, left panel) localizes primarily in points in live cells, while expression of GFP without a targeting sequence yielded signal throughout the parasite (Fig. 5B, right panel).

DISCUSSION

The African trypanosome inhabits two very different environments, requiring adaptation to conditions found in these different hosts. In the mammalian bloodstream, the parasite is bathed in its primary carbon source, glucose, while in the tsetse fly glucose is rapidly depleted during bloodmeal digestion. In the fly, the parasites persist by metabolism of amino acids, taking advantage of the abundance of proline produced by the fly to power its flight muscles.

Both PF and bloodstream parasites monitor glucose abundance to make developmental decisions. During differentiation from BSF to PF, parasites switch surface coats from VSG to procycalin in a process that can be triggered *in vitro* by growth in low glucose medium (Milne, et al., 1998). In the fly, PF parasites respond to changes in glucose concentrations by altering surface molecule expression. PF parasites very early in the differentiation progression express both EP- and GPEET- procyclins, but switch to express predominantly GPEET-procycalin shortly after initiation of the infection (Acosta-Serrano, et al., 2001, Vassella, et al., 2001, Vassella, et al., 2000). This switch is coincident with the fall of glucose levels during digestion of the bloodmeal. This progression can be recapitulated by growing parasites in very low (0.03 mM) glucose conditions or by using RNAi to silence glycolytic enzymes (Morris, et al., 2002). These observations suggest that glucose levels, presumably reflected in the rate of glycolysis, are being monitored to regulate GPEET-procycalin expression.

The molecular mechanisms that govern the expression of the procyclins likely differ, as both *cis*- and *trans*- factors have been identified (Vassella, et al., 2000, Walrad, et al., 2009). The 3' UTR of GPEET-procycloin contain a glucose response element required for the upregulation of GPEET-procycloin expression in response to low glucose medium and during its development in the fly (Vassella, et al., 2004). EP-procycloin expression is likely not regulated by glucose, as culturing parasites in low glucose does not ablate EP-procycloin expression (Morris, et al., 2002). Furthermore, deletion of the *TbGalE* gene, which encodes an epimerase that interconverts UDP-Glc and UDP-Gal in glycosomes, leads to a 10-fold increase in the expression of EP- but not GPEET-procycloin, suggesting that Gal metabolism is also involved in controlling the expression and copy numbers of the procycloin molecules (Roper, et al., 2005).

We have demonstrated here that TbAMPK β and γ play a role in surface molecule expression, as silencing of the genes leads to upregulation of GPEET-procycloin expression. The change in procycloin expression is only detectable when RNAi cells were grown in low glucose media, suggesting that the incomplete penetrance of the RNAi of the AMPK subunits was not alone sufficient to trigger GPEET expression.

The physical connection between glucose and surface molecule expression is problematic, as the majority of glucose metabolism is compartmentalized in the glycosome – how is a signal transmitted from the glycosome to the rest of the cell? In other eukaryotes, AMPK plays a key role in responding to cellular glucose metabolism, being activated to trigger changes throughout the cell in response to changes in glycolysis (Hardie, 2008). AMP generated as a result of glucose metabolism could activate a *T. brucei* complex that is homologous to mammalian AMPK (Fig. 7). Hexokinase and phosphofructokinase both consume ATP, which may be regenerated by a glycosomal adenylate kinase (ADKD) (Ginger, et al., 2005). In other systems, this enzyme synthesizes ATP from ADP with the concomitant production of AMP and the cells use the ATP:AMP ratio as a marker for the status of energy levels (Hardie, 2008).

Immunolocalization of TbAMPK β indicates a widespread distribution, which could reflect the role of the subunit as part of a signaling complex. Although TbAMPK β lacks a C-terminal tripeptide peroxisomal targeting sequence (PTS1) (Opperdoes, 1987) or a PTS2-type targeting sequence, the subunit partially co-localizes with glycosomes (Fig. 5A), where its activity may be influenced by ADKD (Ginger, et al., 2005). Additionally, the β subunit localizes to the flagellum, where it is positioned to interact with a previously described flagellar AK (Pullen, et al., 2004). TbAMPK β subunit localization (and function) may be dependent on the protein's myristoylated state, akin to mammalian AMPK- β 1 (Resh, 1999, Warden, et al., 2001). It is also possible that the as of yet unidentified α subunit provides cellular targeting information to the complex. The partial localization of TbAMPK β with glycosomes positions the complex to potentially monitor the status of glycolysis in the cell, which has been previously demonstrated to play a central role in the modulation of surface molecule regulation.

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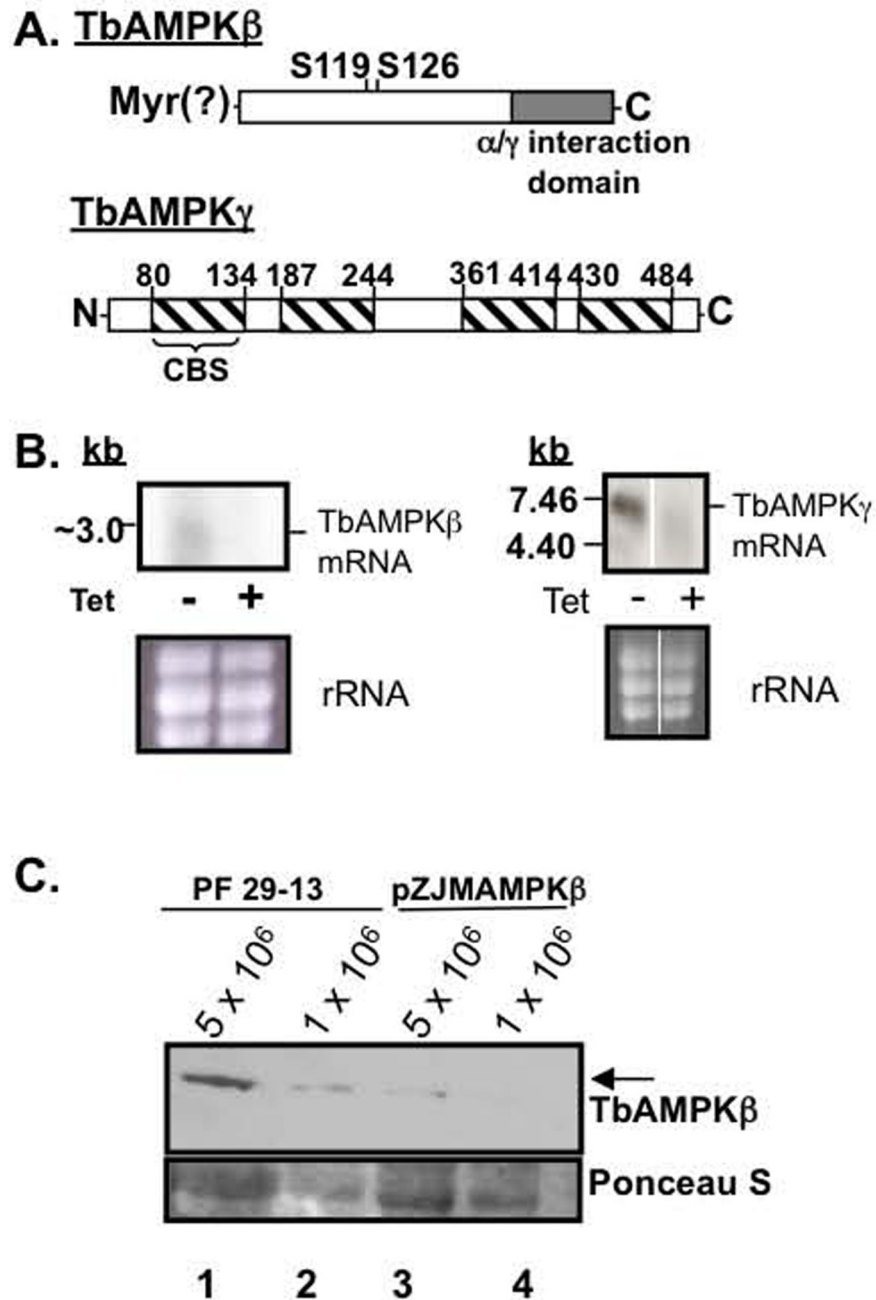


Fig. 1. Targeting *TbAMPK β* and *TbAMPK γ* by RNAi impacts transcript and protein levels. (A) *TbAMPK β* and *TbAMPK γ* diagram. *TbAMPK β* contains a putative N-myristoylation sequence (MGNTSAE), two conserved Ser residues (corresponding to Ser101 and Ser108 from rat AMPK β 1), and a β/γ interaction domain. *TbAMPK γ* contains 4 CBS domains that have been implicated in AMP binding (Cheung, et al., 2000, Daniel and Carling, 2002). (B) Analysis of RNAi of *TbAMPK β* and *TbAMPK γ* by northern blot. Total RNA from parental (–) and tetracycline induced (+) (for 40 h) cells containing the construct pZJM(*TbAMPK β*) or pZJM(*TbAMPK γ*) was purified from 5×10^7 parasites and electrophoresed on a formaldehyde 1.5% agarose gel. Ribosomal RNA levels were estimated by ethidium bromide staining to ensure

equal loading of RNA. (C.) Western blot performed on 5×10^6 or 1×10^6 cell equivalents of PF 29-13 (lanes 1–2) or cells induced 4 days to silence TbAMPK β with pZJMAMPK β (lanes 3–4) were resolved by SDS-PAGE, transferred to nitrocellulose and probed with affinity purified TbAMPK β antibodies. The bottom panel is a Ponceau S-stained band used to estimate loading.

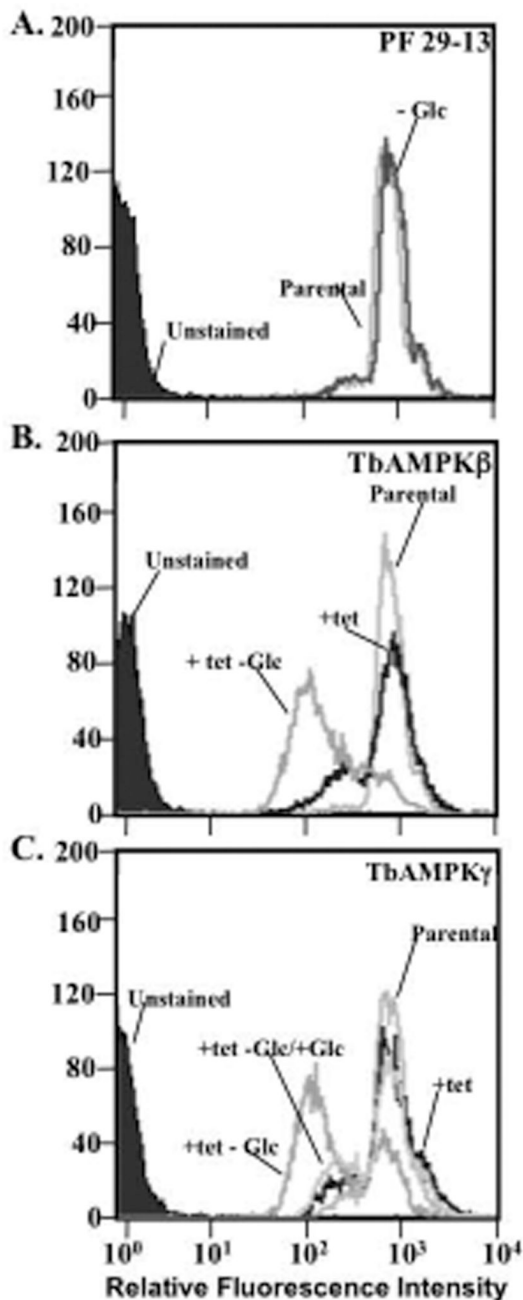


Fig. 2.

Silencing TbAMPK β or γ causes a change in surface molecule expression that is enhanced by growth in reduced glucose medium. (A.) Living PF 29-13 trypanosomes were cultured for two weeks in normal SDM-79 (parental, light grey line) or reduced glucose medium (-Glc, dark grey line) and then incubated with 10 μ g/ml fluorescein-conjugated Con A for 15 min at room temperature in cytoM. Parasites were then analyzed by flow cytometry (10,000 cells/assay). (B.) Cell line PF 29-13 (parental, light grey line), cells induced to silence TbAMPK β for 7 days (+tet, black line) and cells induced to silence TbAMPK β for 7 days grown in reduced glucose medium for 7 days (+tet -Glc, grey line) were analyzed after Con A-FITC staining. Laser intensity was adjusted to yield autofluorescence from unstained cells (purple shade) of

~3 fluorescence intensity units. (C.) Cells in which TbAMPK γ was silenced for 7 days (+tet, black line), trypanosomes induced to silence TbAMPK γ for 7 days grown in reduced glucose medium (+tet, -Glc, grey line), and cells induced to silence TbAMPK γ for 7 days grown in reduced glucose with glucose supplemented (5 mM) (+ tet, -Glc/+Glc, grey line) for 7 days were analyzed.

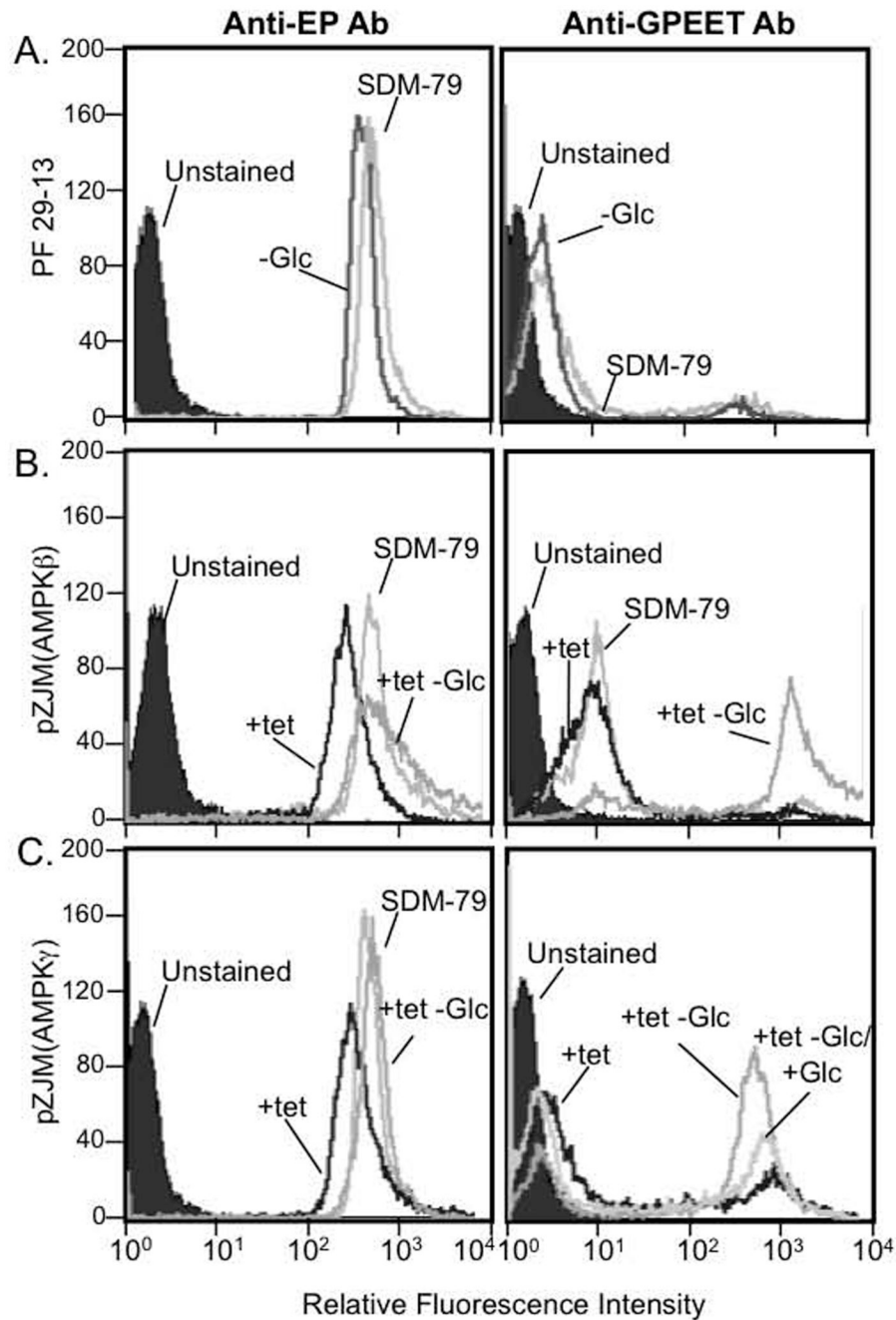


Fig. 3. Trypanosomes deficient in either TbAMPK β or TbAMPK γ express GPEET-procycalin. Parasites were grown in normal SDM-79 (light grey line) or reduced glucose medium (-Glc, dark grey line), and cells induced to silence TbAMPK subunits for 7 days (+tet). Induced cells were also grown in reduced glucose medium (+tet -Glc), or in media with glucose supplemented (5 mM) (+ tet, -Glc/+Glc). Parasites (5×10^6) were fixed and stained with antibodies to EP-procycalin (TBRP1/247) or GPEET-procycalin (monoclonal 5H3). Primary antibodies were detected with FITC-conjugated anti-mouse secondary antibody and cells (10,000) analyzed by flow cytometry.

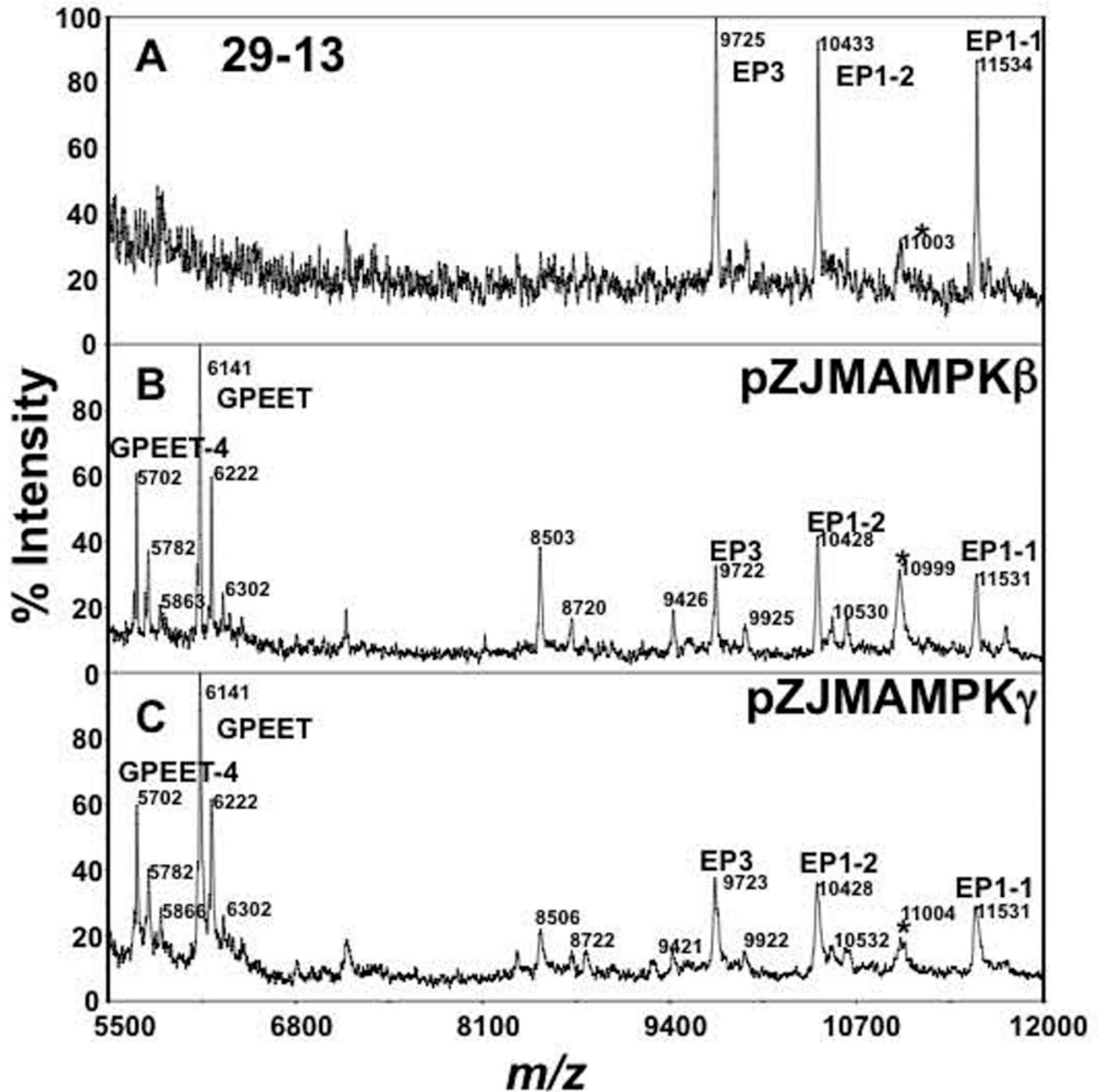


Fig. 4. Mass spectrometry analysis of procyclins. Butanol extracts of (A) PF 29-13, (B) TbAMPK β , and (C) TbAMPK γ silenced cells grown in low glucose medium were dephosphorylated with aqueous HF and aliquots analyzed by MALDI-TOF-MS in positive mode. Some of the unlabelled ions in B and C correspond to fragments of EP-procyclins that lack 10 amino acids from the N-terminus (Acosta-Serrano, et al., 2000). Asterisks represent a contaminant that has been previously assigned as KMP-11 (Acosta-Serrano, et al., 1999).

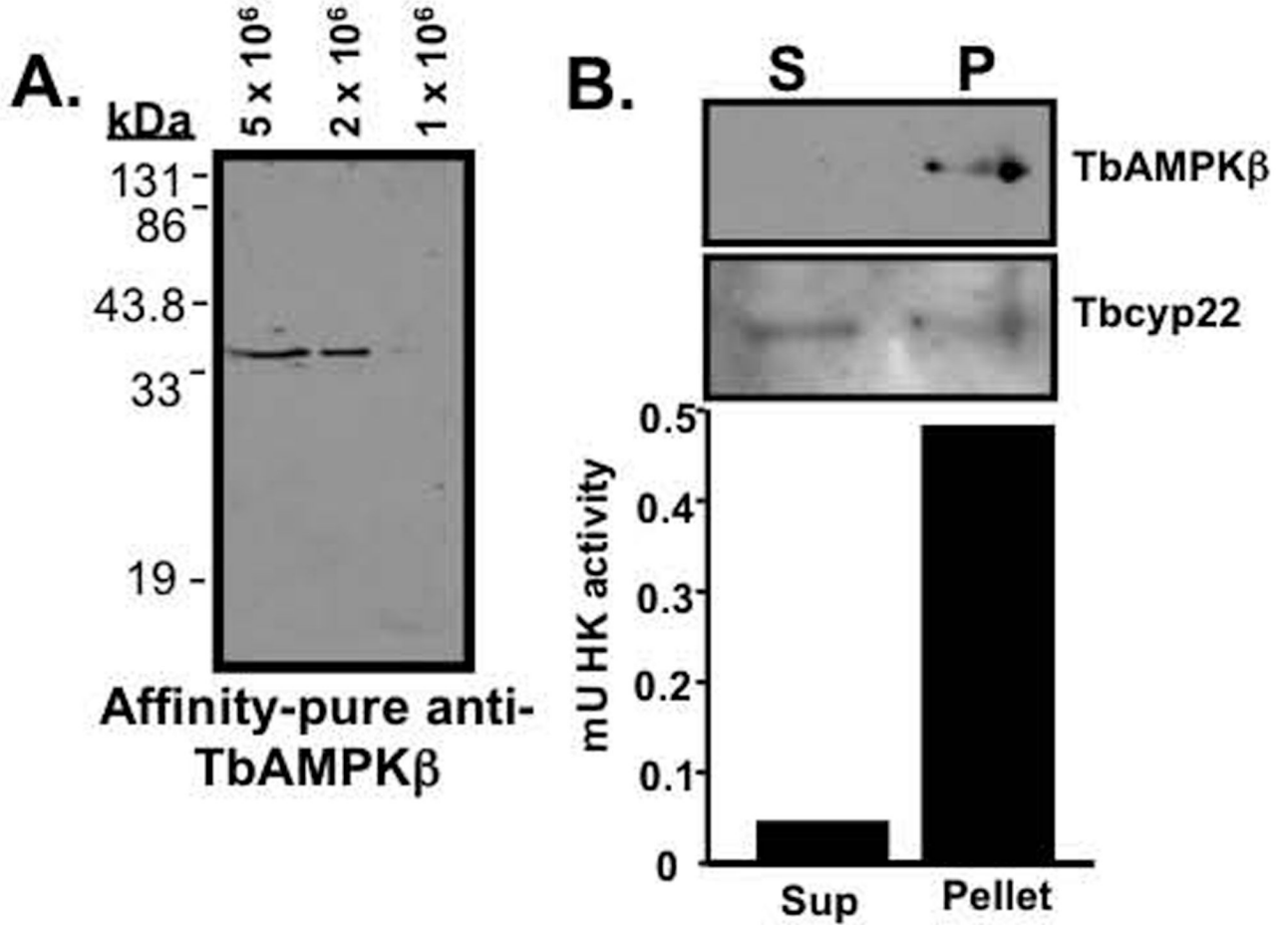


Fig. 5.

TbAMPK β has an organellar digitonin fractionation. (A.) Affinity-purified antibodies raised against TbAMPK β recognize a single polypeptide in total *T. brucei* cell lysates. (B.) TbAMPK β partitions with the particulate fraction of digitonin permeabilized cells. Following fractionation, equal volumes of supernatant and resuspended pellet (1×10^6 cell equivalents/lane) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to either TbAMPK β (1:100) or Tbcyp22 (1:100). Primary antibodies were detected with HRP-conjugated goat anti-mouse antibodies (1:10,000). To explore hexokinase activity, fractions were assayed in a coupled hexokinase reaction, with the conversion of NAD to NADH by glyceraldehyde-3-phosphate dehydrogenase monitored by spectrophotometer.

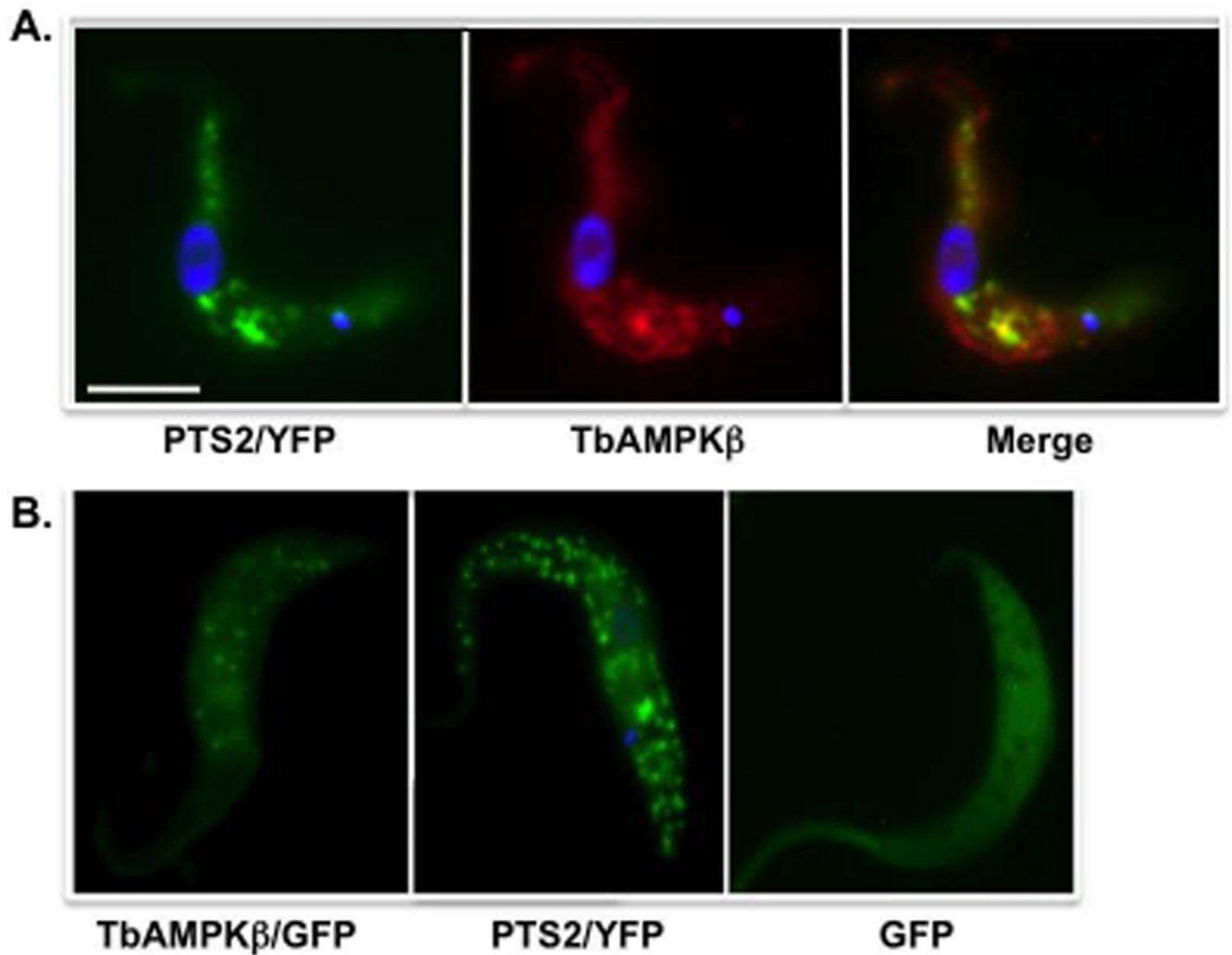


Fig. 6. TbAMPK β is localized to punctate bodies that partially co-localize with glycosomes. (A.) Immunofluorescence using affinity-pure polyclonal sera raised against recombinant TbAMPK β . Transgenic PF 29-13 harboring pXS2(PTS2YFP) were fixed, permeabilized, and stained with a mouse anti-GFP monoclonal antibody (left panel) or affinity-purified polyclonal anti-TbAMPK β antibodies (center panel). Primary antibodies were detected with FITC-conjugated goat anti-mouse or mouse anti-rabbit antibodies, respectively. Scale bar = 5 μ m. (B.) Expression of TbAMPK β fused to GFP in live cell using pLew111(2T7)GFP β (middle panel) Transformed trypanosomes were induced to express the recombinant fusion protein for 4 days by addition of tet (1 μ g/ml). For comparison, live cells expressing glycosomally targeted YFP (left panel) or GFP without a targeting sequence (right panel) are included. Cells were washed, resuspended in PBS and spotted on slides after mixing with an equal volume of antifade reagent.

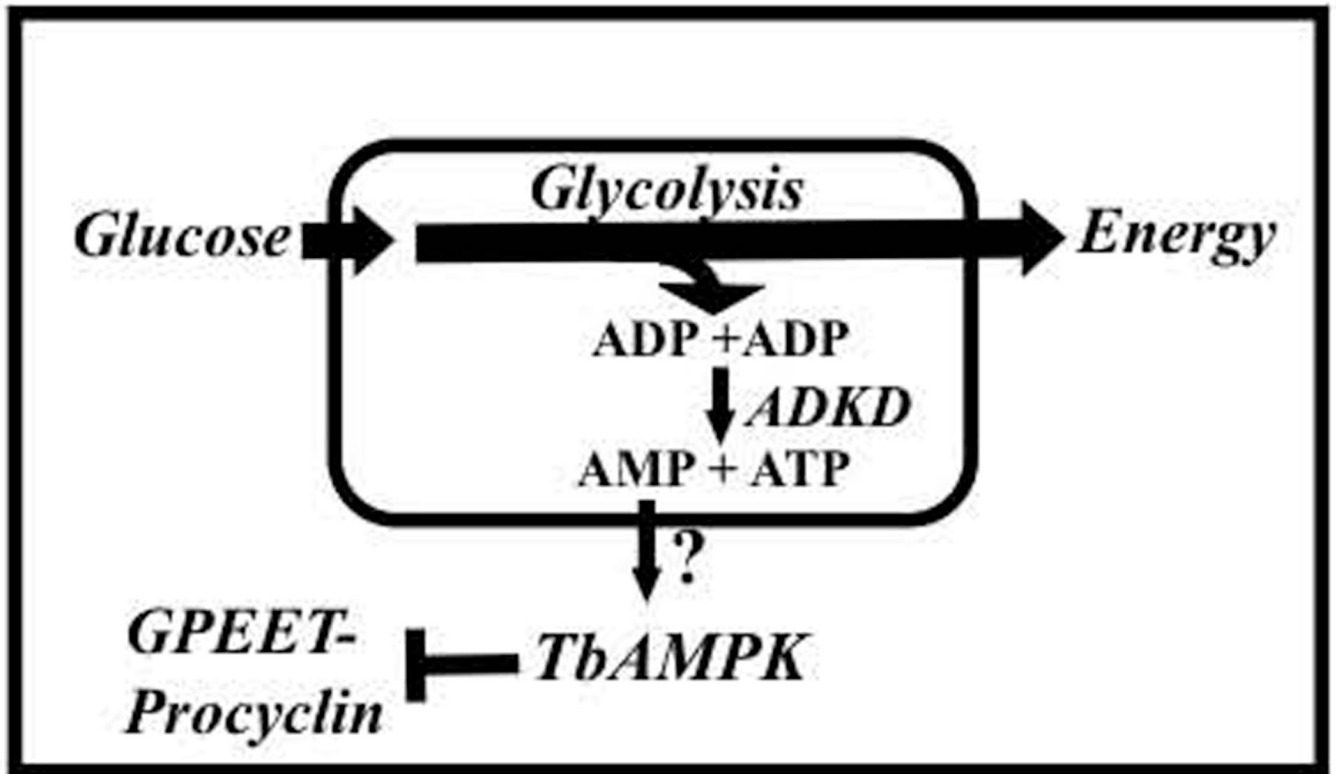


Fig. 7.

A schematic representation of the connection of TbAMPK to surface molecule expression. The pathway labeled with a question mark remains to be resolved, as extra-glycosomal AMP may function to activate TbAMPK. ADKD, glycosomal adenylate kinase.