

# Developmentally regulated instability of the *GPI-PLC* mRNA is dependent on a short-lived protein factor

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Received January 11, 2005; Revised February 4, 2005; Accepted February 23, 2005

## ABSTRACT

The expression of the vast majority of protein coding genes in trypanosomes is regulated exclusively at the post-transcriptional level. Developmentally regulated mRNAs that vary in levels of expression have provided an insight into one mechanism of regulation; a decrease in abundance is due to a shortened mRNA half-life. The decrease in half-life involves *cis*-acting elements in the 3' untranslated region of the mRNA. The *trans*-acting factors necessary for the increased rate of degradation remain uncharacterized. The *GPI-PLC* gene in *Trypanosoma brucei* encodes a phospholipase C expressed in mammalian bloodstream form, but not in the insect procyclic form. Here, it is reported that the differential expression of the *GPI-PLC* mRNA also results from a 10-fold difference in half-life. Second, the instability of the *GPI-PLC* mRNA in procyclic forms can be reversed by the inhibition of protein synthesis. Third, specifically blocking the translation of the *GPI-PLC* mRNA in procyclic forms by the inclusion of a hairpin in the 5' untranslated region does not result in stabilization of the mRNA. Thus, the effect of protein synthesis inhibitors in stabilizing the *GPI-PLC* mRNA operates in *trans* through a short-lived factor dependent on protein synthesis.

## INTRODUCTION

Protein coding genes in *Trypanosoma brucei* and other kinetoplastid protozoa are arranged in arrays (1,2) and tens of genes are co-transcribed from distant promoters (3–7). Individual mRNAs are monocistronic and are co-transcriptionally processed from a pre-mRNA by two steps, a *trans*-splicing reaction that adds a capped 39 base mini-exon (8) to the 5' end

of the mRNA and a cleavage and polyadenylation reaction at the 3' end. The *trans*-splicing is functionally linked to the polyadenylation of the 5' flanking mRNA (9–11). The *trans*-splicing reaction is mechanistically similar to *cis*-splicing (12) and orthologues of many of the factors required for polyadenylation in crown group eukaryotes are present in the genomes of kinetoplastids (13), but the canonical AAUAAA motif for the site of polyadenylation is not present.

With some exceptions, there does not appear to be extensive clustering of genes into operon-like arrays of obviously functionally linked genes. In contrast, mRNAs derived from adjacent genes are often present at different levels and can show different developmental expression patterns [see (14) for an example]. In trypanosomes, there is regulation of transcription of the two dominant developmentally regulated cell surface proteins, the variant surface glycoprotein and procyclin (15–18), but these are transcribed by RNA polymerase I and represent a special case (3,19–21). The vast majority of the protein coding genes are transcribed by RNA polymerase II and there is no definitive evidence for transcriptional control of these genes (22). If adjacent genes are transcribed at the same rate, then the regulation of individual mRNA levels must be post-transcriptional. The mechanism of post-transcriptional regulation that has attracted most investigation is mRNA half-life and especially the involvement of regulation of half-life in differentially expressed mRNAs that are more abundant in one life cycle stage than in another (22). In trypanosomes, there are two life cycle stages amenable to experimental manipulation: the bloodstream form trypanosome, normally resident in a mammal, and the procyclic form present in the insect midgut. The bloodstream form differentiates into the procyclic form in the insect and the process can be reproduced in culture (23). Most effort has concentrated on mRNAs that are more abundant in procyclic forms and the identification of the *cis*-acting sequences necessary for instability in bloodstream forms (24–26). This work has identified U-rich elements (UREs) within the 3'UTR of three mRNAs that are required for instability of these mRNAs in

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bloodstream forms (27,28). The pathways by which unstable mRNAs are degraded have received less attention. There is evidence for involvement of the exosome (29), a complex of 3' to 5' exonucleases (30), and other nucleases as both 5' to 3' and 3' to 5' exonuclease intermediates have been observed (31). The most striking inhibition of degradation of unstable mRNAs occurred after inhibition of protein synthesis; procyclic form-specific *EP* procyclin mRNA in bloodstream form trypanosomes (32–34) and logarithmic phase-specific *GP63* mRNA in stationary phase *Leishmania chagasi* promastigotes (35).

The *GPI-PLC* gene encodes a non-essential glycosylphosphatidylinositol-specific phospholipase C expressed in bloodstream form, but not procyclic form trypanosomes (36–38). The mRNA is moderately abundant in bloodstream forms, but almost undetectable in procyclic forms (37,39), and there is no difference in the transcription rate in the two life cycle stages (39). In this paper, the differential expression of *GPI-PLC* mRNA in the two life cycle stages is investigated and it is shown that the developmental regulation of steady-state mRNA levels is underpinned by a 10-fold reduced half-life in procyclic forms. Inhibition of protein synthesis causes a rapid stabilization of *GPI-PLC* mRNA in procyclic forms resulting from an increase in mRNA half-life. However, specifically blocking the translation of the *GPI-PLC* mRNA in procyclic forms does not lead to stabilization of the mRNA. Thus, it is likely that the stabilization of the *GPI-PLC* mRNA caused by inhibition of protein synthesis results from the dissipation of a polypeptide with a very short half-life that is necessary for the degradation of the unstable *GPI-PLC* mRNA in procyclic forms.

## MATERIALS AND METHODS

### Trypanosomes

The stock Lister 427 was used throughout (40). Bloodstream forms expressing either VSG MITat 1.5 (118) or MITat1.2 (221) were grown in culture in HMI-9 medium (41) containing 10% foetal bovine serum. *In vitro* differentiation of bloodstream forms to procyclic forms was performed using DTM with 15% foetal bovine serum containing 3 mM citrate and 3 mM *cis*-aconitate at 27°C (23). Procyclic forms derived *in vitro* from bloodstream forms were grown in DTM containing 15% foetal bovine serum. Established procyclic form cell lines (Lister 427) were grown in SDM-79 containing 10% foetal bovine serum (42). For the tetracycline-inducible expression of the *GPI-PLC* gene, Lister 427 procyclic forms with the plasmids pLEW29 and pLEW13 integrated into the tubulin locus (427 29-13) were used (43) (a gift from Professor George Cross, Rockefeller University). Sinefungin was used at a final concentration of 2 µg/ml and cycloheximide at 50 µg/ml, this latter concentration is sufficient for maximal inhibition of [<sup>35</sup>S]-methionine incorporation into trichloroacetic acid precipitable material (44).

### Transgenic trypanosomes

The over-expression of *GPI-PLC* mRNA in procyclics was achieved using a plasmid derived from pLEW100 (43). First,

the *EP* procyclin 5'UTR and luciferase gene were removed using a complete BamHI and partial XmaI digest and replaced with a polylinker containing BstBI and HindIII sites to produce p1865. Second, a ClaI HindIII fragment containing all of the *GPI-PLC* gene (37) plus processing signals for the flanking genes (Figure 4a) was cloned into the BstBI and HindIII sites of p1865 to produce p1885. The plasmid p1885 was linearized with NotI and electroporated into Lister 427 29-13 procyclic forms and integrants selected using zeocin.

*GPI-PLC*  $-/-$  trypanosomes were generated from bloodstream form trypanosomes expressing VSG MITat 1.5 using constructs described previously (38) except that a bleomycin-resistance gene was used in place of a neomycin-resistance gene. The *GPI-PLC* gene was returned to the endogenous locus using the construct shown in Figure 4b. The neomycin-resistance cassette contained the *EPI* procyclin mini-exon addition signal and 5'UTR, and the  $\beta$ -tubulin 3'UTR and polyadenylation site. It was located 400 bp to the 3' side of the putative *GPI-PLC* polyadenylation site.

### Cell harvesting

For most experiments, cells—typically 10 ml of procyclic form culture at  $5 \times 10^6$  cells/ml—were harvested by centrifugation at 1200 g for 7 min, the pellet was resuspended in 1 ml phosphate-buffered saline, transferred to an Eppendorf tube and cells recovered by centrifugation at 8000 g for 30 s, the supernatant was removed and the cell pellet lysed in the appropriate buffer from the RNA preparation kit (see below). This procedure took between 9 and 10 min from culture to lysis. For the determination of *GPI-PLC* mRNA half-life in procyclic cells (Figure 1c), cells were harvested by centrifugation at 6000 g for 1 min, the supernatant was aspirated away and the cell pellet lysed in the appropriate buffer from the kit. This procedure took ~3 min from culture to lysis.

For western blot analysis, cells were washed twice with 0.5 culture volumes of phosphate-buffered saline prior to lysis in SDS-PAGE sample buffer (45).

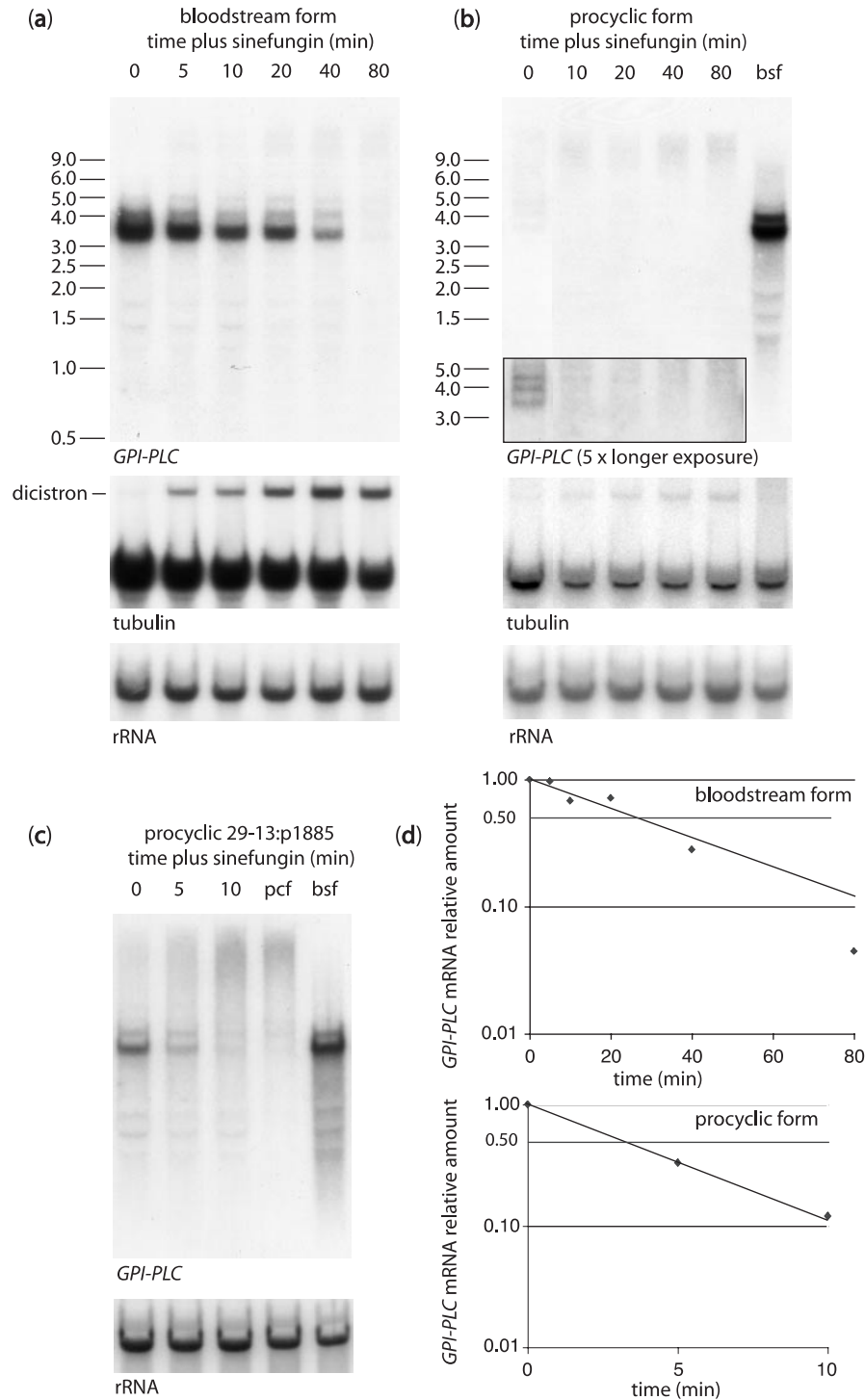
### RNA and protein analysis

RNA was prepared using the RNeasy kit from Qiagen and analysed using vertical agarose gels after denaturing the RNA with glyoxal (46). Northern blotting was as described in (47). After hybridization, all blots were washed in 15 mM sodium chloride 1.5 mM trisodium citrate (0.1× SSC), 0.1% sodium dodecyl sulphate at 60°C. Routinely, blots were probed sequentially for *GPI-PLC*, then  $\alpha$ - and  $\beta$ -tubulin sometimes including *EP* procyclin at the same time, then rRNA. All time courses were performed twice and gave the same results within the accuracy of the phosphorimager measurements (see below).

SDS-PAGE (45) and western blots (48) were performed using standard procedures.

### Phosphorimager analysis

Data were collected using a Molecular Dynamics Storm 840 and analysed using the accompanying ImageQuant



**Figure 1.** Estimation of *GPI-PLC* mRNA half-life following inhibition of mRNA maturation with sinefungin. Northern blots showing decay of *GPI-PLC* mRNA after sinefungin addition in (a) bloodstream forms and (b) procyclic forms and (c) transgenic procyclic forms expressing a *GPI-PLC* gene from a strong promoter. In each case, the same blot was probed with  $\alpha$ - and  $\beta$ -tubulin to show the sinefungin-dependent appearance of dicistronic tubulin mRNAs and then for an rRNA to estimate comparative loading. The probe is indicated under each blot. The inset in panel (b) shows a five times longer exposure of region of the blots containing the *GPI-PLC* mRNA. (d) The *GPI-PLC* mRNA and rRNA were quantitated in (a) and (c) using a phosphorimager and the *GPI-PLC* mRNA determined as a concentration relative to the amount of rRNA. RNA size standards are shown in panels (a) and (b).

software. Individual bands were measured and background subtracted. Loading differences were measured using the signal from an rRNA overprobe of the same blot and the mRNA signal adjusted for loading. The error in the

measurement procedure was estimated by comparing values for the same sample loaded two or three times on the same gel. The range of values fell within  $\pm 15\%$  of the mean value.

## RESULTS

### A difference in mRNA half-life underlies the developmentally regulated expression of the *GPI-PLC* gene

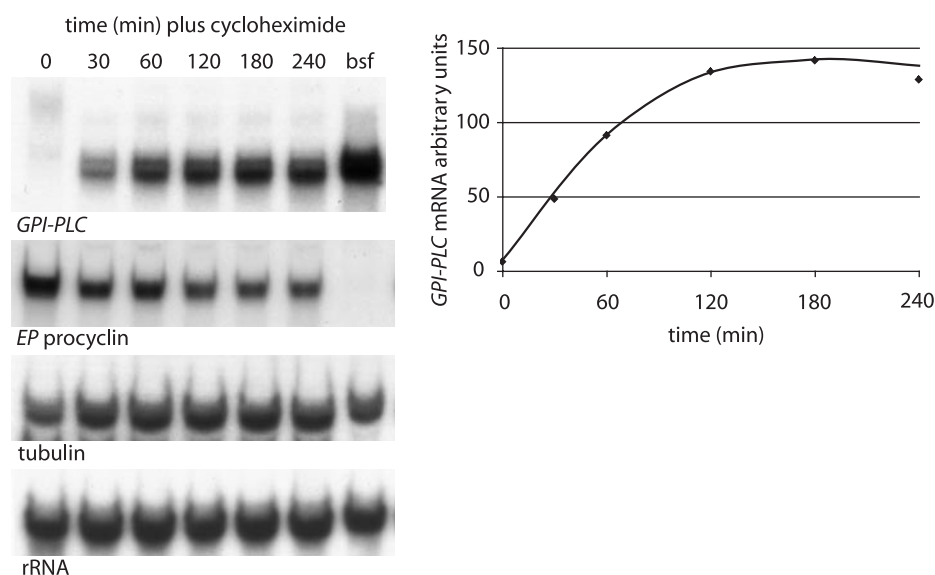
Expression of the *GPI-PLC* gene is developmentally regulated, the mRNA is readily detected in bloodstream form trypanosomes, but is barely detectable in procyclic forms (37). As the *GPI-PLC* gene is located in a presumed polycistronic transcription unit on chromosome 2 (2) and is flanked by two constitutively expressed genes *HSP100* and  $\beta'$ -*COP* (49,50), it appeared likely that the developmental regulation of the *GPI-PLC* mRNA is post-transcriptional. To test this, the half-life of the *GPI-PLC* mRNA in the two developmental stages was estimated by determining the rate of decay after blocking mRNA maturation using sinefungin, which blocks methylation of the 5' cap on the mini-exon donor RNA, thus inhibiting *trans*-splicing which in turn blocks polyadenylation (9,11,51). Over the course of the experiments, the sinefungin had no effect on cell motility and the cells retained normal morphology as judged by phase contrast microscopy. One effect of sinefungin was to cause an immediate accumulation of incompletely processed tubulin mRNAs as previously described (11), most visible as dicistronic RNAs on the northern blot (Figure 1a). The use of sinefungin to follow mRNA decay was used in preference to an inhibitor of transcription, such as actinomycin D, as the point of action in blocking mRNA maturation removes any need to consider the fate of partially processed transcripts. The effect of sinefungin was very rapid and allowed the measurement of mRNA half-lives of as little as 3 min (see below).

The major form of the *GPI-PLC* mRNA detected on northern blots was 3.6 kb (Figure 1a). The half-life of the 3.6 kb *GPI-PLC* mRNA was  $30 \pm 5$  min in bloodstream forms (Figure 1d). In procyclic forms, the *GPI-PLC* mRNA was

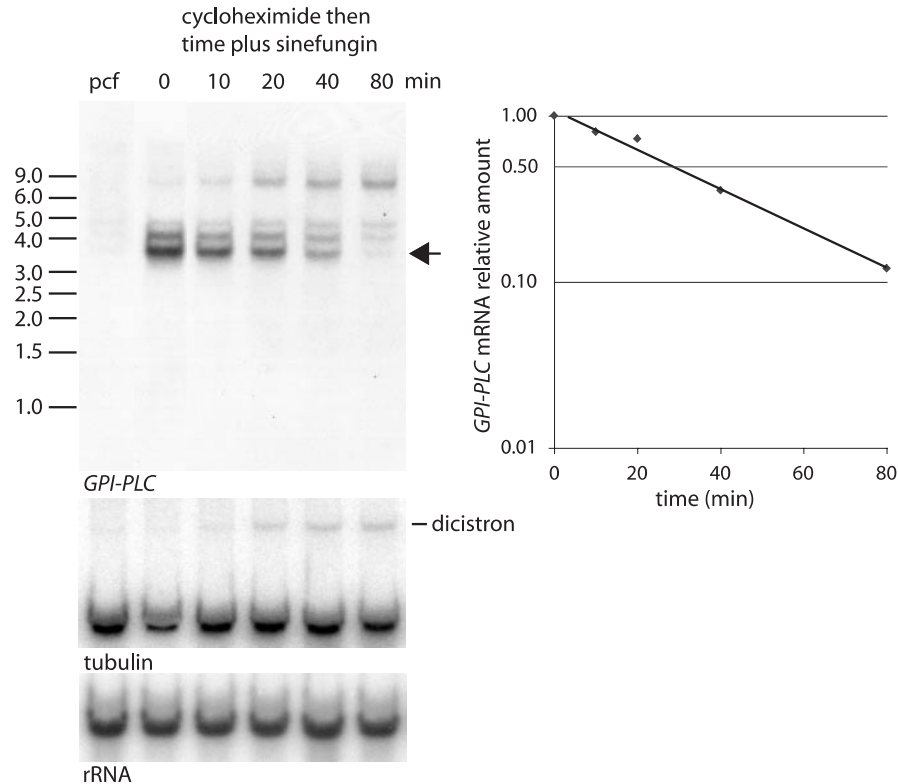
detected only after a long exposure of the northern blot and was absent at the 10 min sinefungin time point suggesting a very short half-life (Figure 1b). The low steady-state levels in procyclic forms made an estimate of the half-life technically difficult, so a procyclic cell line with a *GPI-PLC* transgene expressed from the strong *EP* procyclin promoter (43) was produced with the aim of increasing the steady-state level of the *GPI-PLC* mRNA at the beginning of the time course. In addition, the cell harvesting procedure was altered so that cells were lysed in 3 min from culture instead of the 10 min taken in the previous experiments. The over-expression strategy was successful and permitted the estimation of half-life after addition of sinefungin (Figure 1c). The half-life of the *GPI-PLC* mRNA was  $3 \pm 1$  min (Figure 1d). Thus, the half-life of *GPI-PLC* mRNA is approximately 10-fold greater in bloodstream forms than in procyclics.

### Inhibition of protein synthesis in procyclic forms leads to a rapid accumulation of *GPI-PLC* mRNA due to an increase in half-life of the mRNA

Inhibition of protein synthesis in bloodstream form trypanosomes leads to a rapid accumulation of the normally unstable *EP* procyclin mRNA (32–34). A similar effect was observed when procyclic forms were treated with cycloheximide and the steady-state levels of *GPI-PLC* mRNA determined (Figure 2). There was readily detectable accumulation 30 min after cycloheximide addition, and the mRNA reached a steady-state level after 2 h. A similar stabilization of the *GPI-PLC* mRNA was also observed with anisomycin, another protein synthesis inhibitor (data not shown). The effect of protein synthesis inhibitors on the *GPI-PLC* mRNA was not a general phenomenon; over the time course of cycloheximide treatment, the levels of  $\alpha$ - and  $\beta$ -tubulin mRNAs increased but less than 2-fold whereas the levels of *EP* procyclin mRNA decreased (Figure 2).



**Figure 2.** Inhibition of protein synthesis results in the appearance of *GPI-PLC* mRNA. Northern blot showing a time course after the addition of cycloheximide. The same blot was probed for *EP* procyclin and  $\alpha$ - and  $\beta$ -tubulin to demonstrate that mRNA stabilization is not a general consequence of blocking translation and for an rRNA to estimate comparative loading. The probe is indicated under each blot. The *GPI-PLC* mRNA and rRNA were quantitated using a phosphorimager, and the *GPI-PLC* mRNA determined as a concentration relative to the amount of rRNA and is expressed in arbitrary units.



**Figure 3.** Estimation of the half-life of cycloheximide-induced *GPI-PLC* mRNA in procyclic forms. Cells were incubated with cycloheximide for 2 h, and then sinefungin added and samples were taken over a time course and analysed by northern blotting. After probing for *GPI-PLC*, the same blot was probed with  $\alpha$ - and  $\beta$ -tubulin to show the sinefungin-dependent appearance of dicistronic tubulin mRNAs and then for an rRNA to estimate comparative loading. The probe is indicated under each blot. The predominant *GPI-PLC* mRNA indicated with an arrow and rRNA were quantitated using a phosphorimager and the *GPI-PLC* mRNA determined as a concentration relative to the amount of rRNA. RNA size standards are shown.

The data from this experiment were also used to estimate the ratio between the bloodstream and procyclic form steady-state levels of *GPI-PLC* mRNA. The result, corrected for rRNA as a measure of loading, was that the *GPI-PLC* mRNA is at 50-fold higher levels in bloodstream than in procyclic forms. After cycloheximide treatment, the difference was reduced to just over 2-fold.

The half-life of *GPI-PLC* mRNA after inhibition of protein synthesis treatment by incubation with cycloheximide for 2 h was determined by addition of sinefungin and preparation of RNA over an 80 min time course (Figure 3). At the end of the time course, the cells had retained motility and appeared morphologically normal by phase contrast microscopy. The sinefungin resulted in an accumulation of incompletely processed tubulin mRNAs, again most visible as the dicistron on a northern blot (Figure 3). The rate of accumulation of the tubulin dicistron was similar to procyclic cells treated with sinefungin without prior cycloheximide treatment (Figure 1b) suggesting that the 2 h treatment with cycloheximide had little effect on transcription. After addition of sinefungin, the half-life of the *GPI-PLC* mRNA was  $30 \pm 5$  min. Thus, the inhibition of protein synthesis had resulted in a 10-fold increase in the half-life of the *GPI-PLC* mRNA in procyclic forms.

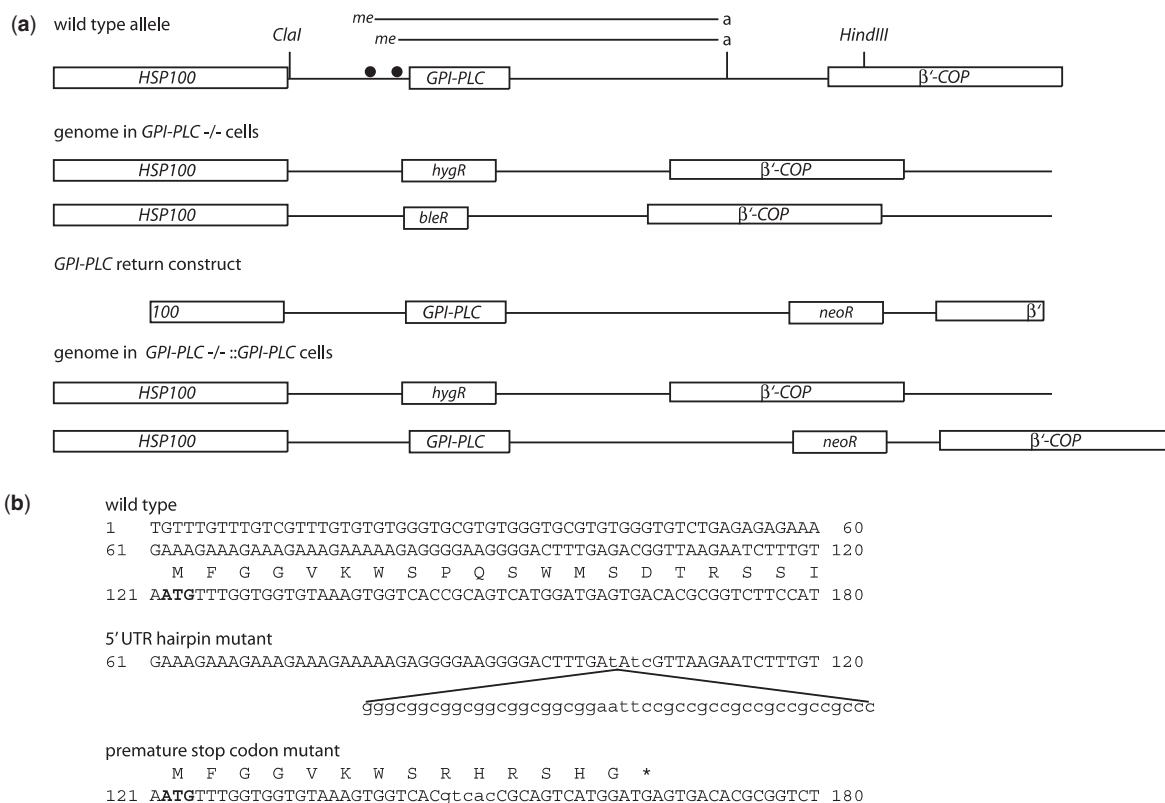
#### Blocking the translation of the *GPI-PLC* mRNA in procyclic forms does not result in stabilization

The *GPI-PLC* gene is non-essential (38) and bloodstream trypanosomes derived from the Lister 427 cell line with

both copies of the *GPI-PLC* gene deleted proliferate at the same rate in culture as wild-type cells (52) (H. Webb and M. Carrington, unpublished). The strategy used to specifically block translation of the *GPI-PLC* mRNA was to reintroduce versions of the *GPI-PLC* gene that could not be translated into the endogenous locus of *GPI-PLC* null bloodstream trypanosomes (Figure 4). The bloodstream trypanosomes were then differentiated to procyclic forms *in vitro* and the steady-state level of the *GPI-PLC* mRNA was compared in the two life cycle stages.

To make the *GPI-PLC* null cell line, the entire *GPI-PLC* mRNA coding sequence, including 5' and 3'UTR, was replaced with an antibiotic-resistance cassette. The two alleles were replaced sequentially, first using a hygromycin and then a bleomycin-resistance gene (Figure 4). Once the *GPI-PLC* deletants had been verified, a range of wild-type and modified *GPI-PLC* genes were returned to the locus (Figure 4b). The re-introduction construct returned the entire *GPI-PLC* mRNA coding sequence, restored the intergenic sequence to the 5' side of the *GPI-PLC* gene and introduced a neomycin-resistance cassette in the intergenic sequence between the *GPI-PLC* gene and the downstream gene.

Three *GPI-PLC* genes were returned to the endogenous locus: (i) a wild-type *GPI-PLC* gene as a control for expression; (ii) a *GPI-PLC* gene with a hairpin in the 5'UTR; (iii) a *GPI-PLC* gene containing a stop codon instead of codon 15 to assay for nonsense-mediated decay of the *GPI-PLC* mRNA. The hairpin added to the 5'UTR contained 23 bp (Figure 4),



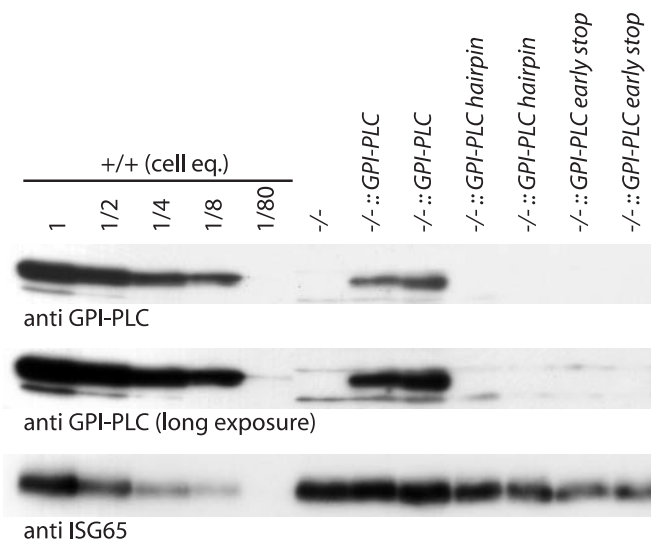
**Figure 4.** (a) Diagram showing the *GPI-PLC* genomic locus which is flanked by the *HSP100* and  $\beta'$ -*COP* genes (49) and the *GPI-PLC* gene replacement strategy. The two major mRNAs are shown above the *GPI-PLC* gene with the mini-exon addition sites indicated by filled circles and the approximate location of the polyadenylation site by a vertical line. The ClaI HindIII restriction enzyme fragment cloned into the expression vector p1865 is indicated. The starting cells are *GPI-PLC*<sup>-/-</sup> and targeted insertion of a *neo*<sup>R</sup> gene results in the return of a *GPI-PLC* gene to the endogenous locus. (b) Sequence detail around the initiation codon of the *GPI-PLC* showing the location and sequence of the hairpin introduced into the 5'UTR and the location of the premature stop codon.

and was based on constructs used in other systems that have been shown to be sufficient to block translation (53).

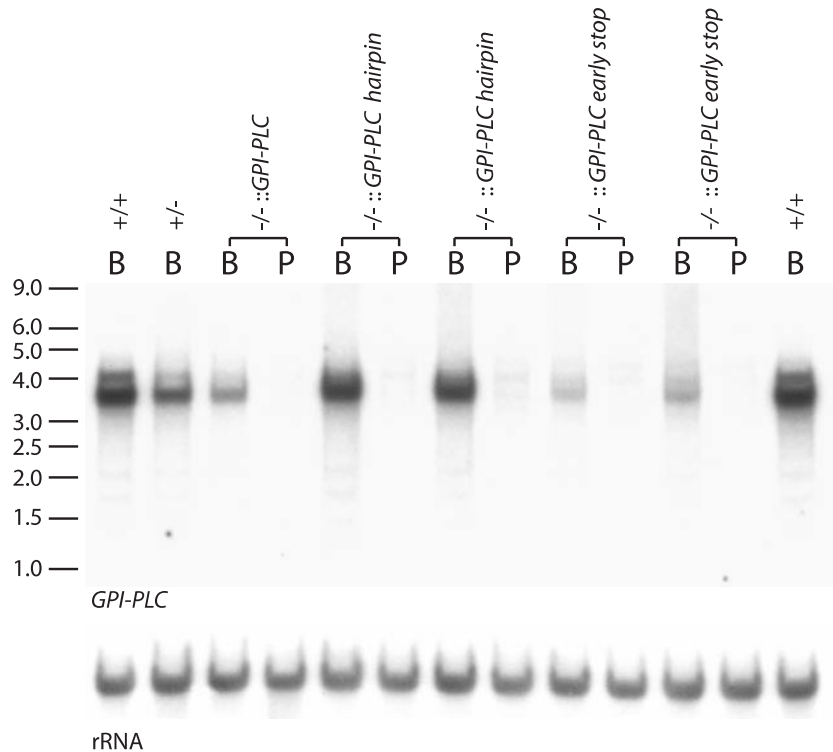
The level of expression of GPI-PLC protein was determined by western blotting bloodstream form clones containing a transgene (Figure 5). A titration of wild-type cell lysate was used to calibrate the sensitivity of the assay and on a long exposure of the western blot, it was possible to detect GPI-PLC when 1/80 of the cell equivalents of wild-type cells (+/+) were loaded indicating that the assay was sensitive down to ~1% of wild-type levels. Expression from the wild-type *GPI-PLC* transgene (-/-::*GPI-PLC*) was readily detected, but no expression was detected from either the *GPI-PLC* gene containing a hairpin in the 5'UTR (-/-::*GPI-PLC* hairpin) or, as expected, from the *GPI-PLC* gene containing a premature stop codon (-/-::*GPI-PLC* early stop) (Figure 5). These results demonstrate that the hairpin in the 5'UTR of the *GPI-PLC* mRNA blocked translation.

Each of the bloodstream trypanosome clones was differentiated to procyclic forms *in vitro* and RNA prepared from both life cycle stages for each clone. Two distinct clones containing the *GPI-PLC* transgene with a 5'UTR hairpin and two containing a *GPI-PLC* transgene with a premature stop codon were analysed. The level of the *GPI-PLC* mRNA was determined by northern blotting (Figure 6).

*GPI-PLC* mRNA was detected in all bloodstream form clones containing a transgene. In all cases, the mRNA was a similar size



**Figure 5.** Expression of GPI-PLC protein from *GPI-PLC* transgenes returned to the endogenous locus. The expression of GPI-PLC was tested in the parental *GPI-PLC*<sup>-/-</sup> cell line and two independent clones of cell lines containing transgenes encoding wild-type *GPI-PLC* or *GPI-PLC* with a hairpin in the 5'UTR or *GPI-PLC* with a stop codon replacing codon 15. A titration of  $2 \times 10^6$  (1 cell equivalent) to  $2.5 \times 10^4$  bloodstream form cells (1/80 cell equivalent) is shown as a measure of sensitivity. The antiserum used is indicated under each blot, anti-ISG65 was used as a loading control.



**Figure 6.** Comparison of *GPI-PLC* expression from transgenes. RNA from both bloodstream (B) and procyclic (P) forms of the same cell lines was analysed by northern blotting. The probe is indicated under each blot. One clone containing a wild-type *GPI-PLC* transgene was compared with two independent clones of each of two cell lines containing either *GPI-PLC* with a hairpin in the 5'UTR or *GPI-PLC* with a stop codon replacing codon 15. *GPI-PLC* +/+ and +/- bloodstream form RNA is shown for comparison, rRNA was used as a loading control and RNA size standards are shown.

to that from *GPI-PLC* +/+ cells and had the characteristic pattern of two distinct mRNAs indicating that the transcript was correctly processed. The steady-state level of mRNA in the clone containing the wild-type *GPI-PLC* transgene (-/-::*GPI-PLC*) was less than in a heterozygote (+/-). The only difference between the transgene and the endogenous locus is the antibiotic-resistance cassette to the 3' side of *GPI-PLC* gene, which may affect the efficiency of polyadenylation of the *GPI-PLC* mRNA. The steady-state level of *GPI-PLC* mRNA in bloodstream forms with the transgene containing a premature stop codon (-/-::*GPI-PLC* early stop) was similar to that from the wild-type transgene indicating that there is little or no nonsense-mediated decay of the *GPI-PLC* mRNA. There were increased levels of the *GPI-PLC* mRNA in bloodstream trypanosomes containing a transgene with a hairpin in the 5'UTR (-/-::*GPI-PLC* hairpin) presumably due to interference with the normal mRNA turnover pathway.

None of the transgenes produced a significant steady-state level of *GPI-PLC* mRNA in the procyclic forms indicating that the developmental regulation of *GPI-PLC* mRNA was still operating for all the transgenes. Thus, specifically blocking the translation of the *GPI-PLC* mRNA does not lead to stabilization of the mRNA in procyclic forms.

## DISCUSSION

The regulation of mRNA stability is the major level of regulation of gene expression in trypanosomes. Developmentally regulated mRNAs, such as *GPI-PLC* which is moderately

abundant in bloodstream trypanosomes, but almost absent in procyclic forms, provide an opportunity to investigate the regulation of stability. The major findings of this paper are that (i) the half-life of the *GPI-PLC* mRNA is approximately 10-fold greater in bloodstream forms than in procyclic forms; (ii) the inhibition of protein synthesis causes a rapid stabilization of *GPI-PLC* mRNA in procyclic forms; (iii) the stabilization of the *GPI-PLC* mRNA in procyclic forms after inhibition of protein synthesis results from an approximately 10-fold increase in mRNA half-life; (iv) the inclusion of a hairpin in the 5'UTR of the *GPI-PLC* mRNA is sufficient to block translation, but does not stabilize the *GPI-PLC* mRNA in procyclics; (v) a *GPI-PLC* mRNA with a stop codon in place of codon 15 is not subject to nonsense-mediated decay but is not stabilized in procyclic forms.

The steady-state amount of any mRNA in a particular cell will be determined by the rate of synthesis and the rate of decay. Phosphorimager measurements of the amount of any mRNA are standardized against rRNA to allow for differences in the amount of RNA loaded in each gel. This step effectively converts the estimate of mRNA amount into a concentration, and thus the measurements of steady-state levels are affected by the cell volume and ribosome density. In trypanosomes, the relative abundance of the same mRNA in the two life cycle stages will be affected by: (i) relative transcription rate, which is probably higher in bloodstream forms grown at 37°C than in procyclic forms grown at 27°C; (ii) cell volume, procyclic forms are roughly twice the volume of bloodstream forms (iii) ribosome density, no comparison of ribosomal content of the two cell types has been reported and (iv) mRNA

half-life. The considerations other than half-life become apparent when a constitutive expressed mRNA is analysed. The actin mRNA is equally abundant in bloodstream and procyclic forms (54), but the half-life in bloodstream forms (31) is several-fold shorter than in procyclic forms (27). Presumably, the difference in half-life is necessary to compensate for the higher transcription rate and smaller cell volume of bloodstream forms.

The steady-state level of *GPI-PLC* mRNA is 50-fold higher in bloodstream than in procyclic forms. It is probable that this difference in steady-state levels can be accounted for by a half-life that is 10-fold greater in bloodstream forms combined with the effect of the higher transcription rate in and smaller volume of bloodstream forms, both factors amplifying the difference in steady-state levels. There is an interesting problem to be addressed in the future: why the low level of *GPI-PLC* mRNA in procyclic forms produces no detectable GPI-PLC activity (36). The possible explanations are that there is translational control of the mRNA in procyclic forms and/or the GPI-PLC protein has a short half-life.

At least two *GPI-PLC* mRNAs were usually detected on a northern blot; the predominant band of 3.6 kb corresponds to the mature mRNA, whereas the precise origin of all of the larger *GPI-PLC* mRNAs is less clear. All originate from the single *GPI-PLC* gene present in heterozygotes (Figure 6) and so do not represent allelic variants. One of the larger *GPI-PLC* mRNAs results from the use of an alternative *trans*-splicing site in the 5'UTR and can be reproduced by transferring the sequence to a reporter gene (H. Webb and M. Carrington, submitted). It is not clear whether the different *GPI-PLC* mRNAs represent different mature mRNAs resulting from the use of alternative *trans*-splicing and/or polyadenylation sites, or whether the larger mRNAs are partially processed precursors that are yet to complete maturation to the final *GPI-PLC* mRNA. After sinefungin treatment, the turnover rate of the different *GPI-PLC* mRNAs is not the same (Figures 1a and 3) and the major, 3.6 kb, mRNA is turned over more rapidly than the larger forms, either it has a longer half-life or the larger mRNA are precursors of the 3.6 kb mRNA.

The *GPI-PLC* mRNA is unstable in procyclic forms with a half-life of 3 min. Inhibition of protein synthesis increases the half-life to 30 min. Previously, it had been shown that inhibition of protein synthesis in bloodstream forms led to accumulation of the normally unstable *EP* procyclin mRNA (32–34) as fully mature and polysome-associated mRNA (33). The *GPI-PLC* mRNA that accumulates after protein synthesis inhibitor treatment appears to be fully processed, judged by mobility after northern blotting. The stabilization of *GPI-PLC* mRNA after treatment with cycloheximide indicated that the normal rapid turnover of the mRNA is translation dependent. The two possibilities were that the translation of the *GPI-PLC* mRNA itself is necessary for the rapid turnover or that translation of another mRNA is necessary and that the polypeptide product is effective for only a very short period. To distinguish between these two possibilities the translation of the *GPI-PLC* mRNA was blocked in two ways: the insertion of a hairpin in the 5'UTR or the inclusion of a premature stop codon. The production of an mRNA from each of the transgenes was verified in bloodstream forms, all produced readily detectable mRNA but only the wild-type transgene produced detectable

*GPI-PLC* protein. The cell lines were differentiated to procyclic forms and the expression from the transgenes determined. This step contained the assumption that the *GPI-PLC* mRNA with a hairpin in the 5'UTR was also blocked for translation in procyclic forms, but it is unlikely that this would be affected by developmental stage.

In many eukaryotes, mRNAs that are not translated are degraded by the nonsense-mediated decay pathway (55), but this pathway did not appear to act on the *GPI-PLC* mRNA containing a premature stop codon. Neither the inclusion of the premature stop codon, which allowed translation as far as codon 14, nor a hairpin in the 5'UTR designed to prevent ribosome assembly on the initiation codon stabilized the *GPI-PLC* mRNA in procyclic forms. Thus, the rapid turnover of the *GPI-PLC* mRNA in procyclic forms is dependent on the translation of an mRNA other than *GPI-PLC* mRNA itself and the cycloheximide is acting *in trans*.

Only one other example of dependency of the rapid turnover of an mRNA on the translation of a *trans*-acting factor has been unambiguously demonstrated. In mammalian cells, the transferrin receptor (TfR) mRNA is stable when cells are depleted of iron but degraded when iron is plentiful. The rapid turnover of the TfR mRNA that occurs in the presence of iron can be blocked by the addition of cycloheximide (56). The action of the cycloheximide is to block the turnover of the *trans*-acting factor IRP-2, which along with a second *trans*-acting factor, IRP-1, probably stabilizes the TfR mRNA by binding to iron responsive elements within the 3'UTR. The labile factor affected by cycloheximide could be necessary for targeting IRP-2 to the proteasome as treatment with the proteasome inhibitor MG132 also stabilized the TfR mRNA (56).

In other examples, where rapid mRNA turnover can be blocked by protein synthesis inhibitors, it is the translation of the mRNA that is necessary for turnover. In the case of  $\beta$ -tubulin mRNA, a polysome-associated nuclease is involved (57). The *c-fos* mRNA is unstable and the turnover regulated by AU-rich elements (AREs) in the 3'UTR and by protein coding region determinants. Blocking translation of the major coding region determinant by the inclusion of a stem loop stabilizes the mRNA (58,59). In yeast, the turnover pathway followed by mRNAs containing AREs involves a shortening in poly A tail length followed by removal of the 5' cap and subsequent hydrolysis by a 5' to 3' exonuclease. *Trans*-acting factors bound to AREs are believed to modulate the rate at which deadenylation occurs though whether translation of the mRNA itself is necessary for deadenylation is not clear [reviewed in (60) and (61)]. The U-rich elements identified in the 3'UTRs of unstable mRNAs in trypanosomes are believed to function in a similar manner to AREs (22,28), but at this stage it is not known whether any *cis*-acting sequences in the *GPI-PLC* mRNA are similar to U-rich elements.

In conclusion, it has been shown that the developmentally regulated expression of the *GPI-PLC* gene in trypanosomes can be accounted for by the regulation of mRNA half-life and that the rapid turnover of the *GPI-PLC* mRNA in procyclic form trypanosomes is dependent on the continuous translation of a *trans*-acting factor. The dependency of the rapid turnover of the *GPI-PLC* mRNA in trypanosomes and TfR mRNA in mammalian cells on a labile factor suggests the presence of an evolutionarily conserved pathway and a better understanding



of the pathway in the future will come from the identification of its components.

## ACKNOWLEDGEMENTS

We would like to thank Christine Clayton for fruitful discussions. This work was funded by the Wellcome Trust. R.B. and N.K. held MRC PhD studentships, L.E. holds a BBSRC PhD studentship. Funding to pay the Open Access publication charges for this article was provided by St John's College, Cambridge.

*Conflict of interest statement.* None declared.

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