An FKBP Destabilization Domain Modulates Protein Levels in *Plasmodium falciparum*.

Christopher M. Armstrong and Daniel E. Goldberg*
Howard Hughes Medical Institute, Washington University School of Medicine, Departments of Molecular Microbiology and Medicine, St. Louis, MO 63110, USA

**corresponding author:* goldberg@borcim.wustl.edu Tel. (+1) 314 362 1514; Fax (+1) 314 367 3214.

Abstract

To enhance the repertoire of molecular tools for studying malaria parasite biology, we have adapted a ligand-regulatable FKBP destabilization domain for use in *P. falciparum*. The reporter protein YFP and the *Plasmodium* protease falcipain-2 were destabilized in a ligand-reversible manner by fusion of FKBP at either end. The swollen food vacuole phenotype of falcipain-2 knockout parasites could be rescued in a Shld1 ligand-dependent fashion by falcipain-2-FKBP expression.

New tools are needed to study the biology of malaria parasites. Knockout technology is well established in *Plasmodium*, but the study of essential genes is difficult in this haploid organism[1]. The tet-regulatable expression system has been adapted for use in *Plasmodium*[2] but has not yet found wide applicability. RNAi manipulation of these parasites has been reported, but does not appear to be generally functional and typical RNAi machinery is absent from the genome[3]. Gene expression potentially can be down-regulated at multiple points from transcription all the way to post-translational control. Recently a destabilization domain technique has been developed, in which an engineered version of human FKBP12 (FKBP) is fused to the N or C terminus of a protein, promoting degradation of the fusion protein[4]. In the presence of an FKBP ligand, degradation is mitigated, allowing regulation of protein levels in mammalian cells.

We sought to adapt this system for use in *P. falciparum*. The original study et al used an analog of rapamycin they called Shld1 because both of the common FKBP ligands rapamycin and FK506 have pleiotropic effects on mammalian cells. In *Plasmodium falciparum*, FK506 and rapamycin are toxic at levels lower than the concentrations used to stabilize the FKBP12 mutant[5]. In order to ensure that Shld1 is not toxic to *P. falciparum*, we treated parasites with Shld1 for three days and parasitemia was measured using flow cytometery. At the concentrations used for FKBP stabilization, there was no inhibition of parasite growth (not shown).

To allow different genes and promoters to be used facilely in the system, we employed the multi-site Gateway system (Invitrogen) to create plasmids for transfection into the parasites (**Supplementary Fig. 1** online)[6]. To test the system, we used the Hsp86 promoter to drive expression of the FKBP degradation domain fused to the N-

terminal or C-terminal end of yellow fluorescent protein (YFP). 3D7 cells were transfected with plasmid and cultured under drug selection for two to three weeks, to create parasites that possess episomal copies of the constructs.

We grew cells containing FKBP fused to the N-terminal end of YFP in the absence of Shld1 or in the presence of 1 μM Shld1 for 24 hours. Whole cell protein from two independent transfections was isolated from Shld1-treated and untreated parasites and analyzed by western blot. FKBP fused to the N-terminal end of YFP showed a near complete loss of YFP protein when Shld1 was absent, but had high protein levels in the presence of Shld1 (**Fig. 1a**). Two independent C-terminal fusions showed levels of YFP comparable to those of the N-terminal fusions when Shld1 was present. In the absence of Shld1, there was a several fold reduction in YFP, but levels were still detectable in most experiments. Similar results are seen for mammalian cells[4] and Toxoplasma (see accompanying paper).

We treated N-terminal FKBP-expressing parasites with varying levels of Shld1 for 24 hours and measured FKBP-YFP levels by western blot (**Fig. 1b**). At very low levels of Shld1, there was little difference in protein levels compared to in the absence of ligand. As Shld-1 levels increased, protein levels rose to the robust levels seen at 0.5 μM. Higher levels up to 3 μM gave comparable YFP signal to that seen at 0.5 μM (not shown). This suggests that in *P. falciparum*, lower levels of Shld1 are necessary for full expression of the protein than what is seen in mammalian cells. When we fused FKBP to the C-terminal end of YFP, comparable results were seen, down to a plateau at 0.1μM, where YFP levels were comparable to levels seen with no Shld1. This is likely because,

as noted above, the C-terminal FKBP fusion does not lead to complete degradation of the protein.

We also wanted to see how quickly protein levels respond to the presence of Shld1. To do this we grew parasites expressing FKBP fused to the N-terminal end of YFP in the presence or absence of 0.5 μM Shld1 for two days. We then added 0.5 μM Shld1 to parasites that had been grown in the absence of ligand. Western blot analysis showed that 6 hrs after Shld1 was added, FKBP-YFP levels were comparable to those seen in parasites continuously grown in the presence of Shld1 (**Fig. 1c**).

To investigate how quickly protein levels respond to the absence of Shld1, we grew parasites in 0.5μM Shld1 for two days and then removed the compound. We measured FKBP-YFP levels over time by western blot (**Fig. 1d**). After 6 hours, protein levels had dropped substantially and by 20 hours no FKBP-YFP was detectable. These results demonstrate that shld1 acts quickly and can be used to modulate protein levels within a single round of the parasite life cycle.

To assess the effect of the degradation domain on a native *P. falciparum* protein, we generated a construct to fuse FKBP to the C-terminal end of the food vacuole cysteine protease falcipain-2. Fusion protein expression was driven by the hsp86 promoter and the plasmid was introduced and maintained episomally in a falcipain-2 knockout clone. Knockout of the falcipain-2 gene gives rise to a swollen food vacuole phenotype, due to impairment of the parasite hemoglobin degradation pathway[7]. This enabled us to assess the capacity of the FKBP fusion for conditional phenotypic rescue. Transfected parasites were grown for two weeks in WR99210 to select for plasmid-carrying parasites and then were treated with or without 0.5 μM Shld1 for 48 hours. Protein levels were analyzed by

western blot with anti-FKBP antibodies. The analysis showed the presence of falcipain-2 when Shld1 was added to the medium and its absence in medium without ligand (**Fig. 2a**). In the presence of Shld1, correction of the swollen food vacuole phenotype was observed (**Fig. 2b,c**). These results demonstrate that native proteins can be modulated by the destabilization domain and that this should be a useful tool in the study of many *P. falciparum* proteins.

We have used the FKBP destabilization domain system to regulate protein levels in *P. falciparum*. Fusion of FKBP to either the N or C terminus of a target protein allows Shld1-tunable modulation. Having FKBP at the N terminus allows more complete down-regulation, though C-terminal fusion still mediates substantial protein level suppression. This flexibility can be useful, as placing FKBP at one or the other end could effect targeting and/or function. The results with falcipain-2 demonstrate that native *P. falciparum* proteins are subject to modulation by the degradation domain system. Falcipain-2 is synthesized as an integral membrane protein precursor via the secretory pathway, so there is potential for this system to work on a range of proteins, as seen in mammalian cells[4]. Applying this technique to *Plasmodium* proteins should allow for the use of this method in dominant-negative studies and even conditional knockout approaches. It may even be possible to integrate the FKBP sequence at the 3' end of an endogenous coding region[8] to generate a conditional knockdown in situ.

Limitations of the system include: 1) leakiness- the degree of degradation may not be enough to produce a phenotype for some proteins. A combination with the tet system[2] may tighten regulation in such cases. 2) some proteins do not function or mistarget when made as fusion proteins. 3) some proteins may not be degraded well despite FKBP

fusion. 4) our episomal expression was under control of the HSP86 promoter, which gives peak expression in trophozoite and schizont-stage parasites. Whether an early-expressed protein would be well modulated remains to be assessed. Despite these limitations, this work, and that in the accompanying report by Herm-Gotz et al. on Toxoplasma, suggest that FKBP-based modulation of protein levels may find widespread applicability in apicomplexan parasite research.

Acknowledgements

We thank I. Gluzman (Washington University) for help with transfections, E. Istvan (Washington University) for falcipain-2 knockout parasites, T. Wandless (Stanford University) for FKBP plasmids pBMN L106P-YFP iHcRed-t and pBMN YFP-L106P iHcRed-t as well as for Shld1 compound, Jacobus Pharmaceuticals for WR99210, M. Drew (Washington University) for microscopy advice, G. McFadden (University of Melbourne) for plasmids pCHDR-3/4 and PfHSP86 5'-pENTR4/1, MR4 and J. Adams (University of South Florida) for anti-BiP antibody, and M. Meissner (University of Heidelburg) for helpful comments. This work was supported by NIH grant AI47798.

Author Contributions

C.A. designed and executed experiments, wrote manuscript.

D.G. designed experiment and wrote manuscript.

References

1. Crabb, B. S., Rug, M., Gilberger, T. W., Thompson, J. K., Triglia, T., Maier, A. G. & Cowman, A. F. *Methods Mol Biol* **270**, 263-76 (2004).
2. Meissner, M., Krejany, E., Gilson, P. R., de Koning-Ward, T. F., Soldati, D. & Crabb, B. S. *Proc Natl Acad Sci U S A* **102**, 2980-5 (2005).
3. Ullu, E., Tschudi, C. & Chakraborty, T. *Cell Microbiol* **6**, 509-19 (2004).
4. Banaszynski, L. A., Chen, L. C., Maynard-Smith, L. A., Ooi, A. G. & Wandless, T. J. *Cell* **126**, 995-1004 (2006).
5. Bell, A., Wernli, B. & Franklin, R. M. *Biochem Pharmacol* **48**, 495-503 (1994).
6. van Dooren, G. G., Marti, M., Tonkin, C. J., Stimmler, L. M., Cowman, A. F. & McFadden, G. I. *Mol Microbiol* **57**, 405-19 (2005).
7. Sijwali, P. S. & Rosenthal, P. J. *Proc. Natl. Acad. Sci. USA* **101**, 4384-9 (2004).
8. Klemba, M., Beatty, W., Gluzman, I. & Goldberg, D. E. *J Cell Biol* **164**, 47-56 (2004).
9. Liu, J., Istvan, E. S., Gluzman, I. Y., Gross, J. & Goldberg, D. E. *Proc Natl Acad Sci U S A* **103**, 8840-5 (2006).

Figure Legends

Figure 1. Shld1 suppression of destabilization of proteins fused to FKBP. (a and b) Western blots using anti-GFP antibody against whole cell extract from N- or C-terminally FKBP-tagged YFP. (**a**) Parasites were grown for 24 hrs in the presence or absence of 1.0 μM Shld1. 1 and 2 indicate different transfectants and + and – indicate the presence or absence of Shld1. (**b**) Parasites were grown for 24 hrs in the indicated concentrations of Shld1. (**c and d**) Western blots using anti-GFP antibody against WCE isolated from transfected parasites expressing an N-terminal FKBP-YFP fusion protein. Parasites were grown with or without 0.5 μM Shld1. (**c**) Those grown without ligand were then shifted to medium with 0.5 μM Shld1 for the indicated number of hours (Shld1 added). Those grown initially with Shld1 were maintained in Shld1. (**d**) Parasites were maintained in the presence or absence of Shld1 (Shld, no Shld) or Shld1 was removed from culture that

had been grown in the presence of ligand (remove Shld) for the indicated number of hours. An arrow marks the fusion protein band. All western blots were repeated multiple times; in each case a representative blot is shown. Equal amounts of protein were loaded in each lane and an endogenous cross-reacting (lower) band was used as a loading control. BiP gave similar results when used as a loading control.

Figure 2. Conditional phenotypic rescue of the swollen food vacuole phenotype in falcipain-2 knockout parasites. (**a**) Western blot using anti-FKBP antibody against whole cell extract isolated from parasites transfected with plasmids expressing either YFP with C-terminal FKBP or falcipain-2 with C-terminal FKBP. Parasites were grown in the presence (+) or absence (–) of 0.5 μM Shld1 for 48 hours prior to extraction. BiP levels were assessed as a loading control. (**b**) Parasites possessing swollen food vacuoles were quantified by an independent investigator blinded to the parasite identities on blood smears. Error bars: s.e.m; trophozoites counted, *n*+ 100, 105, 104, 107 for 3D7, ΔFP2, – Shld, +Shld respectively. The difference between – and ≠ Shld is significant at p<.0 bu the test. (**c**) Light micrographs of Giemsa-stained parasites. 3D7: parental parasites; ΔFP2: knockout parasites; +Shld and –Shld: ΔFP2 /FP2-FKBP knockout parasites expressing fusion protein falcipain-2-FKBP in the presence or absence of 0.5 μM Shld1. Arrows indicate examples of food vacuoles in each parasite line.

Supplementary Methods

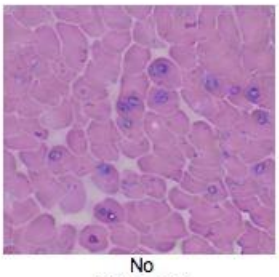
Plasmid construction- The Gateway system (Invitrogen) was used to construct the entry vectors pENTR221-FKBP, pENTR221-YFP, pENTR221-FP2, pENTR2/3-FKBP, and pENTR2/3-YFP. Multisite Gateway was then used with the vectors pCHDR3/4 and PfHSP86 5'-pENTR4/1 to create pCHD-HSP86-FKBP-YFP, pCHD-HSP86-YFP-FKBP and pCHD-HSP86-FP2-FKBP. A human DHFR cassette was used for selection. Plasmid schemes are shown in **Supplementary Fig. 1**.

Transfection- 3D7 parental or falcipain-2 knockout[9] parasite lines were used for transfection. Parasites were grown in RPMI with 0.5 mg/ml Albumax II (Invitrogen) at 2% hematocrit, to 5% parasitemia. Late-stage parasites were used for transfection as has been described [8]. Parasites were grown for two weeks in medium supplemented with 10 nM WR99210 (Jacobus Pharmaceuticals) to select plasmid-containing organisms.

Western blots- parasites were lysed by incubation with 0.1% mg/ml saponin (Sigma) in PBS for 10 min at 25°C, harvested, washed with PBS and lysed in 1% SDS in PBS. Samples were normalized for protein concentration as measured by BCA assay and fractionated on 10% SDS-PAGE gels. They were transferred to nitrocellulose, incubated with anti-GFP (1:5000, Abcam) or anti-FKBP (1:2000, Abcam) for two hrs, then with HRP-conjugated secondary rat anti-mouse antiserum (1:5,000, Amersham), and were developed with ECL+ (Amersham).

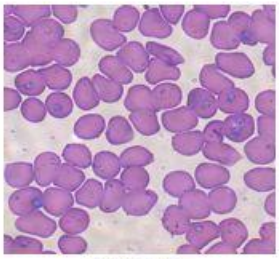
Figure 1

A)



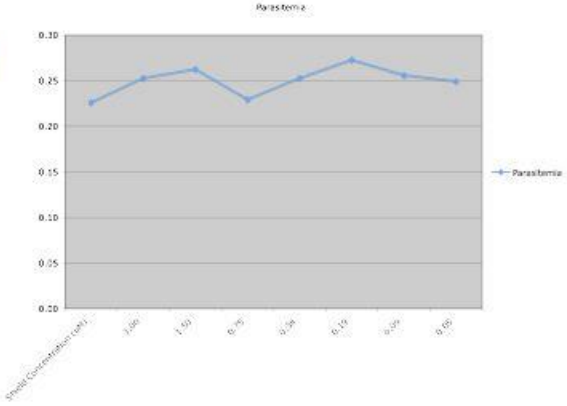
No
Compound

B)



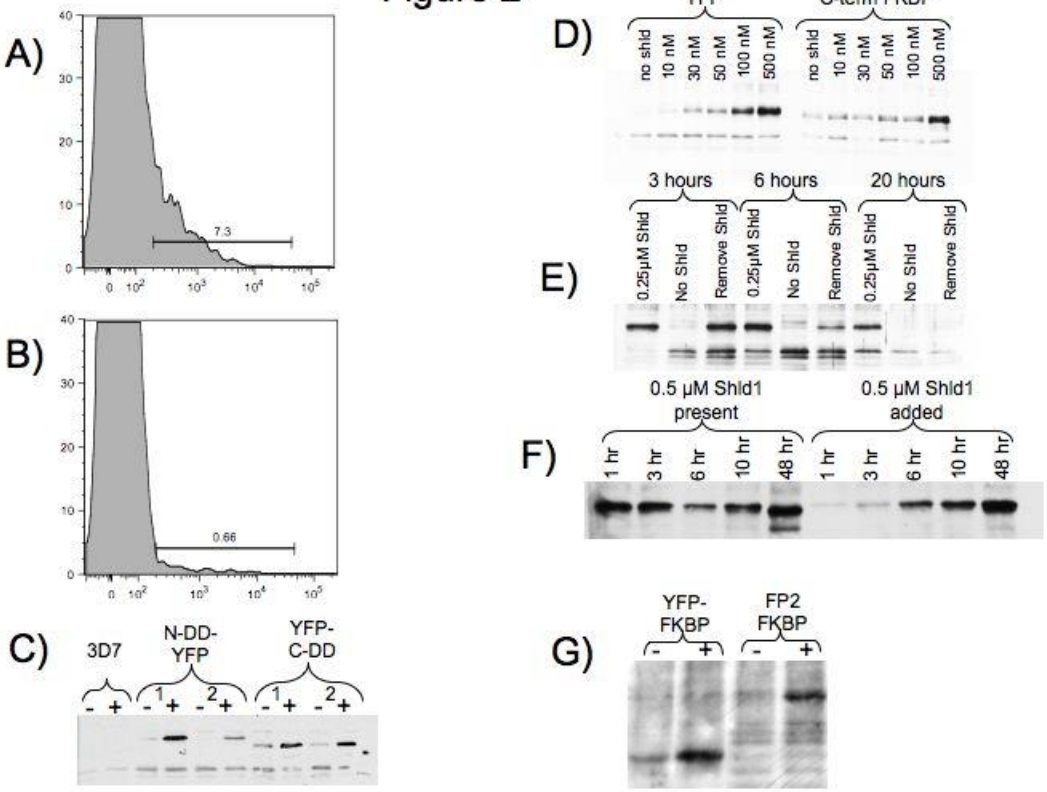
3μM Shld1

C)



D) Here will be a picture of the gateway plasmids

Figure 2


A)
7.3
B)
0.66
C)
3D7
N-DD-YFP
YFP-C-DD
D)
N-term FKBP YFP
YFP C-term FKBP
no shld
10 nM
30 nM
50 nM
100 nM
500 nM
E)
3 hours
6 hours
20 hours
0.25µM Shld
No Shld
Remove Shld
F)
0.5 µM Shld1 present
0.5 µM Shld1 added
1 hr
3 hr
6 hr
10 hr
48 hr
G)
YFP-FKBP
FP2 FKBP

Supplementary Figure 1. Multisite Gateway to create FKBP-YFP. The plasmid pCHD-Hsp86-FKBP-YFP was created by mixing the indicated entry clones in the Multisite Gateway reaction. Plasmids pCHD-Hsp86-YFP-FKBP and pCHD-Hsp86-FP2-FKBP were created in the same way except that pENTR2/3-FKBP was used as the pENTR2/3 vector and either pENTR221-YFP or pENTR221-FP2 was used as the pENTR221 vector.