Functional Reconstitution of the Plasmodium GPI:protein transamidase Complex

It is well document that proteins tethered by a glycosylphosphatidylinositol (GPI) modification play a vital role in the survival and pathogenicity of asexual stages in Plasmodium. The GPI glycolipid serves to anchor many surface proteins to the plasma membrane; for example plasmodial proteins that are directly involved in erythrocyte invasion and therefore parasite proliferation (such as the abundant MSP1 on merozoites). Selective chemical interference of key enzymes involved in either GPI synthesis or transfer of nascent proteins to preformed GPI would hamper this critical step in the lifecycle and so allow eradication. We are interested in the full biochemical characterizing of the enzyme that transfers the precursor target protein to the GPI ie. the Plasmodium GPI:protein transamidase. Below I have included some background information along with difficulties that need to be overcome before the Plasmodium GPI:protein transamidase can be validated as a bona fide drug target.

Background

GPI anchored proteins are found throughout eukaryotes, but seem to be particularly abundant in the parasitic protozoa such as Plasmodium, Trypanosoma, Leishmania and Giardia. Proteins destined to be GPI-

anchored have a number of defining domains (Figure 1). An N-terminal signal sequence directing proteins to the endoplasmic reticulum (ER). Following rapid signal peptide removal within the ER, a C-terminal partially embedded GPI: protein transamidase directing address directs cleavage by the GPI:protein transamidase at a tripeptide specificity region (P_1-P_1´-P_2´) termed the ω site (Figure 1B). The ω site is set in between a linker region and an unstructured region. The preformed GPI-anchor is attached to the nascent protein by a transamidase reaction; initially cleaving the P_1-P_1´ peptide bond of the substrate before reforming the peptide linkage between the terminal amine of the ethanolamine group of the GPI-anchor and the C-terminal carbonyl group at the ω site of the substrate protein (Figure 1C). Numerous GPI anchor prediction algorithms exist online that predict protein substrates for GPI:protein transamidase activity (see www.expasy.org/tools/).

The ω-site defines the substrate preference of the GPI:protein transamidase enzymes and comprises three sequential amino acid residues, namely the P1 (ω+1), P1´(ω+2) and P2´(ω+3). This requirement for GPI:protein transamidase to bind substrates at 3 subsites is unique to clan CD cysteine peptidase [1], which typically have a strict S1 specificity, for example caspase for aspartate residues [2] and asparaginyl endopeptidase for asparaginyl residues [3]. By

comparing the ω-site of nascent GPI proteins between human and parasites (Figure 2) it is evident that there are differences in subsite preference that can be potentially exploited to generate selective inhibitors that target the parasite GPI:protein transamidase over the human orthologue.

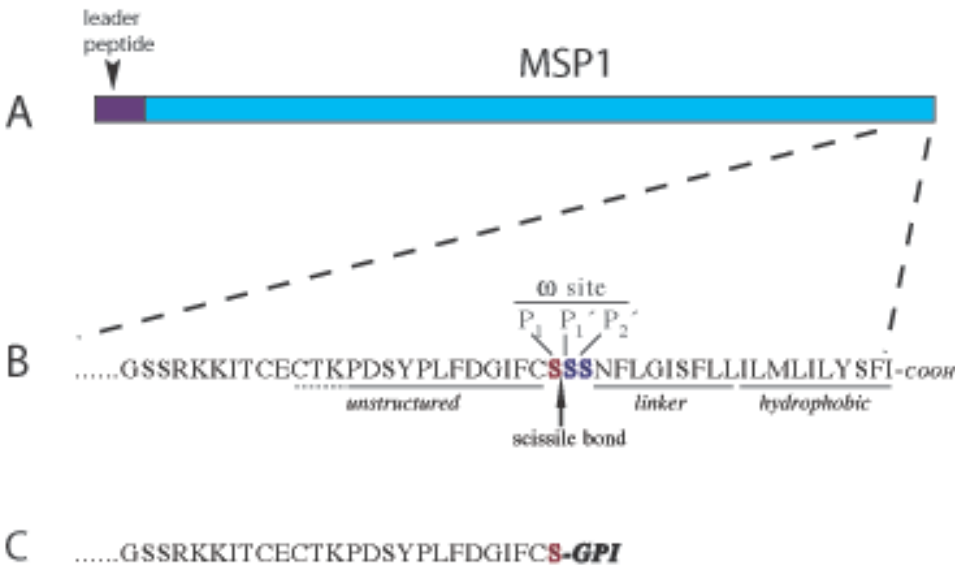
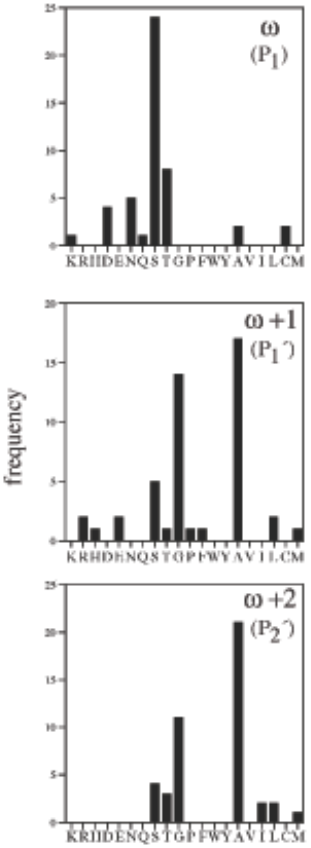


Figure 1. A) Full length MSP1 contains a leader peptide that directs biosynthesis to the ER. B) The C-terminal portion of Plasmodium falciparum MSP1 contains the ω site that direct the parasite's GPI:protein transamidase to the nascent MSP1. The bond that is initially cleaved (scissile bond) is highlighted in red and is the P_1 residue. The P_1' and P_2', which also are involved in binding the enzyme active site, are in blue. C) The end product of the transamidase reaction showing the addition of a GPI anchor to the S1 Ser of MSP1, as so reforming the peptide bind.

Although, GPI anchored proteins have critical functions in Plasmodium, and substrate differences between the human and parasite GPI:protein transamidase do occur, biochemical validation of GPI:protein transamidase has been hampered due to number technical obstacle – listed below.

A



B

Parasite/ GPI anchored proteins	ω site ω, ω+1, ω+2
Plasmodium falciparum	
MSP1_PLAFF	S S S
AMA1-PLAFF	S N S
PF12_PLAFA	N S S
Toxoplasma gondii	
SRS4(076660)	S S S
SUSI(P90614)	G G A
Leishmania	
GP46_LEIAM	V C R
GP63_LEIAM	N T A
GP63(C2; unpublished)	N A A
Typanosoma cruzi	
GP85_TRYCR	A M A
T. brucei	
PAG_TRYBB	A D S
PARA_TRYBB	G A A
VSA1-TRYBB	D S S
VS11_TRYBB	S D S
VS14_TRYBB	D S S
VSM0_TRYBB	D G S
VSWA_TRYBB	S N S
BARP(060946)	S V A
T. congolense	
VSY1_TRYCO	S G S
VSY3_TRYCO	S A A
Giardia lamblia	
VS41_GIALA	S G L
TSA4_GIALA	S G L
Sarcocystis muris	
MSA1_SARMU	A G S

Figure 2. A) Frequency of the ω site (P_1, P_1´ and P_2´) amino acid residue preferences for human proteins that are GPI anchored. The most frequently utilized residues at P_1, P_1´ and P_2´ are Ser-Ala-Ala. B) Comparison of the ω site residues utilized by parasitic protozoa reveals differences at the P_1, P_1´ and P_2´.

Problem 1. There are no sensitive GPI:protein transamidase assays that are amenable to medium/high throughput.

Endopeptidase activity can be sensitively and conveniently measured by using authentic peptide substrates that are bound to a quenched fluorescent moiety. Peptide bond hydrolysis at the fluorochrome releases the quenching and subsequent fluorescence can be monitored dynamically, in real time and is adaptable to high throughput systems. Fluorescent chemical reporters that are commonly used include aminomethyl coumarin, aminocarbamoyl methyl coumarain, β-methoxy napththylamine and ρ-nitroanalide. The aforementioned peptidase assay relies on a peptide hydrolysis step so as to release the fluorochrome, however, with GPI:protein transamidases there is no hydrolysis. GPI:protein transamidases share the initial part of the chemical mechanisms of breaking a peptide bond, with all cysteine peptidase (Figure 3.1). The first step involves the peptidase physically becoming attached to the substrate, which is concomitant with leaving of the amino terminal portion of the substrate (Figure 3.2). Peptidases conventionally transfers the acyl group to the oxygen of water (Figure 3.3) and so complete their 'peptide hydrolysis'. With GPI:protein transamidase the acyl group is not transferred to the oxygen of water,

but instead, the transfer is to the nitrogen of the amine of ethaonolamine of the preformed GPI anchor (Figure 3.4); and so reform a peptide bond. This lack of a convenient chemical leaving group has impeded the study of GPI:protein transamidase enzyme complex, although there are cumbersome, time consuming assays available such as western blotting or those which utilize nucleophile acceptors such as hydroxylamine and hydrazide.

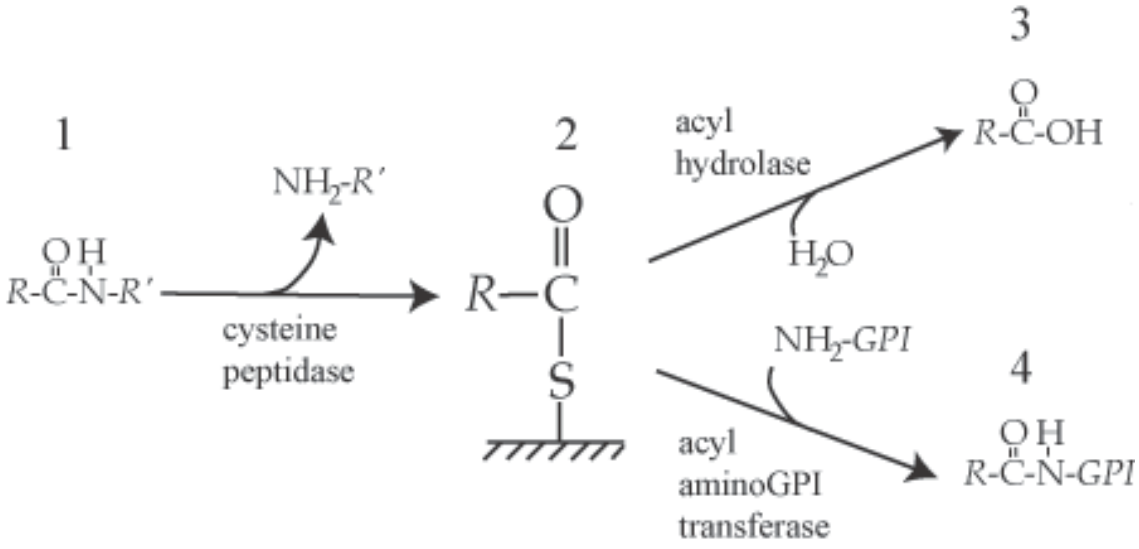


Figure 3. Comparison of the chemical mechanism of peptide bond hydrolysis and GPI:protein transamidase activity. Initial hydrolysis of the substrate by cysteine peptidase activity (1) generates an acylated peptidase intermediate. Acyl transfer to a water molecule (ie. hydrolysis) (3) releases the free acid. GPI:protein transamidases transfer the acyl moiety to the amine of ethanolamine of the preformed glycosylphosphatidylinositol anchor, and so reform a peptide bond.

An assay needs to be developed to measure GPI:protein transamidase activity that takes advantage of the reformed peptide bond between the protein substrate and GPI anchor. For example, assays that exploit close proximity fluorescence resonance energy transfer (FRET; Figure 4).

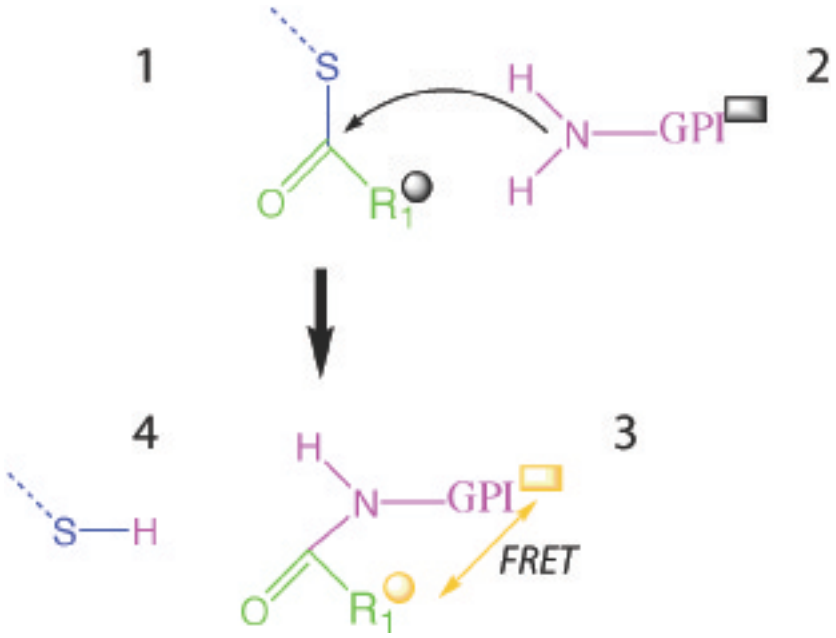


Figure 4. Illustration of the chemical interactions required for a FRET based assay to monitor GPI:protein transamidase activity. (1) The acylated GPI:protein transamidase is attacked by (2) the nitrogen of ethanolamine of the GPI anchor and so reforming (3) a peptide bond and regenerating the thiol of the active site cysteine (4). A FRET donor and acceptor is attached to the substrate (R1, grey circle) and the preformed GPI, or equivalent (grey square), respectively. Once the FRET donor and acceptor are in close special proximity, fluorescence (shown in yellow) is achieved.

Problem 2: The GPI:protein transamidase functions as part of multisubunit complex – and no functional complex has been reconstituted.

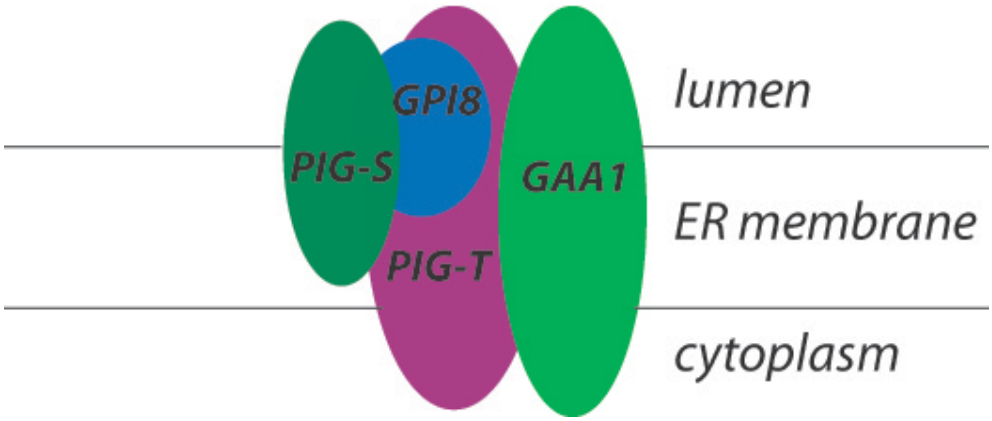


Figure 5. Schematic of the functional complex that is thought to occur in yeast. The GPI:protein transamidase, termed GPI8, is a transamidase only as part of the complex. Modified from [1].

The transamidase step of the GPI:protein transamidase can only take place when all components of a functional complex are brought together (Figure 5); some component may possess hydrophobic membrane bound domains, and so detergents may need to used to maintain the complex; detergents may affect reproducibility and fluorescent assays. Functional recombinant expression of each component of the multisubunit complex has not been demonstrated,

and represents the greatest hurdle that needs to be surmounted before detailed biochemical studies on the GPI:protein transamidase complex can take place.

input required

1. Sensitive and convenient GPI:protein transamidase assay that is adaptable to medium/high throughput analysis, as large cysteine peptidase inhibitor libraries are available.

2. Functional recombinant reconstitution of the GPI:protein transamidase complex.

Literature cited

[1] Mottram JC, Helms MJ, Coombs GH, Sajid M. Clan CD cysteine peptidases of parasitic protozoa. Trends Parasitol 2003;19(4)**:**182-7.

[2] Timmer JC, Salvesen GS. Caspase substrates. Cell Death Differ 2007;14(1)**:**66-72.

[3] Mathieu MA, Bogyo M, Caffrey CR, Choe Y, Lee J, Chapman H, Sajid M, Craik CS, McKerrow JH. Substrate specificity of schistosome versus human legumain determined by P1-P3 peptide libraries. Mol Biochem Parasitol 2002;121(1)**:**99-105.