FUNCTIONAL CHARACTERIZATION OF HETEROLOGOUSLY EXPRESSED

PLASMODIUM FALCIPARUM EBL PROTEINS

by

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(Under the Direction of David S. Peterson)

ABSTRACT

While heterologous expression has replaced biochemical purification as the principle method for obtaining purified, authentic proteins for characterization, no single expression system has proven appropriate for all applications. Heterologous expression of *Plasmodium falciparum* genes has proven especially challenging due to their extreme A+T codon bias and inappropriate post-translational modifications to product made by existing expression systems. These issues have generated interest in developing a novel heterologous expression system for *P. falciparum*. Here, we have transfected the ciliate *Tetrahymena thermophila* with a novel secretion vector bearing the R2 binding region of the *P. falciparum* genes, *ebl-1* and *jsebl*. In addition, we demonstrated successful expression of the R2 region. This is the first demonstration of secreted *P. falciparum* antigens expressed by *T. thermophila*. We believe *T. thermophila* shows promise as a heterologous expression system for *P. falciparum* genes.

INDEX WORDS: *Plasmodium falciparum*, heterologous expression, *ebl-1*, *jsebl*

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CHAPTER 1

INTRODUCTION

Malaria threatens approximately 40% of the world's population and is endemic in over 90 countries. There are 300 – 500 million clinical cases and 2-3 million deaths every year due to the disease. *Plasmodium falciparum* causes the majority of the mortality, primarily in children and pregnant women in sub-Saharan Africa. In fact, the disease is returning to areas once declared malaria-free and entering areas historically free of the disease agent. Malaria is one of humanity's most significant infectious diseases, affecting health, educational achievement, and worker productivity in endemic regions (1, 2).

Plasmodium falciparum has a complex life cycle that requires invasion of multiple human and mosquito cell types. The rapid expansion of merozoites in the asexual, erythrocytic stage of the life cycle causes the classical malarial symptoms. The *ebl* family of micronemal proteins is necessary for the parasite's successful invasion of host erythrocytes. Therefore, understanding this family of proteins may lead to novel strategies to block erythrocyte invasion and infection, and thus prevent disease.

Proteins of the *ebl* family share a number of conserved features, including an N-terminal signal sequence, N-terminal and C-terminal cysteine-rich extracellular domains, as well as transmembrane and cytoplasmic domains (Fig. 1.1). The N-terminal cysteine-rich domain, known as the Duffy-binding-like (DBL) domain, is the functional ligand that mediates erythrocyte binding (3). The DBL domain is conserved both across members of the *ebl* family and between species of the *Plasmodium* genus. The domain

is present as a single copy in the *P. vivax* Duffy binding protein gene (*dbp*) and as tandem repeats, called F1 and F2 – also designated as Region 2, in the *P. falciparum ebl* genes (except for the M1 and M2 repeats in the *ebl* gene *maebl*).
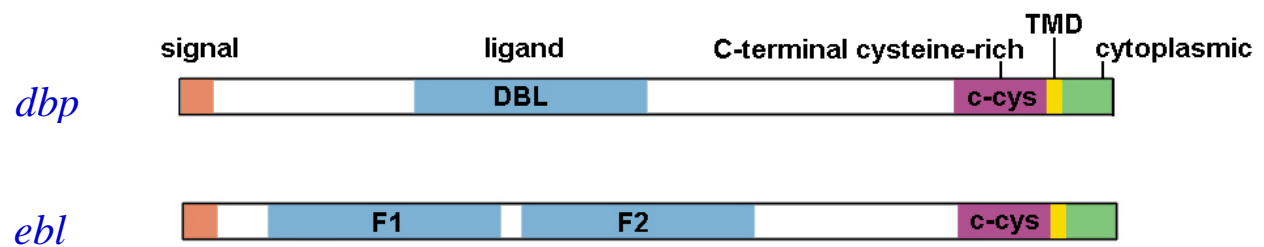
Fig. 1.1. Top: *Plasmodium vivax dbp* gene. **Bottom:** Representative figure of a *P. falciparum ebl* family gene.

In this study, we performed a series of experiments to (a) create a novel expression vector, (b) clone, express, and purify the DBL ligand domain of the *ebl* genes *ebl-1* and *jsebl*, and (c) lay the groundwork for future studies to examine specific binding to human erythrocytes. **Our rationale was that the high sequence conservation of the DBL domain reflects the critical role it plays in host cell invasion.**

CHAPTER 2

SPECIFIC AIM

Erythrocyte invasion is necessary for survival of the malaria parasite. Multiple parasite proteins, initially sequestered in the apical organelles, are specific ligands for cognate receptors on the host cell surface and mediate invasion. The apical organelles, which consist of micronemes, rhoptries, and dense granules, are common to all organisms in the phylum Apicomplexa. Parasite-mediated invasion is necessary because erythrocytes are non-phagocytic. The first step of merozoite invasion of a host erythrocyte is a weak, reversible, attachment to the erythrocyte surface. The parasite then reorients to bring its apical end in contact with the erythrocyte and releases the contents sequestered in the apical organelles. Next, a tight junction forms between the merozoite and the erythrocyte membranes. The parasite is now committed to invasion. The tight junction moves around the merozoite as it enters the erythrocyte and into the parasitophorous vacuole, which likely consists of secretions from the apical organelles and host cell membrane lipids (4, 5).

Significant questions remain as to the nature and sequence of the molecular interactions before, during, and after junction formation. However, the recent implication of apical membrane antigen-1 (AMA-1) in apical reorientation (6), and direct evidence that DBP is necessary for tight junction formation in *P. knowlesi* invasion of human erythrocytes (7), are significant developments in elucidating the invasion pathway.

The situation is even more complex in *P. falciparum* where multiple DBL ligands, specific for multiple host receptors, facilitate multiple invasion pathways. Ligands on the merozoite must recognize and bind to cognate host cell receptors for a tight junction to form and invasion to occur (8). Blocking invasion would prevent disease. Thus, parasite proteins involved in invasion are potential targets for a vaccine or small molecule inhibitors, especially if a domain, well conserved between invasion pathways and strains, proves to be a strong epitope. **Our central hypothesis was that EBL-1 and JSEBL mediate unique invasion pathways. Our long-term goal is to clarify the role of the DBL domain in host cell invasion.**

SPECIFIC AIM: Clone, express and purify functional F1 + F2 domains of EBL-1 and JSEBL. The rationale for this specific aim is that relatively large quantities of purified, functional DBL ligand domains are necessary to study the role of these domains, and possibly identify their cognate receptors.

Objective 1:   Construct an expression vector (pTART) with a multiple cloning sequence (MCS) that includes several GC-rich restriction sites, a FLAG epitope, a 6-His tag, and a *Tetrahymena* specific stop codon.

Objective 2: Amplify the F1 + F2 domains of EBL-1 and JSEBL from *P. falciparum* and insert them into pTART.

Objective 3: Transfect *Tetrahymena* with pTART to produce two clones, one containing the F1 + F2 domains of EBL-1, and the other of JSEBL.

Objective 4:   Using transfected *Tetrahymena* as an expression system, express the F1

+ F2 domains (Region 2) of EBL-1 and JSEBL.

CHAPTER 3

LITERATURE REVIEW

Malaria.

Malaria is without doubt the most important parasitic disease of humans.

Approximately 10% of the world's population experience a clinical case of malaria each

year, resulting in at least 2 million deaths; approximately 3 billion people live at risk of

contracting this disease (9, 10). The situation is worsening due to increasing drug

resistance, the emergence of insecticide resistant mosquitoes, and the reintroduction of

malaria into previously disease-free areas (1). In addition to morbidity and mortality, it

affects economic, educational, and social development in affected areas (10). There is

strong evidence that accelerated economic growth usually follows a decrease in the

prevalence of malaria – that in fact, malaria is an important factor causing poverty. This

negative correlation with economic growth is not found with other endemic tropical

diseases (11, 12).

Malaria at one time was endemic in the United States. In the 16th and 17th

centuries, European colonists introduced *P. vivax* and *P. malariae*, and African slaves

introduced *P. falciparum* to the Americas. In the United States, the incidence of the

disease probably peaked in the 1870s (13), and malaria continued to be endemic until

the middle of the 20th century. There is potential for reintroduction of malaria into the

United States for several reasons. First, competent *Anopholene* vectors are

widespread in the United States; second, there is increased air travel and immigration

from countries where malaria is endemic; third, climatic changes, resulting in a more hot

6

and humid environment, would favor the completion of the sporogenic cycle of the malaria parasite by shortening the cycle to where it is less than the average *Anopholene* lifespan. In fact, there have been reports of autochthonous malaria transmission in the United States in the last several years in New Jersey, New York, Texas, and Virginia. These outbreaks occurred in years that were more hot and humid than normal and in areas that had communities of immigrants from malarious areas (13, 14).

Plasmodium is a member of the phylum Apicomplexa, which also includes *Toxoplasma*, *Babesia*, *Cryptosporidium*, and *Theileria*, which are all intracellular parasites that rely on apical organelles for invasion. Four different *Plasmodium* species cause malaria in humans. These are *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. *Plasmodium vivax* is the most widely distributed, and *P. falciparum* is the most pathogenic. In all cases, humans are the obligatory intermediate host and reservoir and Anopholene mosquitoes are the definitive host and vector. A malarial infection begins with the injection of sporozoites by an infected mosquito taking a blood meal. The sporozoites rapidly invade the liver where they undergo many divisions through schizogony. After about a week, the schizonts release thousands of merozoites into the bloodstream where they invade red blood cells; this is where the asexual, cyclic, erythrocytic stage of the infection and pathogenesis occurs (15).

The clinical manifestation of malaria can range from non-symptomatic parasitemia in endemic areas to death. Uncomplicated malaria presents with flu-like, often periodic, symptoms, which can include fever, chills, sweats, head and generalized body aches, nausea, and vomiting. Severe cases can lead to cerebral malaria or severe anemia, often accompanied by acute respiratory distress and metabolic

acidosis, and can result in death (15). Other organs, such as the kidneys, may also be affected in severe cases. Usually caused by *P. falciparum*, severe malaria most often occurs in all age groups in areas of low transmission, and in young children and pregnant women in areas of high transmission. In addition to causing severe symptoms, malaria in pregnant women often leads to low birth-weight babies and premature births. Thus, the clinical manifestation can depend on geography, species of parasite, and immune status of the human host.

The DBL Domain.

Host cell invasion must occur for a productive infection and survival of the parasite. Thus, invasion represents a process that can be exploited to defeat the organism. It likely takes less than 60 seconds for a merozoite to recognize, attach, and invade a red cell (16), with the invasion process itself (apical reorientation, tight junction formation, and entry) lasting as little as 20 seconds (17). Proteins that mediate invasion are located on the merozoite surface and in the apical complex (18). During reorientation, micronemes are believed to release some of their contents to the merozoite surface (19). The actual invasion process likely involves interactions between ligands at the apical end of the merozoite and host erythrocyte receptors (16).

To fully appreciate the importance of the *ebl* family of proteins, we must first understand the importance of the Duffy-binding-like (DBL) domain, first described in *P. vivax* and *P. knowlesi* as the binding ligand on the Duffy binding protein (DBP) (20, 21). The DBL domain is a conserved, erythrocyte-binding domain shared by evolutionarily distant *Plasmodium* species. The most highly conserved residues in DBL domains are

cysteines and aromatic amino acids, which suggests a functionally conserved, three-dimensional structure (20, 22-24). While the cysteines clearly have a defining role in the DBL domain, analysis of mutations within this region demonstrate that some of the aromatic amino acids are also significant in defining the specificity and affinity of binding (25). Other amino acids in the DBL domain are highly polymorphic. These variable amino acids in the domain may provide unique specificity for binding or aid in immune evasion or both.

The DBL domain is the ligand domain for the DBL superfamily of genes, which includes both the *ebl* and the *var* gene families. The EBL family of proteins is involved in mediating erythrocyte invasion, and the DBL domain is the functional ligand on all EBL proteins, except MAEBL, which recognize different host erythrocyte receptors. The variant *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), encoded by the *var* family, mediates cytoadherence of infected erythrocytes to the endothelium in the peripheral vasculature and to syncytiotrophoblasts in the placenta (26) and is associated with most of the mortality caused by malaria (27). That erythrocyte invasion and cytoadherence are the fundamental pathogenic mechanisms of *P. falciparum* (3, 28) highlights the importance of the DBL domain to this pathogen.

There is convincing evidence that the DBL domain is required for a tight junction to form during erythrocyte invasion. *Plasmodium knowlesi* will attach to, and undergo apical reorientation on human erythrocytes that lack the Duffy blood group at the same rate as on those that have the Duffy blood group. However, on Duffy-negative erythrocytes, the parasite fails to form a tight junction and invasion subsequently stalls (29). Furthermore, *P. knowlesi* which lacks DBP through targeted deletion, will also

attach and undergo apical reorientation, but will fail to form a tight junction and invade

Duffy-positive erythrocytes (7). Since DBP binds to the Duffy antigen/receptor for

chemokines (DARC) in erythrocytes, these data suggest that *P. falciparum ebl* family

ligands are also involved in junction formation.

The *ebl* Gene Family.

The malaria parasite invades multiple cell types in two hosts, the human and

mosquito. In order to complete its life cycle, the merozoite stage must recognize and

invade human erythrocytes. The *ebl* family of proteins is critical to successful invasion

of erythrocytes and thus, to initiating and maintaining a productive infection.

The DBL ligand domain of *ebl* family proteins binds cognate receptors on the host cell

erythrocyte, and is required for invasion to occur. This domain is important in defining

specificity for host cell receptors and represents the best-characterized ligand in

malarial merozoites (22). However, while the ligand's presence is required, its precise

functional role in the invasion process has yet to be determined. There is a single copy

of the DBL domain in *Pv*DBP, the only *ebl* product identified in *P. vivax*. In contrast, the

domain is present as tandem repeats in five of the six known *ebl* genes of *P. falciparum*.

The DBL repeats are designated F1 and F2 in *jsebl*, *pebl*, *eba-175*, *baebl*, and *ebl-1* (8,

20, 22, 30) and contain well conserved disulfide bridges which determine the structure

of the DBL domains (31). Other conserved features of the *ebl* family include an N-

terminal signal sequence, the C-terminal cysteine-rich extracellular domain (c-cys), as

well as transmembrane and cytoplasmic domains (Fig. 3.1).
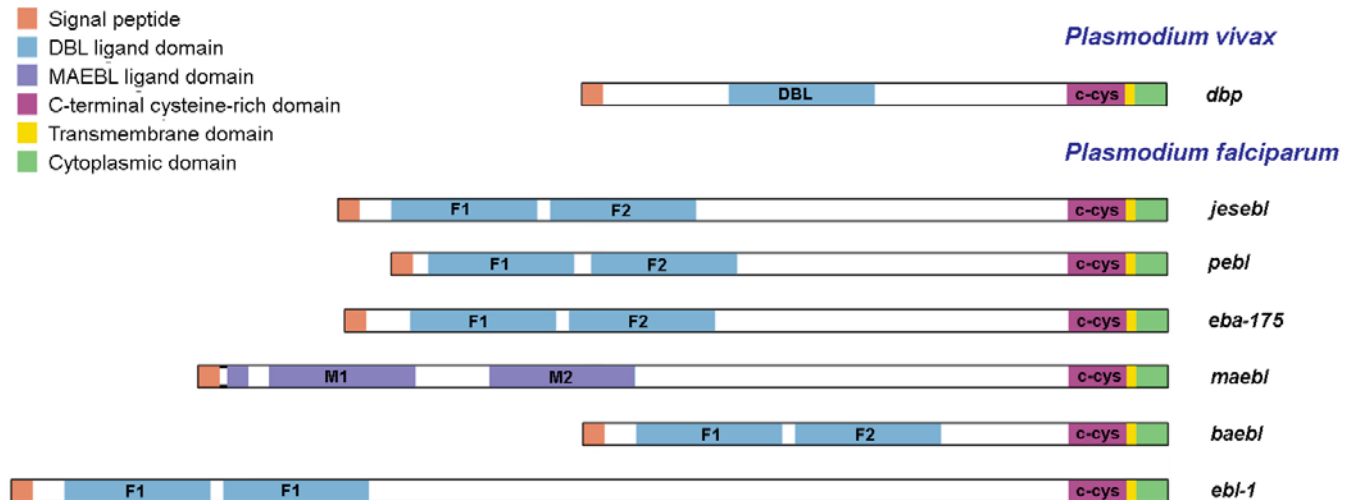
Fig. 3.1. Representation of the *Plasmodium vivax* and *P.falciparum ebl* gene family**.**

The first *P. falciparum ebl* protein identified was EBA-175, an ortholog to *Pv*DBP (32). Other functional members of the *P. falciparum ebl* family include *ebl-1* (33), *jsebl* (34), and *baebl* (35) which are paralogs to *eba-175*. All *ebl* genes have a similar intron–exon division with conserved splicing boundaries. The exons correspond to the products of functional domains. Typically, about one-half of *P. falciparum* genes have introns, with many genes having either none, or perhaps just one between the signal sequence and the rest of the gene. This similarity in gene structure across the *ebl* family suggests that the genes are from a common origin (20). On the other hand, the diversification the family provides may aid in immune evasion by providing for alternate receptors (Table 3.1).

Table 3.1. Characteristics of the *Plasmodium falciparum ebl* gene family.

Gene Name	Chromosome	Product	AA	MW	Receptor	Pathway Resistant	Pathway Sensitive	Comments
jesebl; eba-181	1	JESEBL; EBA-181	1567	181,133	Receptor E	Trypsin	Chymotrypsin Neuraminidase	Receptor NOT Glcyophorin B Cannot by KO'd in strain 3D7 Can be KO'd in strain Dd2 (34)
pebl; eba-165	4	PEBL; EBA-165	1431	164,905	-	-	-	Pseudogene
eba-175	7	EBA-175	1475	171,473	Glycophorin A	Chymotrypsin	Trypsin Neuraminidase	
Maebl	11	MAEBL	2056	243,314	-	-		Chimeric; AMA-1 domains
baebl; eba-140	13	BAEBL; EBA-140	1210	140,607	Glycophorin C	-	Trypsin Neuraminidase	Requires exon 3 of Glycophorin C (36)
ebl-1	13	EBL-1	2647	304,524	-	-		-
-	-	-	-	-	Glycophorin B	Trypsin	Neuraminidase	-
-	-	-	-	-	Receptor X	Neuraminidase	Trypsin	-

The F1 repeat in *Pf*EBL and the DBL in *Pv*DBP are more closely related to each other than either is to the F2 repeat. In addition, all *ebl* family products except for *maebl* have a region paralogous to the F1 repeat. Michon, et al., suggest that the F2 repeat found in the *ebl* family of *P. falciparum*, and its close relative *P. reichenowi*, is the progenitor, by duplication and diversification, to the *var* family DBLs (22). One group reported that in EBA-175, the binding pattern of F2 matches the native protein (37); however, later work by the same laboratory found that COS7 cells expressing BAEBL required the region encompassing both the F1 and F2 repeats to bind erythrocytes (38). In fact, both repeats are probably important in all cases, as Mayor, et al., reported that while the F2 repeat of EBA-175 will bind on its own, the efficiency is much higher when both repeats (F1 + F2) are present (3). In support of this, the crystal structure of EBA-175 R2 shows that while F2 makes a majority of the contacts with its receptor, F1 and F2 form a dimer – and both repeats take part in binding (27, 31). Interestingly, *maebl*

has a chimeric structure with its DBL domain replaced with a cysteine rich region more similar to apical membrane antigen-1 (AMA-1), however, these domains are functionally equivalent to DBL ligand domains with respect to erythrocyte binding (22).

Transcription of the entire *ebl* family occurs during erythrocytic schizont development with *maebl* transcription peaking somewhat earlier than the rest of the family (30). *Pebl* (a.k.a. *eba*-165) is a transcribed but untranslated pseudogene in *P. falciparum* (39); however, in *P. reichenowi*, the *pebl* homologue is a translated, functional gene (40). After translation, DBP, EBA-175, JSEBL, and BAEBL all localize to the micronemes; during invasion, they are believed to be exported to the merozoite surface and then proteolyzed and shed (28). In contrast, MAEBL localizes to the rhoptries and the surface of mature merozoites (22, 34, 41). The difference in evolutionary origin of *maebl's* erythrocyte binding domain, along with the temporal and spatial difference in expression suggests it may have a different function in invasion than the rest of the *ebl* paralogs. However, though its function remains unknown, the sequence conservation of the C-terminal cysteine-rich region suggests it plays a common role in all of the *ebl* family members.

Pathways of Invasion.

Although the EBL family of proteins is very important to the invasion process, disrupting any one member of the family does not typically abrogate invasion as other members of the family can apparently mediate the process.

The most studied erythrocyte receptors for EBL proteins are the DARC for *P. vivax* and glycophorin A for *P. falciparum,* the latter being the most abundant protein on

the human erythrocyte surface. *Plasmodium vivax* is unique in that it is dependent on a single erythrocyte receptor, DARC (20, 42), and cannot invade DARC negative human erythrocytes. This reflects the observation that *P. vivax* possesses only a single *ebl* gene product, *Pv*DBP. On the other hand, *P. falciparum* has multiple pathways of invasion mediated by the other members of the *ebl* family and their cognate receptors (8). The five independent receptors for *Pf*EBL mediated invasion identified to date fall into three broad categories of invasion pathways. These are (a) glycophorin A and sialic acid dependent, (b) glycophorin independent but sialic acid dependent, and (c) sialic acid independent but trypsin-sensitive (43) (Table 1). Both cloned laboratory lines and field isolates (17, 44) are able to utilize multiple pathways of invasion; therefore, this is not a phenomenon caused by long-term *in vitro* growth. In fact, of the at least five pathways of invasion described, four have been identified in wild isolates (45). No one has yet demonstrated a human erythrocyte that both lacks a specific receptor and is resistant to invasion from all *P. falciparum* clones. Conversely, no one has yet demonstrated that any single member of the *ebl* family is necessary in all *P. falciparum* strains for successful invasion of human erythrocytes. Thus, the other members of the *ebl* family are presumably functionally equivalent. The use of different invasion pathways is apparently strain dependent as was demonstrated by Maier et al. (36) who observed that antibody inhibition of BAEBL affected host erythrocyte invasion by strain 3D7 significantly more than by strain W2mef. The fact that sialic acid independent invasion is more common in field isolates from Asia than Africa (17, 44) is also evidence that preference for invasion pathways may be strain dependent. However, we do not

know the mechanism that determines sialic acid dependence or independence, or why strain preferences occur.

The parasite ligand that binds the receptor glycophorin A is EBA-175. Recognition and binding of glycophorin A by EBA-175 requires both the sialic acid moiety and the peptide backbone. This is demonstrated by its lack of binding to glycophorin B, which has the same sialic acid configuration as glycophorin A but lacks an identical peptide backbone (37). JSEBL (EBA-181) and BAEBL (EBA-140) are also sialic acid dependent, and neither binds glycophorin A or B. Therefore, for sialic acid dependent ligands, the peptide backbone of their cognate receptors is probably the key factor determining specificity (34).

Disruption of either the *eba*-175 gene (46) or removal of glycophorin A (37) from the erythrocyte surface provides evidence for the existence of multiple pathways of invasion. Disruption of the EBA-175/glycophorin A pathway almost interrupts invasion for some cell lines, such as W2mef, while only slightly reducing invasion efficiency for other cell lines, such as 3D7. Additionally, both EBA-175 dependent and independent cell lines show reduced invasive efficiency when invading chymotrypsin-treated erythrocytes. As chymotrypsin spares glycophorin A and sialic acid, this result might suggest that multiple ligand-receptor interactions are involved in invasion (47). Other evidence suggesting the necessity for multiple ligand-receptor interactions is the observation that antibodies directed against the F2 repeat of the EBA-175 domain can inhibit sialic acid dependent as well as independent invasion (48). These data, and the general observation that removing any single invasion pathway from a cell line usually reduces invasive efficiency by at least a small amount, even if the removed pathway is

not the cell line's dominant invasion route, supports the hypothesis put forth by Duraisingh, et al., that a minimal affinity provided by multiple ligand-receptor interactions is necessary for invasion to occur (47).

Redundant invasion pathways may be advantageous for several reasons. First, they could provide the parasite an advantage in dealing with the host immune response by providing a means to circumvent a pathway blocked by an immune response that could otherwise prevent a continued infection. Second, they could be an effective response to host cell receptor heterogeneity. Loss of a pathway due to a polymorphism or a new mutation in a host receptor is less likely to prevent a successful infection if the parasite has multiple invasion routes available. Finally, multiple ligand-receptor pairs could contribute to a minimum affinity that might be necessary for invasion to occur (43, 47).

Polymorphisms and Antigenic Diversity.

Polymorphisms and antigenic diversity represent the most significant obstacle to developing a malaria vaccine. The presence of polymorphisms in *ebl* family ligands that can alter both the affinity and specificity of binding, and the antigenic character of the ligand, illustrate this fact. The DBL domain of *ebl* products can be highly polymorphic among different strains, and within some members of the *ebl* family. For example, in *baebl* and *jsebl*, a single nucleotide change causing a nonsynonymous amino acid change can lead to a change in erythrocyte receptor specificity (38, 49). On the other hand, in *Pv*DBP and EBA-175, nonsynonymous substitutions do not lead to changes in ligand specificity; however, in the case of *Pv*DBP, these polymorphisms can lead to

changes in the antigenic character of the ligand. The majority of these polymorphisms

affect charged or polar residues in a region with potential to cause both changes in

protein structure and possibly changes in how the immune system "sees" new epitopes

(50, 51). In the *Pfebl* genes *baebl* and *jsebl*, the majority of known nonsynonymous

polymorphisms in the DBL domain occur in the F1 repeat rather than the F2 repeat,

even though the latter is often considered the more important repeat for host cell

binding. In contrast, Drummond, et al., found the majority of nonsynonymous

polymorphisms in *ebl*-1 occur in the F2 repeat (23, 38, 49). Whether changes in binding

specificity of *ebl*-1 are occurring due to nonsynonymous mutations in the binding

domain is unknown at this time, as the cognate host cell receptor for this ligand has not

been identified.

Recent studies of EBA-175, EBL-1, and EBA-140 have reported a high ratio of

nonsynonymous to synonymous polymorphisms (i.e. K_n/K_s >1) in the DBL domain,

which suggests positive selective pressure for polymorphisms. There are two possible

reasons for positive selective pressure to operate on the DBL domain: (1) to change

receptor specificities in cases where the primary receptor may not be available due to

host cell polymorphisms, and (2), to change antigenic character in order to evade the

host immune response (23, 38, 52). Regardless, there is little doubt that the

polymorphic character of the DBL domain will provide a challenge to vaccine

development.

Heterologous Expression.

Heterologous expression has replaced biochemical purification as the principle method for obtaining purified, authentic proteins for characterization. While there are many heterologous expression systems, there is no known single host appropriate for all applications. *Plasmodium falciparum* presents unique challenges for many of these common systems. Many of the issues stem from both the extreme A+T richness of the genome and the cryptic polyadenylation sites (53). Indeed, A+T base-pairs account for approximately 81% of the overall genome, being present at 76% in exons and 86% in introns (54). The A+T richness leads to significant codon bias – and expression levels often correlate to codon preference. As a result, many heterologous systems do not efficiently express *P. falciparum* genes.

We will start by looking at what makes a good expression system and then examine some possibilities. First, the expressed genes should have similar codon usage as the expression host. Codon usage can affect gene expression and thus, quantities of expressed protein (55, 56). Furthermore, if an mRNA is highly expressed, mistranslation can occur if the message contains codons that are rare to the host (56, 57). Second, the expression host should be able make the correct post-translational modifications which can affect the biological activity, solubility, stability and distribution of the expressed protein. Thus, the expressed protein should be authentic, i.e., the biological properties should be the same as the native protein or active domains. Third, ease of downstream purification is important. If a system is available for secreting expressed proteins, purification is simplified. Secretion also avoids high intracellular levels of protein that may be toxic to the expression host. Fourth, expression levels

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should be high; the expression host should be capable of producing large amounts of the protein. Finally, the expression host should be easy and inexpensive to culture.

Next, we will compare some model heterologous expression systems used to express *P. falciparum* proteins.

1. Mammalian cells: Typically COS7 cells, these are good at post-translational processing, and often produce proteins with biological activity. However, mammalian cells typically produce N-glycosylated proteins which are absent in the asexual intraerythrocytic stage of *P. falciparum* where O-glycosylation typically occurs at low levels. In addition, mammalian cell culture is more difficult, expensive, lower yielding, and has a long generation time as compared to other expression systems.

2. *E. coli:* On the positive side, *E. coli*, a bacterium, is simple and inexpensive to culture, can be grown to a high density, may produce high amounts of protein, and does not glycosylate proteins; thus, N-glycosylation is not an issue. On the negative side, production of authentic proteins from eukaryotes is difficult, as prokaryotes do not make proper post-translational modifications of eukaryotic proteins. As an example, since *E. coli* does not glycosylate proteins at all – it cannot attach a GPI anchor, which is the major carbohydrate modification on *P. falciparum* proteins (58). In addition, *E. coli* often does not form disulfide bonds, or forms incorrect ones. The proteins it does produce typically accumulate as inclusion bodies where

they are insoluble (59) and misfolded. Thus, for functional proteins,

proteins produced in *E. coli* must be solubilized from inclusion bodies,

refolded, and then purified (60) which is a costly and low yielding affair.

Finally, in *E. coli* the codon usage is biased towards G+C codon usage

making mistranslations possible (57); indeed, long stretches of the A+T rich

P. falciparum DNA is unstable in *E. coli* (61).

3. *Dictyostelium discoideum:* There are several advantages to using

Dictyostelium, an amoeba, as an expression host. It is simple and cheap to

culture and can be grown to a high density, at least 10^7/mL (62). The

codon usage is relatively A+T rich (62, 63), and thus, may be closer to that

utilized by *P. falciparum*. It can be transfected by electroporation and the

selection process is relatively inexpensive using the drug G418.

Heterologous proteins can either be expressed in the cytoplasm, GPI

anchored on the surface, or secreted (62), and if cytoplasmic expression is

chosen, cell lysis is simple and mild as there is no cell wall (63). Finally, it

has the ability to correctly fold *P. falciparum* genes (63). There are also

some disadvantages to using *Dictyostelium*. The yield is highly variable

depending on the protein. Van Bemmelen et al. expressed several *P.

falciparum* proteins and found the yield to vary depending on the sequence

– from 0.08 mg/L to 3 mg/L (63). Also, the stop codon in *Dictyostelium* is

almost always UAA, while in *P. falciparum* it is typically UAG and UGA;

however, changing to a *Dictyostelium* stop codon in the expression cassette seems to help expression (62).

4. *Pichia pastoris*: *Pichia,* yeast, is another organism that is simple and cheap to culture. Furthermore, it can make complex post-translational modifications and utilize both intracellular and secretory pathways. It also has a long track record of expressing human, animal, plant and viral proteins in authentic form (56). As *Pichia* secretes very little native protein, purification is simplified. Finally, John et al. successfully used *Pichia* to express domains of the *P. falciparum* proteins AMA-1 and EBA-175 (64). On the other hand, expression of both secreted and cytoplasmic proteins is commonly low (56). *Pichia* also hyperglycosylates and recognizes the same N-glycosylation sites as higher eukaryotes, rather than the O-glycosylation sites on *P. falciparum* products. Although mutants have been isolated that do not glycosylate as heavily, these grow much slower (56). These issues are important as over-glycosylation of *P. falciparum* proteins can render them refractory to crystallization as was observed with EBA-175 (31). Another issue in *Pichia* is that some A+T rich sequences may cause early termination or polyadenylation leading to truncated mRNAs (53).

5. *Toxoplasma gondii*: di Christini et al. used *Toxoplasma*, an Apicomplexan, to express CSP (65). As *Toxoplasma* is phylogenetically closer to *P. falciparum* than the other expression systems, it is more likely to correctly

process proteins if the A+T richness of *P. falciparum* is not an issue for expression. The main concern here is using a pathogen as a heterologous expression system. Additionally, it is a very expensive system as *T. gondii* is cultured in a layer of mammalian cells.

6. <u>*Tetrahymena thermophila*</u>: Several properties of the free-living protozoan *T. thermophila* make it a desirable expression host for *P. falciparum*. It is an alveolate like *P. falciparum* and thus, is evolutionarily related. Transfection is by homologous recombination; that is, it is locus specific (66, 67) and thus facilitates gene replacement. Strain Cu522 has a selectable marker for replacement of *β-tubulin 1* (*btu1*) (66). Transfection into this locus has no negative effect on the transfectant because *β-tubulin 2* (*btu2*) performs the same function and is unaffected by replacement of *btu1*. Thus, one can select for any expression cassette. *Tetrahymena* is simple to culture, subculture, and clone and grows to high density in small or large volumes. The generation time is about 3 hours in optimal conditions and the organism tolerates a wide range of tonicity and temperature (68). The culture medium is also many times cheaper than that required for mammalian culture. The genome is A+T rich; approximately 62% of the base pairs in coding regions are A+T (69). Therefore, the codon usage may be closer to *Plasmodium* than in some other model expression hosts. *Tetrahymena* is non-pathogenic, and finally, it has already been used to express proteins from the parasitic ciliate *Ichthyophthirius multifiliis* and the

apicomplexan *P. falciparum* (70, 71). However, *Tetrahymena* also has some shortcomings as an expression host. *Tetrahymena* constitutively secretes hydrolytic (lysosomal) enzymes (68). However, if this becomes an issue, at least one mutant strain (MS-1) is available that is defective in this regard (72, 73).

In summary, the ideal heterologous expression system will be simple and inexpensive to culture, and produce authentic protein in high quantity. The importance of this is illustrated by the fact that some proteins, such as AMA-1, require authentic conformation to elicit parasite-inhibitory antibody responses (74). A general system for production of authentic *P. falciparum* proteins may have implications for multi-subunit vaccine development and rational drug design as this is the only method that will allow enough protein to be produced to be of use in vaccine production.

Summary.

The burden of malaria is continuing to increase even in the face of the "Roll Back Malaria" campaign. Breaking the cycle of host erythrocyte invasion is a key aspect of preventing disease; hence, understanding every facet of erythrocyte invasion is critical. By the end of these studies, we will have a better understanding of the invasion pathways mediated by EBL-1 and JSEBL and will have created tools to study and understand these and other erythrocyte invasion related phenomena.

CHAPTER 4

FUNCTIONAL CHARACTERIZATION OF HETEROLOGOUSLY EXPRESSED

PLASMODIUM FALCIPARUM EBL PROTEINS[1]

Abstract.

While heterologous expression has replaced biochemical purification as the principle method for obtaining purified, authentic proteins for characterization, no single expression system has proven appropriate for all applications. Heterologous expression of *Plasmodium falciparum* genes has proven especially challenging due to their extreme A+T codon bias and inappropriate post-translational modifications to product made by existing expression systems. These issues have generated interest in developing a novel heterologous expression system for *P. falciparum*. Here, we have transfected the ciliate *Tetrahymena thermophila* with a novel secretion vector bearing the R2 region of the *P. falciparum* genes, *ebl-1* and *jsebl*. In addition, we demonstrated successful expression of the R2 region. This is the first demonstration of secreted *P. falciparum* antigens expressed by *T. thermophila*. We believe *T. thermophila* shows promise as a heterologous expression system for *P. falciparum* genes.

Introduction.

Malaria is the most important parasitic disease to affect humans. Greater than 40% of the world's population lives at risk of contracting malaria, resulting in 300-500 million new clinical cases each year and 2-3 million deaths. Most of the deaths, caused by *Plasmodium falciparum*, the most virulent species infecting humans, occur in children and pregnant women living in Sub-Saharan Africa (9, 10). Despite efforts to control the disease, the incidence of malaria is increasing due to resistance to established therapies and insecticides, and the difficulty of developing an efficacious vaccine (1, 75, 76).

The malaria parasite has a complex life cycle that requires it to invade several cell types in its vector and definitive host, the *Anopheles* mosquito, and in its human intermediate host. Invasion of host erythrocytes is a required step in the life cycle and the erythrocyte-binding-like (*ebl*) family of proteins is important to this process (8). It is the cyclic expansion of merozoites in the asexual, erythrocytic stage of its life cycle that causes the characteristic symptoms of malaria.

Structural and functional studies of parasite antigens identified as potential vaccine candidates, and their cognate receptors are important for vaccine and rational drug development. These studies require relatively large amounts of pure authentic antigen derived from the parasite. This need has generated much interest in both existing heterologous expression systems (55, 56, 60, 77), often in combination with the construction of synthetic genes (53, 74, 78), and in attempts to develop new systems. While no known heterologous system is appropriate for all applications (79), the expression of *P. falciparum* proteins has proven especially problematic. Although these problems are in large part due to its extreme A+T codon bias, other issues include high cost, low yield, incorrect disulfide bond formation, inappropriate post-translational modifications, and the G+C biased codon usage of many of the existing expression systems.

In 1999, Gaertig et al. (71) addressed some of these issues by successfully expressing a surface antigen from *Ichthyophthirius multifiliis*, a protozoan parasite infecting fish, in the related ciliate *Tetrahymena thermophila*. Peterson et al. (70) extended this work by expressing the full-length circumsporozoite protein (CSP) of *P. falciparum* on the cell surface of *T. thermophila*. The significance of this study lies in

the observation that while others have expressed full-length CSP, this was the first instance in which the signal for GPI anchor attachment was functional. CSP is a surface membrane protein; however, when this protein was expressed in other heterologous systems, an apparent failure to recognize and process the cleavage and attachment signal of the GPI anchor resulted in CSP remaining in intracellular organelles rather than making its way to the cell surface (62, 65, 80). Alley et al. (under revision) further extended this work by expressing the F2 domain, the principal binding motif, of the EBL protein erythrocyte-binding antigen-175 (EBA-175). They accomplished this in a similar manner to previous work by transfecting *T. thermophila* through replacement of the paclitaxel sensitive β-tubulin 1 (*btu1*) gene with the F2 domain of eba-175 (70, 71). However, while the previous work placed the transgene under the control of the *btu1* promoter, in the EBA-175 study they used a vector that has a metallothionein (MT) promoter rather than a *btu1* promoter. The MT promoter is both inducible and more robust than the *btu1* promoter (81). Additionally, Alley et al. (under revision) modified their vector to include the part of the *I. multifiliis* immobilization antigen (i-antigen) gene that contains the region encoding the N-terminal signal sequence and the C-terminal glycosylphosphatidylinisotol (GPI) anchor sequence. These sequences drove the surface expression of the F2 domain of the eba-175 they transfected into *T. thermophila*. Finally, Alley et al. (under revision) tested for authentic function by determining that the expressed product could bind glycophorin A which is the cognate receptor for EBA-175.

The goal of the current work is to express Region 2, which encompasses the F1 and F2 domains of the EBL proteins EBL-1 and JSEBL in *T. thermophila*. We started

by attempting to bypass a plasmid vector by determining the minimum sequence needed for homologous recombination into the UTR regions. In fact, the minimum sequence necessary was apparently much longer than can be synthesized on a primer. Thus, we constructed a new vector, pTART, which included a His-tag, a FLAG epitope, and several GC-rich cutting sites. Furthermore, pTART is a secretion vector. This should make purification simpler, and allow for experimentation that would not be possible with a protein expressed on the surface of another cell.

The rationale for this work is to continue the development of a system for the heterologous expression of authentic *P. falciparum* genes in *T. thermophila*. Here, we show evidence of *T. thermophila* expressing and secreting Region 2 of the EBL proteins EBL-1 and JSEBL. We believe *T. thermophila* may represent a promising method for producing functional *P. falciparum* protein without the need for refolding protocols (60) or codon optimization.

Materials and Methods.

Minimum sequence needed for homologous recombination

In order to obtain a gene with regions homologous to the 5' UTR and 3' UTR on either side of the *btu1* gene we used the plasmid pBIticsp7a (70). This plasmid contains the full-length CSP gene flanked by the 5' UTR and 3' UTR of the *btu1* gene. In order to test two different lengths of homology, we designed four primers, approximately 100 bp and 500 bp both upstream and downstream of the gene in the UTR, amplified the intervening region, and used the amplicons to transfect *T. thermophila*.

For the 500 bp of homologous UTR we used the primer pair CSP500-5 5'–TTG ATACTTGTTTACGTTGTATTATTTTG–3', and CSP500-3 5'–TAACAAATTTATTTA TAGTACTGAAAAGC–3'. For the 100 bp of homologous UTR we used the primer pair CSP100-5 5'–CTCAATAGTAATATTTAAGAAGAGAGGG–3', and CSP100-3 5'– ATGTTATGTGAATGAAGTTAATTGGG–3'. Extensions in both cases were carried out at 65° C due to the A+T rich nature of the *P. falciparum* genome (82). Annealing temperature for the CSP500 primer pair was 56° C and for the CSP100 primer pair was 60° C. We amplified DNA with Ex Taq polymerase (TaKaRa Mirus Bio Corp., Madison, WI), as it has proofreading activity and appears to work well with *P. falciparum* DNA in our hands. Finally, we gel purified the amplicons using a Zymoclean Gel DNA Recovery Kit (ZYMO Research, Orange, CA).

Construction of expression vectors

We derived the pTART secretion vector from the pMTT-Tvec surface expression vector (Alley et al., under revision) by removing the multiple cloning site (MCS), the c-myc epitope, and GPI anchor signal sequence and replacing them with another MCS with four G+C rich restriction sites, a FLAG epitope for identification, and a 6-His tag for purification.

To make our *ebl-1* expression construct, we first amplified Region 2 of *eba-1* from the *P. falciparum* clone Dd2 with the primer pair ebl-1F1-5' 5'–ATA<u>CCATGG</u>ATT GTGGGAAGAAAATAAAGGAAATG–3', and ebl-1F2-3' 5'–ATA<u>CCGCGG</u>TACATACCA TACAAGCCTCTTTATATTTATTAG–3'. The forward primer and reverse primer have an *Nco I* and a *Sac II* restriction site incorporated respectively (underlined) to facilitate

ligation into the MCS of the secretion vector pTART. Extensions were carried out at 65°

C and annealing at 54° C. Finally, we digested both the Region 2 amplicon and pTART

with *Nco I* and *Sac II*, ligated the amplicon into pTART to produce an expression

construct encoding the entire Region 2 of the *P. falciparum* ebl-1 gene, and cloned the

construct in *Escherichia coli* (XL1-Blue).

To make our *jsebl* expression construct, we first amplified Region 2, the

erythrocyte binding region, of *jsebl* from the *P. falciparum* clone 3D7 with the primer

pair jsebl-F1-5' 5'–ATA<u>CGGCCG</u>ATTGTA AAGGAAAAAGAAGCACACATAC–3', and

5'–ATA<u>CCGCGG</u>TACATGAACATCTTT CCTTAAAGAGTTT–3'. The forward primer

and reverse primer have an *Eag I* and a *Sac II* restriction site incorporated respectively

(underlined) to facilitate ligation into the MCS of the secretion vector pTART.

Extensions were carried out at 65° C and annealing at 54° C. Finally, we digested both

the Region 2 amplicon and pTART with *Eag I* and *Sac II*, ligated the amplicon into

pTART to produce an expression construct encoding the entire Region 2 of the *P.

falciparum jsebl* gene, and cloned the construct in *Escherichia coli* (XL1-Blue).

Sequencing of expression constructs

We had MWG Biotech (High Point, NC) sequence the constructs using the

primer set pTART_seq5' 5'–CAATGATCCATATAAAATAATGTAAATAGTG–3',

jseblR2_seq5'1 5'–TTGTGACGATGTAAAAAATAGTTATTTAG–3', jseblR2_seq5'2 5'–

TTAAATTGT TCTGAGTGTAAGGATATTG–3', pTART_seq3' 5'–ACAGTAAGATGC

TTA AGTAAA AATAATAAAG–3', jseblR2_seq3'1 5'–

AAGTTTTTCTACGTTGATCTACTT CG–3', and jseblR2_seq3'2 5'–AACAGTATCTGT

TTGAGCATTAAATG–3' for the *jsebl* construct. For the *ebl-1* construct, we used the

primer set pTART_seq5' 5'–CAA TGATCCAT ATAAAATAATGTA AATAGTG–3',

pTART_seq3' 5'–ACAGTAAGATGC TTA AGTAAA AATAATAAAG–3', ebl1R2_seq5'1

5'–ACAAGAATG ATAGTATAAATAATAAGTTT AAAG–3', ebl1R2_seq5'2 5'–

AAATTGTTCGATGGATAG AGGAG–3', ebl1R2_seq3'1 5'–

CACATATTTTCTCTAATTGCTTGACTT–3', and ebl1R2_seq3'2 5'–TTTTAATCC

TTCCTCGTTACCG–3'. Several primers were needed for each direction as each run

only provides 600 – 800 bp of sequence data.

Cell culture, transfection, and selection

We used the CU522 strain of *T. thermophila* as our transfection host. This strain

contains a single Lys350Met substitution in *btu1* that renders the microbe sensitive to

the presence of paclitaxel (66), a microtubule stabilizing drug, and thus amenable to

negative selection for replacement of this gene. Replacement of *btu1* is possible as the

btu2 gene encodes a nearly identical protein that is functionally equivalent to the protein

encoded by *btu1* but lacks the Lys350Met substitution and consequently the sensitivity

to paclitaxel. Briefly, we cultured cells in SPP medium supplemented with 100 units ·

mL^{-1} penicillin, 100 ug · mL^{-1} streptomycin, and 0.25 ug · mL^{-1} amphotericin B in a

shaking incubator at 175 rpm and 30° C. We replaced the host *btu1* coding region with

the MT promoter and Region 2 of either jsebl or ebl-1 by biolistic bombardment of

starved vegetative cells followed by paclitaxel selection as previously described (71).

Concentration and partial purification of expressed protein

We set up the concentration and partial purification of expressed protein by starting with one culture of *T. thermophila* transfected with Region 2 of *jsebl*, another culture transfected with Region 2 of *ebl-1*, and a negative control culture using a non-transfected strain CU522. We adjusted all the cultures to be at a concentration of $2 \cdot 10^{5}$ cells $\cdot$ mL^{-1} and induced protein production by the addition of $CdCl_2$ at a concentration of 5 µg $\cdot$ mL^{-1}. Next, we incubated the cultures in a shaking incubator for 12 hrs at 175 rpm and 30° C. After the incubation, we centrifuged 30 mL of each culture at 750 $\cdot$ *g* for 5 minutes. The pellet of cells was frozen for future use and the supernatant filtered through a 0.45 µm syringe filter to remove any remaining cells. The supernatant was then concentrated using an Amicon Ultra Centrifugal Filter (Millipore Corp., Billerica, MA) with a molecular weight cutoff of 30 kDa. We attempted further purification based on the 6-His tag on the expressed regions trying two different columns; the first was the His-Select Spin Column (Sigma, St. Louis, MO) and the second was the His SpinTrap (GE Healthcare, Piscataway, NJ).

Western Analysis

As the purification based on the 6-His tag failed in every instance, we tested for expression using both filtered supernatant and concentrated, filtered supernatant of Cu522 (negative control) and Cu522 transfected with *ebl-1* and Cu522 transfected with *jsebl*. We separated the samples on 8% - 16% gradient gels (Pierce Biotechnology, Rockford, IL). As antibodies against EBL-1 and JSEBL are not available, after SDS-PAGE, we subjected the samples to Western Analysis by probing with Anti-FLAG

polyclonal antibodies (Sigma, St. Louis, MO) diluted 1:500 followed by an Alkaline

Phosphatase conjugated Goat anti-rabbit secondary (Sigma, St. Louis, MO) diluted

1:5000. The target proteins were detected colorimetrically using an NBT/BCIP

substrate.

Results.

Minimum sequence needed for homologous recombination

The rationale for this initial goal is two-fold. First, it is well established that longer

regions of *P. falciparum* DNA can be problematic to clone in *E. coli*. Problems include

lack of recombinant clones, deletions and rearrangements of the insert. While there is

no absolute size of *P. falciparum* DNA at which problems will definitely be observed,

any future attempt by us to clone or express an entire *ebl* gene would likely be affected

by this issue. Second, elimination of this cloning step would significantly speed up the

process of obtaining *T. thermophila* transfectants. Our approach here was to PCR the

P. falciparum CSP gene from the plasmid pBIticsp7a with flanking UTR from the *T.*

thermophila btu1 gene with either 100 bp or 500 bp of UTR. Ideally, we had hoped that

flanking regions of 100 bp would suffice to direct homologous integration. This would

allow us to synthesize oligos containing the homologous targeting region and the *P.*

falciparum gene specific region for amplification. All attempted transfections using the

P. falciparum CSP gene from the plasmid pBIticsp7a with flanking UTR from the *T.*

thermophila btu1 gene were unsuccessful. Homologous regions longer than 500 bp are

likely needed for homologous recombination in *T. thermophila* (Fig 4.1). Due to

technical limitations concerning long oligo synthesis, homologous regions of approximately 100 bp or shorter would need to successfully recombine to be of practical use.
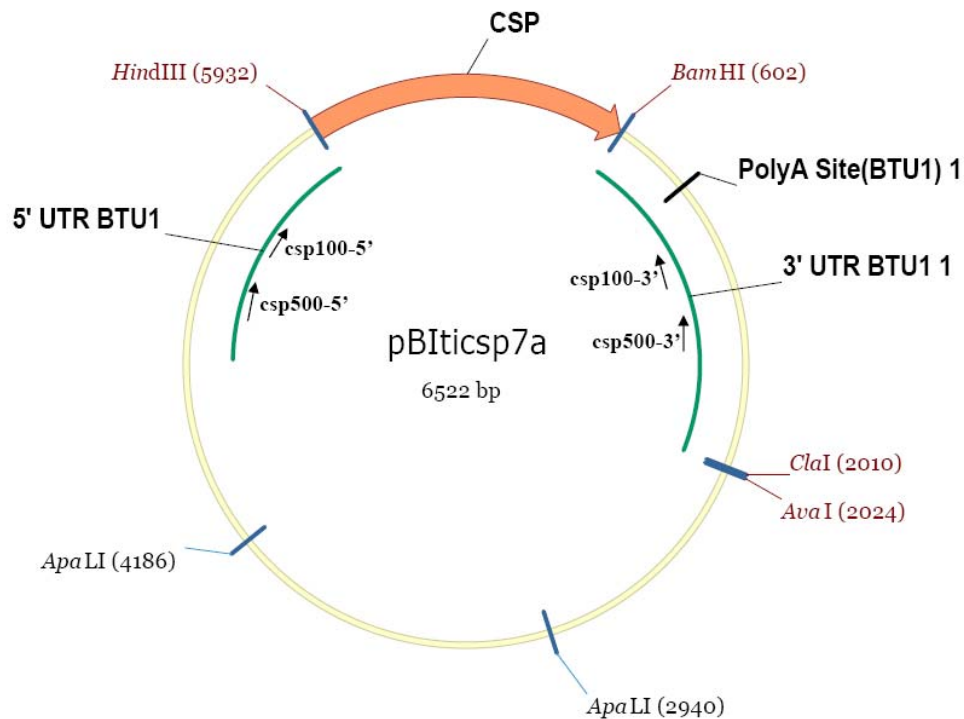

Fig. 4.1. Minimum sequence needed for homologous recombination

Construction of expression vectors

As discussed in the introduction, previous work with the *Tetrahymena* system utilized an expression vector that provided for surface expression of *P. falciparum* domains. To better facilitate purification of expressed regions we sought to construct a vector that would provide secreted protein. We constructed the pTART secretion vector by starting with the pMTT-Tvec surface expression vector and removing the MCS, the c-myc epitope and the GPI anchor signal sequence. To provide for more flexible cloning options we designed the new MCS to have four G+C rich restriction sites that are

unlikely to be found in A+T rich *P. falciparum* sequences, a FLAG epitope for

identification, and a 6-His tag for purification. Additionally, it has an *I. multifiliis* I-antigen

signal sequence to target the protein to the secretory pathway. The protein should then

be secreted rather than retained at the membrane surface as it lacks a GPI anchor

signal sequence or a transmembrane domain. Finally, the vector has a *T. thermophila*

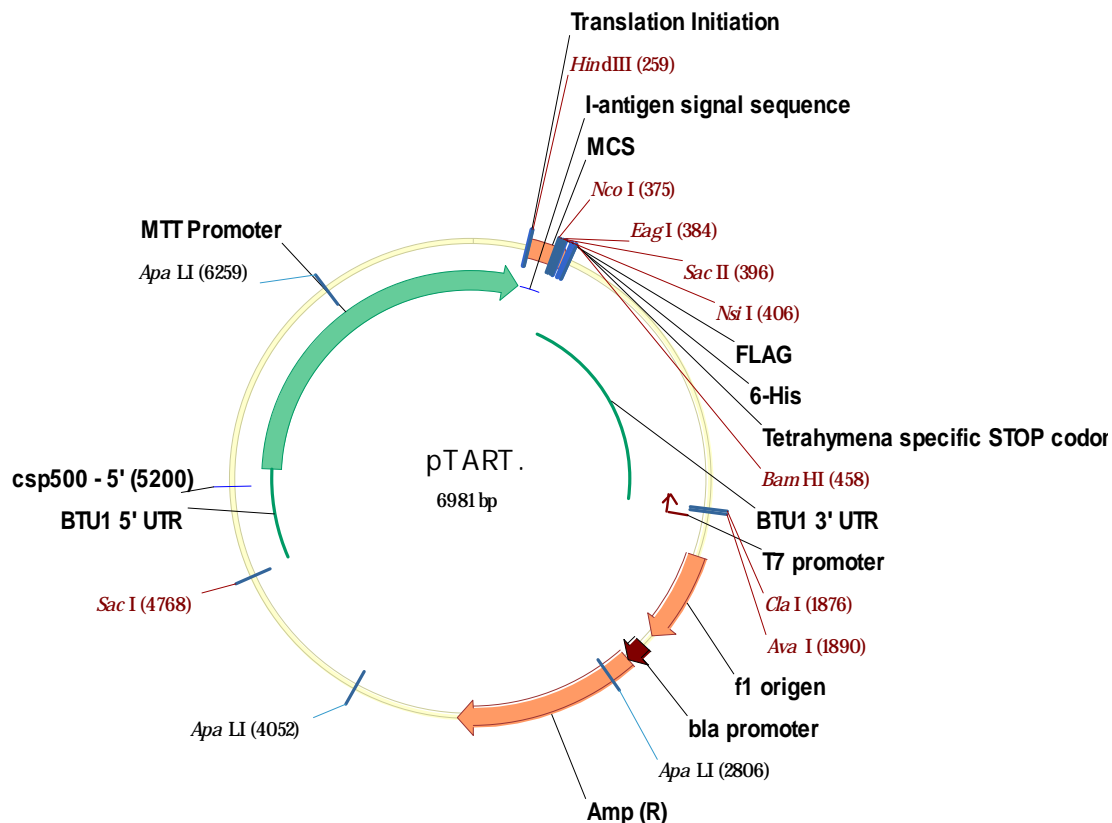specific stop codon. (Fig 4.2).

Fig. 4.2. The secretion vector pTART.

Next, we cloned Region 2 of the EBL-1 and JSEBL proteins into the pTART

secretion vector. We selected the entire Region 2 of the EBL-1 and JSEBL proteins to

express because, while previous studies show the F2 domain of the related protein

EBA-175 is both necessary and sufficient for binding the red cell, the F1 domain

nevertheless contributes to binding (31). Region 2 is comprised of the F1 and F2

domains (Fig. 4.3). We designed the primers by downloading the gene sequences for

EBL-1 and JSEBL from PlasmoDB and choosing primer sites on either side of Region 2

of the respective genes. After PCR, a single band was observed in both instances,
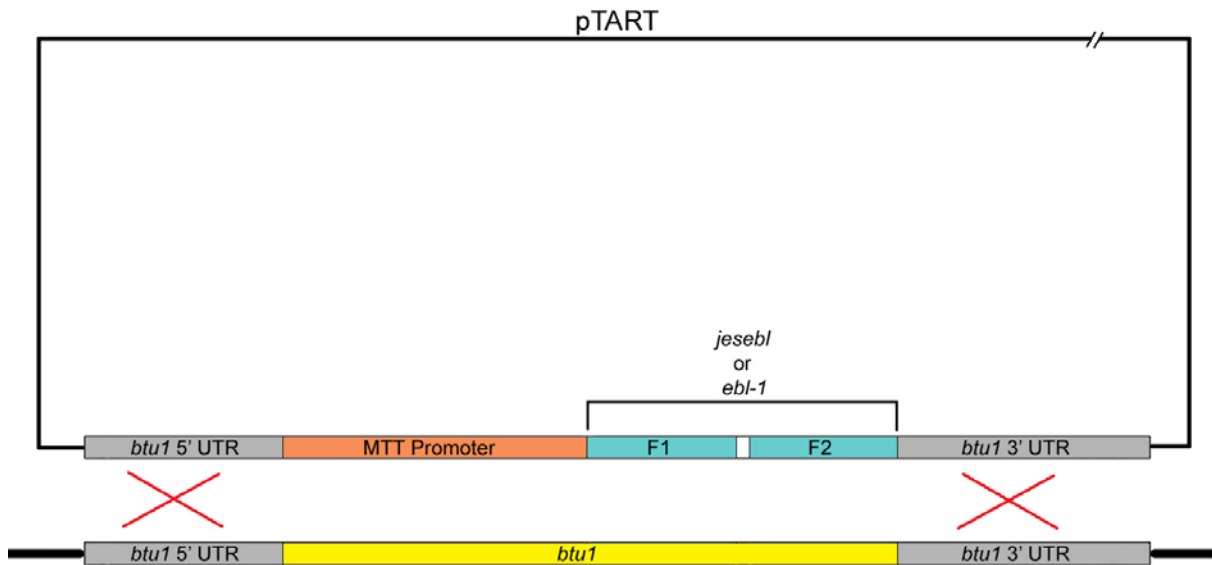
which we then cloned into pTART.

Fig. 4.3. Schematic representation of the pTART expression vector replacing the *Tetrahymena btu1* gene with the F1 + F2 domains of *jsebl* or *ebl-1*.

Sequencing of expression constructs

We obtained the full nucleotide sequence of both strands of the insert, and

confirmed the identity of the sequences by comparing the translated amino acid

sequences to those published in GenBank. We verified the existence of an open

reading frame for the R2 region using Vector NTI.

Transfection and selection of transgenic T. thermophila

We introduced the *P. falciparum* gene sequences into starved, vegetative *T. thermophila* via biolistic bombardment using gold particles coated with linearized expression vector. The expression vector integrated into the *btu1* locus by homologous recombination facilitated by the (500 bp) 5' and (1400 bp) 3' UTR of the *btu1* gene flanking the MT promoter and either Region 2 of *jsebl* or *ebl-1* respectively. Biolistic bombardment of starved, vegetative *T. thermophila* targets the macronucleus, which contains approximately 45 copies of every gene. During vegetative, asexual reproduction, the macronucleus divides amitotically, and randomly distributes genes from the macronucleus into each daughter cell resulting in phenotypic assortment (66). Only partial replacement of the *btu1* genes occurs initially, and paclitaxel selects against the presence of *btu1*; thus, by phenotypic assortment, we preferentially selected cells containing progressively more copies of the transgenes by keeping the cultures under continuous selective pressure. We screened for, and confirmed, the presence of the transgenes by PCR (Fig. 4.4).
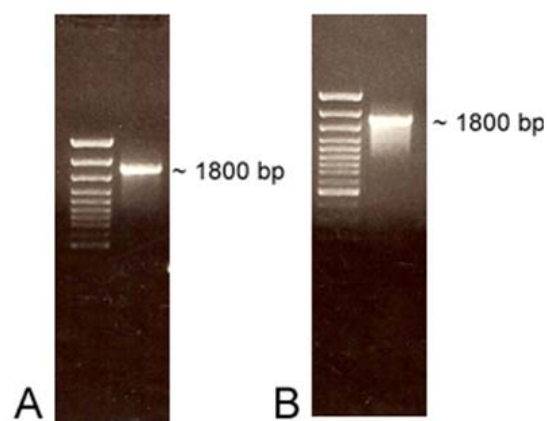

Fig. 4.4. PCR of *ebl-1* and *jsebl* transgenes. **A.** *ebl-1*. **B.** *jsebl*.

Western Blot analysis

For both the *ebl-1* and *jsebl* transfectants, PCR demonstrated the transgene's incorporation into the macronuclear genome. To assess expression, we conducted western blot analysis of one clone expressing EBL-1 and one clone expressing JSEBL. We assayed neat supernatant, supernatant concentrated approximately 85 X, and pelleted cells to determine whether protein was being produced, and if so, if it was secreted. On western blots, the concentrated supernatants revealed a band of approximately 70 kDa in both cases that is consistent with expected gene products containing a FLAG epitope and Region 2 of EBL-1 and JSEBL respectively. In contrast, the pelleted cells, the straight supernatants, and the negative control, *Tetrahymena* strain Cu522 that lacked a transgene, did not show specific staining. In both cases the positive control, a FLAG-BAP (an N-terminal Met-FLAG fusion protein of *E. coli* bacterial alkaline phosphatase (BAP)) protein that migrates as a 45-55 kDa band showed specific staining (Fig. 4.5).
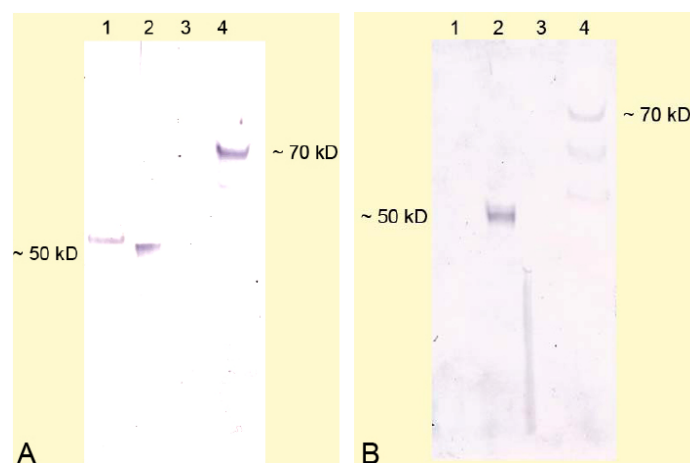

Fig. 4.5. Western Analysis of heterologous expression. **A.** EBL-1. **B.** JSEBL. In both cases lane 1 is the negative control; lane 2 is the positive control; lane 3 is supernatant; lane 4 is concentrated supernatant.

Discussion.

To date, our work supports the continued development of *T. thermophila* as a system for the heterologous expression of *P. falciparum* genes. While earlier work has demonstrated the expression and localization of *P. falciparum* genes to the surface of *T. thermophila* (70), these are the first experiments that demonstrate secretion of heterologously expressed *P. falciparum* proteins.

We began this work by attempting to bypass cloning in *E. coli*, as the long A+T rich sequences of *P. falciparum* are inherently unstable in *E. coli*. Our strategy involved attempting to determine the minimum sequence needed for homologous recombination into the target site with an eye to being able to use a length short enough to be synthesized as part of a primer. We found that more than 500 base pairs are needed for homologous recombination into *T. thermophila*, which forced us to abandon this strategy in favor of making a secretion vector that would be of general use for cloning *P. falciparum* sequences for transfecting into *T. thermophila*. Although the R2 regions we cloned into our vector were stable, it is likely that an attempt to express EBL-1 and JSEBL in their entirety would have failed. To date, there are no reports of the expression of an entire protein containing a DBL domain. This is unfortunate, as structural studies relying only on the expression of DBL domains alone may not provide a complete picture of the structure of the entire molecule. The vector, pTART, contains an I-antigen signal sequence for targeting, a His-tag to aid in purification of the expressed protein, a FLAG epitope for identification, and several GC-rich cutting sites that are unlikely to be found in the AT-rich *P. falciparum* genome. We chose to make a secretion vector rather than a vector designed for surface expression as a limitation to

surface expressed proteins is the relative difficulty in purification. However, efforts at

purification using the 6-His tag failed every time it was attempted. We believe this could

have been due to the tag being buried in the protein, and thus not available for binding

to the nickel column. If time and availability permitted, we would have liked to have tried

our purification system with a positive control 6-His protein. It may be possible to use

the FLAG epitope for purification. Alternatively, a version of the vector with a N-terminal

6-His tag could be constructed. It remains to be seen whether the purification difficulties

are due to a structural feature of the DBL domain

In spite of our inability to purify the protein on a Nickel column, we were able to

detect both expressed EBL-1 and JSEBL on Western Analysis using filtered,

concentrated supernatant. We filtered the supernatant through a 0.45 μM filter to

remove remaining *T. thermophila* to rule out signal due to possible surface expression.

Concentrating the supernatant approximately 85 X through an Amicon Ultra-15 (mw cut-

off = 30 kD) was enough to give a strong signal of the expected molecular weight where

the unconcentrated supernatant gave no signal. However, we had an unresolved issue

with run-to-run variation, with some runs (growth, induction, filtration, concentration,

Western blot) giving either no signal or bands of much smaller size. Out of eight runs, 3

showed specific binding – however most of these also showed binding of much smaller

bands. This may have been due to lysosomal proteases secreted by *T. thermophila*

(68). We could have tested this hypothesis by using an available mutant strain of *T.*

thermophila that is defective in protease secretion (72, 73). Additionally, while some

Western blots produce bands of the correct estimated size, we were not able to test for

authenticity of the expressed protein due to both time constraints and the issue of run-

to-run variation mentioned above. However, after solving the variability issue, invasion inhibition assays to determine authenticity will be the next logical step. Another approach to determine proper function would be an assay to measure binding of the expressed domain to erythrocytes.

We did not assay for the complete replacement of all the approximately 45 copies of the *btu1* gene in the macronucleus. Even though complete replacement is possible (66), it is apparently not necessary to express high levels of protein. However, complete replacement would remove the requirement for continuous drug pressure and would be something to consider if long-term culture became necessary.

In summary, several features of *T. thermophila* make it attractive as a heterologous expression system for *P. falciparum* genes. First, *T. thermophila* is an alveolate like *P. falciparum* and is thus evolutionarily closer than other common expression systems. Second, *T. thermophila*, like *P. falciparum*, has an A+T rich genome and thus is likely to have similar codon usage (69). Finally, *T. thermophila* is non-pathogenic, free-living, can be grown to high density in large volumes, is inexpensive to culture, and, has a relatively short doubling time of 3 hours (71). *Tetrahymena thermophila* represents a promising system for the heterologous expression of secreted, authentic proteins that is needed for the structural and functional studies required to identify promising parasite antigens for rational drug design and vaccine development.

CHAPTER 5

CONCLUSION

Our work justifies continuing the development of *T. thermophila* as a means to heterologously express *P. falciparum* genes as our experiments have demonstrated secretion of *P. falciparum* protein domains.

Our initial attempts to bypass cloning in *E. coli* altogether were not successful. Thus, we were unable to express EBL-1 and JSEBL in their entirety because long A+T sequences of *P. falciparum* are inherently unstable in *E. coli*. This is unfortunate, as structural studies relying only on the expression of DBL domains may not provide a complete picture of the structure of the entire molecule.

However, we were successful in creating a secretion vector that will be of general use for cloning *P. falciparum* sequences. The vector, pTART, contains an I-antigen signal sequence for targeting, a His-tag to aid in purification of the expressed protein, a FLAG epitope for identification, and several GC-rich cutting sites that are unlikely to be found in the AT-rich *P. falciparum* genome. We chose to make a secretion vector rather than a vector designed for surface expression because a limitation of surface expressed proteins is the relative difficulty in purification. We were successful in cloning at least 1800 base pairs of P. falciparum DNA into the pTART vector. Furthermore, as these were the DBL domains of *ebl* genes it seems reasonable to expect that we could also express the similar DBL domains of *var* genes. *Var* is a family of approximately 60 genes whose products are responsible for cytoadhesion of infected erythrocytes to

peripheral microvasculature. Each possesses two to several domains each of which are potential ligands for a number of receptors (83).

Efforts at purification using the 6-His tag failed each time attempted. We believe this could have been due to the tag being sterically buried in the protein, and thus not available for binding to the nickel column. An alternative version of the vector with a N-terminal 6-His tag could be constructed and tried. Alternatively, we could create a new C-terminal 6-His tag vector with a spacer region providing greater separation of the heterologous protein region from the 6-His tag. This may allow the tag to remain available for binding to the nickel. If this were to solve the purification problem, the next logical steps would be to determine proper function through invasion inhibition and binding assays.

We were able to detect both EBL-1 and JSEBL on Western Blot Analysis although there was some run-to-run variation, with some runs (growth, induction, filtration, concentration, Western blot) giving either no signal or bands of much smaller size. We believe we could have ultimately solved this problem by either manipulating the variables involved in each run or exploring the use of a strain of Tetrahymena defective in protease secretion.

In summary, *Tetrahymena thermophila* represents a promising system for the heterologous expression of secreted, authentic proteins that is needed for the structural and functional studies required to identify promising parasite antigens for rational drug design and vaccine development.

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