Potential drug target proteins involved in liver stage of malarial parasite and various approaches of their expression as recombinant protein

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Abstract- Malaria parasite proteins like circumsporozoite protein (a surface protein), are expressed during liver stage of plasmodium parasite life cycle and are involved in host modulation and the virulence. These proteins could serve as good candidate for the drug development against malaria parasite. Malaria parasite traffic its virulence proteins into the hepatocyte cytosol for its development in hepatocyte. These parasite proteins modify the hepatocyte cytosol for maximum benefit of parasite. This process changes the permeability of the hepatocyte for uptake of various nutrients and factors required for parasite survival in hepatocyte. Trafficking of parasite proteins into hepatocytes depends on a conserved pentameric motif, known as PEXEL-motif. Marti et al. identified 22 sporozoite stage proteins in P. falciparum that had putative PEXEL motif. It has been identified that protein that contains PEXEL motif can be a potential drug candidate.

Index Terms- circumsporozoite protein, liver stage, Malaria, PEXEL motif.

I. INTRODUCTION

Malaria is an infectious disease caused by protozoan intracellular obligate parasite, wide spread in tropical and subtropical regions which extensively modify and remodel the host cell for their own need. According to the center for disease control and prevention, it has been reported that each year 300-500 million people are infected by the malaria parasite and results in 3-5 million people death (1). Malaria parasite Plasmodium, belongs to the phylum Apicomplexa that include other important intra-cellular pathogen such as Toxoplasma, Babesia, Theileria, Eimeria and Cryptosporidium which is characterized by presence of chloroplast like Apicoplast, An apical polar ring which serve as microtubule organizingcentre and secretory vesicles named as roptries and microneme. Both the organelles, roptries and microneme are released at the anterior tip of the parasite and their content are involved in apicomplexan motility, host cell invasion and generation of non-phagosomalparasitophorus vacuole where the parasite reside and replicate inside host cell. Malaria belongs to the poverty and is hindrance in economic development of the country

In the mammalian host, invasion of the hepatocytes is the first step towards developing malaria disease. Invasion by infective sporozoites is complex process and not understood very well. During early liver stage development a sporozoite surface protein (CSP) is introduced in the hepatocytes cytoplasm. CSP then shuttles into and out of nucleus of hepatocytes and changes the host transcription profile. CSP export into the cytoplasm of infected hepatocyte requires the presence of PEXEL / HTS motifs. The transport of CSP into the hepatocyte nucleus is then mediated by importins α3/β1 that binds to the nuclear localization signal (NLS) of CSP. The NLSs of CSP and NFκB p50 share the same importins. The entry of NFκB-p50 into the nucleus is strongly inhibited in cell lines expressing CSP, and in infected hepatocytes. Microarray data, from a N-terminal truncated CSP expressing cell line, showed that 40 NFκB targets were significantly down regulated. Presence of CSP in the host modifies thousands of host gene transcription that govern diverse biological processes such as

metabolite transport, cell cycle, immune responses, cell growth, cell attachment, apoptosis and hypoxia and the overall effect is to enhance EEF growth.

II. THE LIFE CYCLE OF MALARIA PARASITE

It is very complicated and is completed in two hosts. The one is human and other vertebrates, which act as intermediate host for the malaria parasite where asexual part of life cycle occur and other one is mosquitoes, where sexual part of life cycle take place and act as definitive host for the parasite. Malaria in humans is caused by four species of Plasmodium (2).

1. Plasmodium vivax - benign malaria, incubation period 12-20 days
2.Plasmodium falciparam - malignant malaria, incubation period 8-15 days
3. Plasmodium malariae- benign malaria, incubation period 18-40 days
4.Plasmodium ovale- benign malaria, incubation period 11-16 days

Plasmodium berghei and Plasmodium yoelii are mouse malaria parasites. Among four human malaria species, P. falciparum, one of the deadliest parasites, causes severe disease in humans resulting in more than million deaths per year. Malignant malaria can kill but other forms are less likely to prove fetal.

Life cycle

The complete life cycle of the malaria parasite is completd in three stages:
1) Sporogonic cycle take place in mosquito.
2) Exo-erythrocyticcycle take place in the Liver of infected person.
3) Erythrocytic cycle take place in the erythrocyte of infected person.

Malaria infection starts when the mosquito takes their blood meal (3) and injects the sporozoite into the host skin. The sporozoite enters into blood stream, reach to the Liver where they invade the hepatocytes and developed into Exo-erythrocytic form (EEF). EEF grow and differentiate into thousands of merozoites which are released by rupturing of the hepatocyte and invade the RBC to continue the Erythrocytic cycle and cause the symptoms of the disease. Some merozoites developed into gametocyte, which are taken up by mosquito during their blood meal. Inside the mosquito, gametocyte fused to form the zygote which further developed into invasive ookinetes. Ookinetes reach to the space between midgut epithelium and basal lamina of mosquito where they develop into oocysts. Thousands of sporozoites developed in oocyst and released into the heamolymph. They invade salivary gland and remain viable but do not replicate.

Plasmodium Virulence Proteins

When the sporozoites invade the hepatocytes, they hide themselves in a specialized vacuolar compartment called parasitophorus vacuole (4). The membrane of this vacuole is derived from host cell plasma membrane and physically separates the parasite from host cell. Further development of parasite take place inside this parasito-phorus vacuole. Malaria parasites export hundreds of proteins to remodel the host cell in order to get access to nutrients, solute, ions and other things required for the parasite survival and its propagation inside the host cell (5)

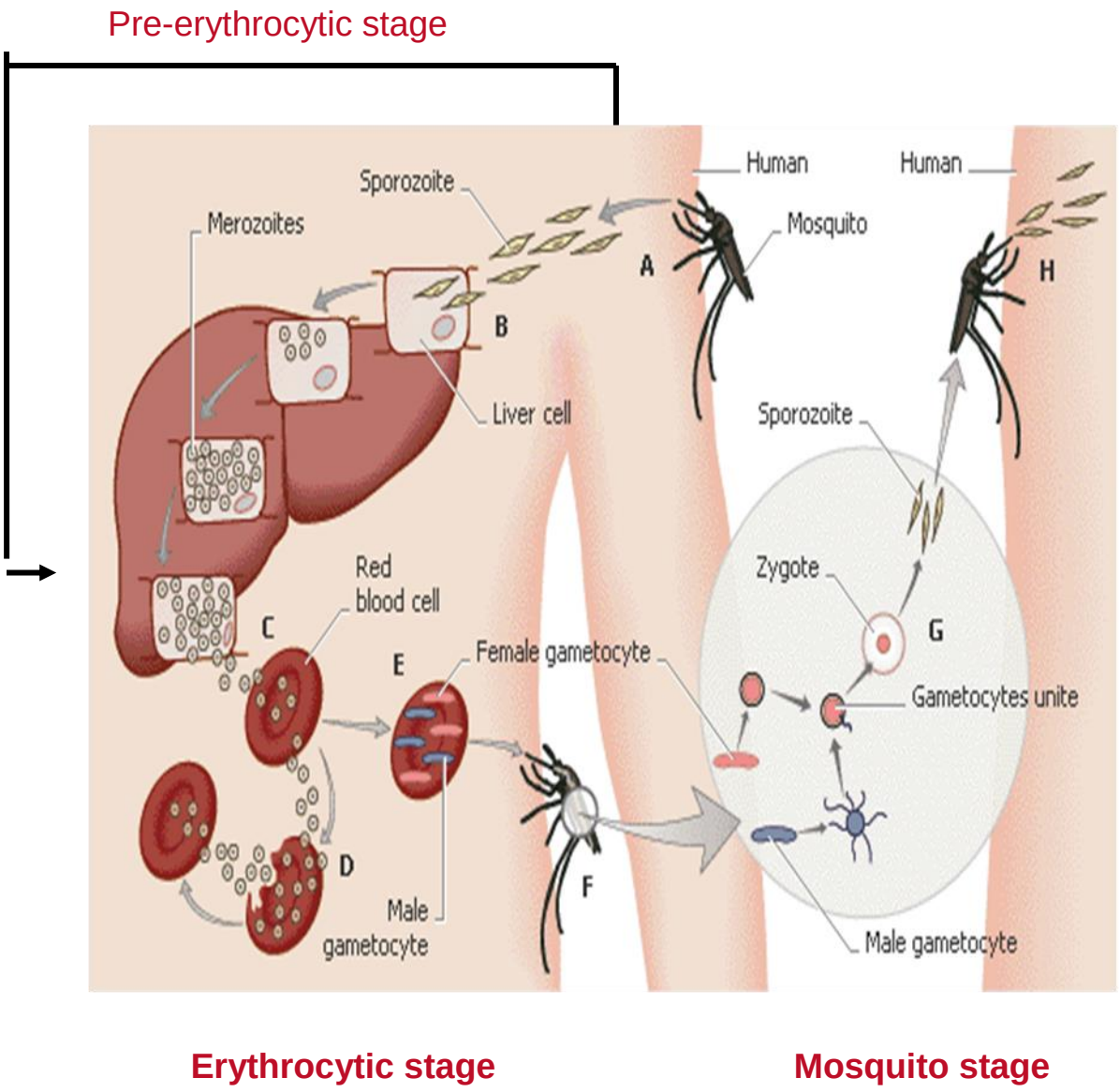

Figure-1.Life cycle of malarial parasites.

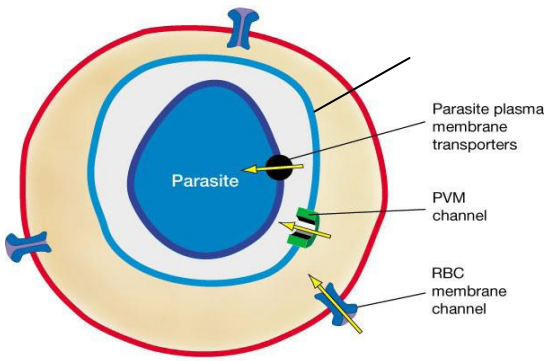

Figure-2. Infected erythrocyte

Parasite exported protein have to cross the two barriers in order to reach the host cytosol. These two barriers are parasite plasma membrane and PV-membrane (6). The parasite protein crosses the plasma membrane by vesicular transport (7) and reach to the space between PVM and parasite. In the infected RBCs, export of these parasite proteins into host cell depend on plasmodium export element (pexel) or vacuolar transport signal (VTS) (8).

Marti et al. found that virulence proteins of infected RBCs have a trafficking signal. The first part of the signal is SS; responsible of export into the PV and second is a motif that targets the protein for onward transport into the erythrocyte proper. Marti et al. termed that motif as PEXEL (Plasmodium Export element) motif. It is also known as VTS (Vacuolar Transport Signal) or HT (Host Targeting) signal. Marti et al. also found the following features of the conserved pentameric motif –

(1) Positively charged hydrophilic amino acid in position 1, (Arg and Lys).
(2) A hydrophobic amino acid in position 3 (mainly Leu)
(3) Less conserved amino acid in position 5 (predominantly Asp, Glu and Gln).
(4) Non charged any amino acid in position 2 and 4

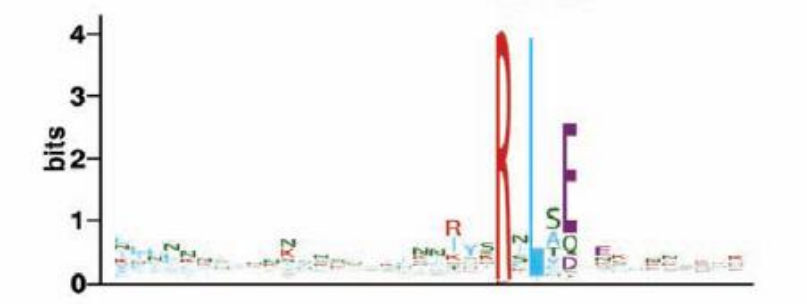

Figure 3.Pexel motif in Plasmodium erythrocytic stage [Consensus: (R/K) X (L) X (E/D/Q)].

The presence of PEXEL containing protein also in hepatocyticstages, indicates that the machinery for protein translocation across the PV membrane is common in different host cell type. Plasmodium sporozoites have surface protein, known as circumsporozoite (CS) protein. CS is also present in the plasma membrane of EEFs (Exoerythrocyte form) and has been detected in the cytoplasm of the infected hepatocytes. Previous reports suggest that CS plays central role in several stages of parasite life cycle. It is useful for sporozoite development in the oocysts, Invasion of hepatocytes and for EEF development (9). It is a master regulator of plasmodium pre-erythrocytic stages development.

CS has the PEXEL motif by which it traverses the PV membrane. CS protein also has a NLS (Nuclear Localization Signal) and a putative NES (Nuclear export signal) at C-terminus (9). NLS in CS uses alpha3 importin to enter into the nucleus. Binding of CS to importin alpha3 was abolished after deletion of the NLS. It has been proved that NLS has a role in the enhancement of EEF development. Hence plasmodium has PEXEL motif, to introduce the CS protein into the hepatocyte cytoplasm and a NLS to enter its nucleus. Once inside hepatocyte cytoplasm, CS out competesNFkB (involved in inflammation) nuclear import, thus down regulating the expression of many genes; controlled by NFkB. Thus CS inside hepatocyte creates a favorable niche for parasite growth. CS currently is a target antigen for vaccine against liver stage of malaria and it is in a phase-II clinical trials and have shown partial protection (~50%).

Liver stage of Malaria Parasite

Liver stage of the malaria parasite is asymptomatic, considered the most promising target for vaccine and good target for prophylactic drugs because the parasite number is limited and intervention at this stage prevent the progression of disease to blood stage (10). The long lasting protection against the malaria is achievable with radiated or genetically attenuated sporozoite which cannot complete liver stage development and thus disease does not progress to symptomatic blood stage (11). The one licensed drug against LS is primaquine though it is contraindicated in pregnant women and individuals with Glucose-6phosphate deficiency, but primaquine can kill the P.vivaxhypnozoites in liver (12). A comprehensive liver stage gene expression analysis shows that approximately 2000-genes are expressed at this stage which aids in liver stage development of which 66% have orthologs in P. falciparum. Gene expression profile of LS and intraerythrocyte stages are more similar than the mosquito stage spoorozoite (13). The proteome analysis of liver stage shows that approximately 712 proteins are expressed at this stage of which 90% have orthologs in P.falciparum. Liver stage is the best stage for the prevention of malarial parasite because in this stage parasitic load is small (under 500).

Marti et al. identified 22 sporozoite stage proteins in P. falciparum that had putative PEXEL motif. Many of these proteins are hypothetical and their function or localizations are not known. These proteins most likely function only after invasion of hepatocyte and PV formation.

III. EXPRESSION OF RECOMBINANT PROTEIN

Too little is known about the mechanism of folding of proteins in different organisms to predict which host – vector system might be best for given protein, and few methods are available to increase the efficiency of folding or to prevent the aggregation, denaturation, and/or degradation of foreign proteins expressed in the environments that are unnatural to them (14).

Five major expression systems have been developed to a point where they are reasonably portable from one laboratory to another. The host cells for these systems are E. coli, Bacillus subtilis, Saccharomyces cerevisiae, cultured insect cells, and cultured mammalian cells.

Because of the vast knowledge about its genetics, biochemistry, and molecular biology, E. coli is the system of first choice for expression of many heterologous proteins. Genetic manipulations are straightforward, cultures of E. coli are easily and inexpensively grown, and many foreign proteins are well tolerated and may be expressed at high level (15).

Choosing an expression system

Factors that influence the choice of a system for expression of a particular protein in E. coli are listed below.

- The size of the protein. Small cytosolic proteins and polypeptide (< 100 residues in length) are best expressed in E. coli as fusion proteins composed of carrier sequences linked by a standard peptide bond to the target protein. Cytosolic proteins >100 residues in length are the most problematic proteins to express in either system. In E. coli, these proteins are often unstable or form insoluble inclusion bodies.
- The amount of protein needed. If only small quantities of target protein are required- for example, when screening a series of site-directed mutants for enzymatic activity- there is little point in trying to optimize production.
- Whether active protein is required. If the purpose of expressing the target protein is simply to obtain material for raising antibodies, there is no point in trying to obtain active protein. Instead, expression system can be used that facilitate purification of target protein, irrespective of its state of biological activity or denaturation.

Choosing a promoter and vector system

The following categories of expression vectors are described on basis of the typeof promoter.

- Expression Vectors containing an IPTG-inducible Promoter. Several different vectors based on the lac operon are used for high – level expression of foreign proteins in E. coli, including: the trp-lac (tac) promoter (16), the trp-lac promoter (17), the lac promoter (18).
- Expression vectors containing the bacteriophage T7 promoter. The pET series of vectors, originally developed by Studier et al. (1990) and since expended, allow regulated expression of foreign genes by bacteriophage T7 RNA polymerase.
- Expression vectors containing bacteriophase λ pL promoter. In vectors of this class, the bacteriophase λ pL promoter is regulated by a temperature sensitive repressor, cIts857, which represses pL- driven transcription at low temperature but not at elevated temperature.

Cloning & Expression

The vector

GST fusion proteins are constructed by inserting a gene or gene fragment into the multiple cloning sites of pGEX vectors. Expression is under the control of the tac promoter, which is induced by the lactose analog, isopropyl b-D thiogalactoside (IPTG). All pGEX vectors are also engineered with an internal lacIq gene. The lacIq gene product is a repressor protein that binds to the operator region of the tac promoter, preventing expression until induction by IPTG, thus maintaining tight control over expression of the insert.

The Host

Although a wide variety of E. coli host strains can be used for cloning and expression with the pGEX vectors, there are specially engineered strains that are more suitable and that may maximize expression of full-length fusion proteins. Strains deficient in known cytoplasmic protease gene products, such as Lon, OmpT, DegP or HtpR, may aid in the expression of fusion proteins by minimizing the effects of proteolytic degradation by the host (19-22). Using E. coli strains that are not protease-deficient may result in proteolysis of the fusion protein, seen as multiple bands on polyacrylamide gels (SDS-PAGE) or Western blots. E. coli BL21, a strain defective in OmpT and Lon protease production, has been specifically selected to give high levels of expression of GST fusion proteins. An alternative strain for cloning and maintenance of the vector (e.g. JM105) should be used, as BL21 does not transform well. However, one should not use an E. coli strain carrying the recA1 allele for propagation of pGEX plasmids. There have been reports that these strains can cause rearrangements or deletions within plasmid DNA.

Insert DNA

Insert DNA must possess an open reading frame. Whether subcloned from another vector or amplified by PCR, the insert must have ends that are compatible with the linearized vector ends. Using two different restriction enzymes will allow for directional cloning of the insert into the vector. Directional cloning will optimize for inserts in the correct orientation.

Expression

Expression in E. coli yields fusion proteins with the GST moiety at the amino terminus and the protein of interest at the carboxyl terminus. The protein accumulates within the cell's cytoplasm. GST occurs naturally as a 26000Da protein that can be expressed in E. coli with full enzymatic activity.
After clones expressing the fusion protein have been selected, growth conditions should be evaluated for optimal expression. Media, growth temperature, culture density, induction conditions and other variables should

be evaluated. The presence of inclusion bodies may affect optimization of expression. High-level expression of foreign fusion proteins in E. coli often results in formation of inclusion bodies. Inclusion bodies comprise dense, insoluble aggregates that are failed folding intermediates (23-24).

Formation of inclusion bodies can be advantageous in purifying an active form of an expressed fusion protein that otherwise may be unstable in the soluble fraction. However, the steps needed to solubilize and refold the fusion protein can be highly variable and may not always result in high yields of active protein A variety of growth parameters can be investigated for good expression, either solely or in combination, that may provide a good yield of non-degraded fusion protein in the soluble fraction. Steps to investigate include:

- Lowering the growth temperature to between 20 °C and 30 °C
- Increasing aeration
- Altering induction conditions.

In general, induction at lower cell densities (A600 = 0.5) usually results in greater yields of the fusion protein in a soluble form. However, in some cases it may be beneficial to grow the cells to a higher cell density (> 1 A600 unit) for a shorter period of time, or simply to induce for a shorter period of time. Growing the cells to a higher cell density and either omitting induction by IPTG or reducing the IPTG concentration to 0.1 mM leads to lower yields, but more of the fusion protein is likely to be obtained in an intact form.

IV. PURIFICATION

GST fusion proteins are purified from bacterial lysates by affinity chromatography using immobilized glutathione. GST fusion proteins are captured by the affinity medium, and impurities are removed by washing. Fusion proteins are eluted under mild, non-denaturing conditions using reduced glutathione. The purification process preserves protein antigenicity and function. Cleavage of the protein from GST can be achieved using a site-specific protease whose recognition sequence is located immediately upstream from the multiple cloning sites on the pGEX plasmids. Fusion proteins can be detected using colorimetric or immunological methods.

V. CONCLUSION

In malarial parasite life cycle, liver stage parasite proteins are also involved in virulens and with application of various approaches towards cloning and expression of these genes as recombinant proteins in suitable expression system, potential anti malarial drug can be targated by understanding host-parasite interactions during malaria liver stages development, identifying Plasmodium proteins that modulate the hepatocyte cellular functions, knockout of parasite genes, target identification for inhibitor design, Known and novel compounds assay (ex vivo and in vivo) to test inhibitor/ drug efficacy, Discovery of new antigens from liver-stage parasites and evaluation in animal models. A multi-disciplinary approach is the need to address above issues.

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