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SUMMARY OF THE THESIS

The malaria parasite invades and infects erythrocyte during asexual stage of its life cycle, where it extensively modifies the host cell. The process of invasion involves secretion of apical organelles (rhoptries, micronemes, mononemes and dense granules) which play important role in attachment, invasion, formation of the parasitophorous vacuole, and subsequent growth of the parasite within various cell type. Because of their role, proteins of all these organelles are attractive targets for vaccine and drug development. The secreted contents of major apical organelles, rhoptries, contain two main protein complexes, RhopL, (low molecular weight (LMW) complex having rhoptry-associated proteins, RAP-1, RAP-2 and RAP-3) and RhopH (high molecular weight (HMW) complex having RhopH1, RhopH2 and RhopH3) (Sam-Yellowe et al., 1993; Kaneko, 2007). Monoclonal antibody, mAbD2 generated during earlier studies in laboratory reacted with both *P. berghei* parasite and infected erythrocyte membrane. Immunoprecipitation studies in combination with N-terminal sequencing revealed that this antibody is directed against 105 kDa RhopH3 member of HMW complex of rhoptries which further associates with 146 kDa protein. Labeling studies using mAbD2 further showed that unlike 146 kDa molecule, RhopH3 is translocated to erythrocyte membranes (Bhattacharjee, 2003). With this background, in this study, attempts were made to characterize RhopH3 and associated molecule(s).

Anti-RhopH3 antibodies were selected from panels of antibodies against *P. berghei* and *P. falciparum* parasites and respective infected erythrocyte membranes, generated during earlier studies. Selection was made based on their reactivity with mAbD2 (anti-*P. berghei* RhopH3 antibody) purified RhopH3. Two mAbs, B6 and G6, showed good reactivity with 105 and 52 kDa proteins and low reactivity with 80 and 66 kDa proteins, affinity purified from mAbD2 column. Isotype analysis showed that like mAbD2, mAbs B6 and G6 are also IgG1.

Affinity matrices, prepared with these antibodies (B6 and G6) isolated primarily components of 146 and105 kDa along with minor bands at 66 or 52 kDa positions. Similar elution profile of mAbs (B6, G6 and D2) and cross-reactivity among their affinity purified antigens indicated that like mAbD2, mAbs B6 and G6 are also directed towards *P. berghei* RhopH3. This was further confirmed by 100% identical N-terminal sequence (KDYFNGVLN) of 105 kDa molecule purified using one of these antibodies, mAbB6.

Localization of RhopH3 in parasite, PVM and the membranes of *Plasmodium* infected red blood cells was revealed by following observations:

i) both antibodies (mAbs B6 and G6) recognized *P. berghei* infected erythrocytes in indirect immunofluorescence assay. Punctated fluorescence, characteristic of rhoptry staining, showed the localization of RhopH3 in merozoite rhoptries.

ii) confocal microscopy revealed co-localization of anti-NRBC membrane antibodies with anti-RhopH3 antibodies in unpermeabilized infected erythrocytes, thus demonstrating presence of RhopH3 on *P. berghei* infected cell surface.

iii) both antibodies reacted with PVM of IRBCs treated with tetanolysin, which selectively permeabilizes IRBC membrane, thus exposing PVM antigens facing erythrocytic cytoplasm in infected erythrocytes.

Affinity purification of RhopH3 always resulted in co-purification of 146 kDa molecule. Coupled with this, following observations confirmed that RhopH3 associated 146 kDa protein is RhopH2:

i) three peaks from peptide mass fingerprint of 146 kDa moleule matched with theoretical digest of RhopH2.

ii) anti-*P. yoelii* RhopH2 antibody, mAb25, reacted with this protein.

iii) overall similarity of 94% and an identity of 86.4% were observed in case of *P. berghei and P. yoelii* RhopH2

During purification of RhopH3 over mAbD2 affinity column, the presence of protease in RhopH3 containing eluate was indicated by the appearance of degraded forms of RhopH3 (80, 66 and 52 kDa). Beside this, eluate efficiently degraded casein, azocasein and FITC-casein, thus confirming the presence of protease along with RhopH2 and RhopH3. Zymographic analysis showed that protease activity was primarily confined to two positions: 107 kDa (very close to the size of RhopH3) and >205 kDa. Further, similar activity could be seen in *P. berghei* IRBC extract without any detectable activity in NRBC, indicating origin from parasite. Solubilization of immune-complex in 2% SDS resulted in disappearance of >205 kDa form while keeping 107 kDa form intact. Further upon storage of these beads, the intensity of >205 kDa protease activity decreased with a concomitant increase of 107 kDa protease, thus indicating that > 205 kDa protease might be oligomeric form of 107 kDa protease.

Although 107 kDa protein migrated at very close position to that of RhopH3, better resolution of eluted fraction on low percentage SDS-PAGE followed by zymography, staining and immunoblotting experiments indicated that 107 kDa protease has identity distinct from RhopH3. The 107 kDa protease was not precipitated by either sepharose alone or when conjugated to BSA or myoglobulin, ruling out non-specific interactions with the column during affinity purification. Although, the protease activity co-eluted with RhopH3, yet the enzyme also exhibited interactions with other immunoglobulin indicating that enzyme might have dual binding sites, one for immunoglobulins and other for RhopH3.

Characterization of the protease revealed that its activity was not affected by the inhibitors of serine, aspartic, cysteine and threonine classes of proteolytic enzymes, while presence of metal chelators *i.e.* EDTA, EGTA and 1', 10' phenanthroline lead to reduction in enzyme activity. Drastic reduction in the activity was observed in absence of calcium ions and background activity of protease in absence of $CaCl_2$ was completely blocked by metal chelators. Further, protease activity was observed in the presence of various divalent metal ions with maximal activity in the presence of calcium ions. The optimal activity of enzyme was observed at pH 9.5. These characteristics showed that 107 kDa protease is divalent metal dependent alkaline protease.

Although, 107 kDa protease reported in this study resembled with MMP-9 and insulysin in few characteristics, yet in initial experiments of immunoblotting, non-reactivity of anti-MMP-9 and IDE antibodies, raised either against same protein from same species (for MMP-9) or against conserved epitope (for insulysin) indicated that 107 kDa protease might be different from these two enzymes.

Purification of RhopH3 in the presence of 10 mM EGTA (chelator of metal ions thus inhibiting metalloprotease) yielded good recovery of intact RhopH3, unlike degraded forms which were often seen in the presence of Ca^{2+} ions. Further, degradation of MSP-1 in presence of EGTA was not effected which indicated that RhopH3 might be a substrate for 107 kDa metalloprotease.

During overexpression studies of RhopH3, expression of full length and central region of RhopH3 could not be observed in *E. coli pLysS* host cells. *E. coli* BL21(DE3) cells containing N-terminal region of RhopH3 *i.e.* $rRhopH3_{1\text{-}200}$ or $rRhopH3_{16\text{-}392}$ genes cloned in pET vector, upon induction with 1mM IPTG, over-expressed 24 and 44 kDa proteins respectively in accordance with the expected size. However, majority of $rRhopH3_{16\text{-}392}$ was

present in inclusion bodies, even on culturing at low temperature (16°C). In attempts to purify and refold protein, it was observed that rRhopH3$_{16-392}$ protein was sparingly soluble in 8 M urea, while 6 M guanidine hydrochloride (GnHCl) readily solubilized it. Refolding of GnHCl solubilized protein in the presence of oxidized and reduced glutathione (GSH-GSSH), low molecular weight additive (L-arginine) and under rapid dilution conditions resulted in the formation of protein aggregates, while rRhopH3$_{1-200}$ purification under denaturing conditions using Ni-NTA affinity column, yielded 24 kDa protein.

Sequence analysis of RhopH3 showed that it is a well conserved protein having homologs in *P. berghei, P. chabaudi, P. yoelii, P. vivax and P. falciparum*. The conserved blocks were clustered at the N-terminus and central region, while C-terminal region was most variable. Out of 10 cysteine conserved across *P. berghei*, *P. yoelii* and *P. falciparum*, 8 were located in N-terminal region and 2 in central region. Computational analysis of PbRhopH3 (885 residue) *via* BcePred and ABCpred showed the presence of as many as 86 epitopes with high threshold score (>0.6). Anti-rRhopH3$_{1-200}$ antiserum exhibited good reactivity with both recombinant protein and native RhopH3. Immunofluorescence studies with anti-rRhopH3$_{1-200}$ antiserum showed characteristic punctated fluorescence of rhoptries. Further, antiserum recognized ~105 kDa rhoptry protein of *P. falciparum* indicating formation of antibodies against conserved domains.

Immunoblotting studies with *P. yoelii* and *P. chabaudi* lysate showed that all the three mAbs D2, B6 and G6 recognize RhopH3 epitopes which are conserved across rodent species. While, later two mAbs also reacted with *P. falciparum* RhopH3, indicating that unlike mAbD2 which is primarily reactive to rodent parasite RhopH3, other two mAbs B6 and G6 recognize epitopes of RhopH3 conserved across rodent as well as human malaria parasite *P. falciparum*. Further, significant *in vitro* growth inhibition (43%) of *P. falciparum* was shown by mAbB6.

Screening of random peptide phage library, showed that mAbG6 reactive mimotopes align in the amino acid region 285-334 of RhopH3. While mapping growth inhibitory mAbB6 epitope, yielded many peptides with consensus sequence [F-][-Y]SHS[H-]M[NST]X[FW]T-[LVFW]E[DST][IVFW][NP]LGGGS[A-][E-]. The mimotopes upon clustalW alignment indicated that 512-541 region of RhopH3 contains epitope of mAbB6. Alignment of this region with RhopH3 protein from *P. yoelii*, *P. chabaudi*, *P. vivax* and *P. falciparum* showed that this region is conserved among all these species of malaria parasite.

In nutshell, studies described here identified first time following:

i) 107 kDa alkaline metalloprotease present in *Plasmodium* parasite which possibly interacts with RhopH3 and immunoglobulins. Also, it may be involved in the degradation of RhopH3.

ii) Region between aa 512-541 of RhopH3 contains epitope which is recognized by *P. falciparum* growth inhibitory antibody and conserved among rodent and human malaria parasite. This epitope/mimotope can be exploited as target for the development of anti-malaria peptide vaccine.