CHAPTER-II

EhCaBP5 interacts with Myosin IB and involved in initiation of phagocytic cup formation

3. 1 Abstract

After successful determination of the structure and analyzing the crystal structure, we performed structure based alignment using (Dali server). This shows that EhCaBP5 structure is similar to essential light chains (ELC) of myosin. It was expected that EhCaBP5 could serve as ELC of *E. histolytica* myosin and it could interact with myosin of *E. histolytica*. Surface plasmon resonance studies confirm our hypothesis that IQ motif peptide of myosin IB was found to be interacting with EhCaBP5, indicating EhCaBP5 might be light chain of myosin IB. Involvement of EhCaBP1, EhCaBP3 and myosin IB in phagocytosis (Jain et al., 2008, Aslam et al., 2012,) raised the question whether EhCaBP5 is linked in phagocytosis or not. To answer this question we checked the cellular co-localization of EhCaBP5 and myosin IB, as well as interaction of these proteins by surface plasmon resonance. The cell localization experiments shows co-localization of EhCaBP5 and myosin IB together in phagocytic cup formation during process of erythrophagocytosis. Immunoprecipitation of EhCaBP5 from total *E. histolytica* cellular extract also pulls out myosin IB and this interaction seems to be calcium independent. Interestingly confocal imaging of *E. histolytica* showed that EhCaBP5 and myosin IB are part of phagosomes. EhCaBP5 leaves phagosome after its formation suggesting EhCaBP5 may be playing a regulatory role.

3.2 Introduction

Phagocytosis is a process in which various types of cells like macrophages and neutrophils and many unicellular eukaryotes have the ability to engulf particles of size greater than 0.5mm. It was first discovered by Elie Metchnikoff more than 100 years ago (Ilya Mechnikov). Since then it has been recognized as a very crucial component of the innate and adaptive immunity. This process has evolved for host defense through tissue remodeling and homeostasis (Flannagan et al., 2012). Phagocytosis is a process whereby particles are taken in through mechanism superficially similar to endocytosis.

Stages of phagocytosis are

1. Initiation
2. Pseudopod formation
3. Phagosome formation.

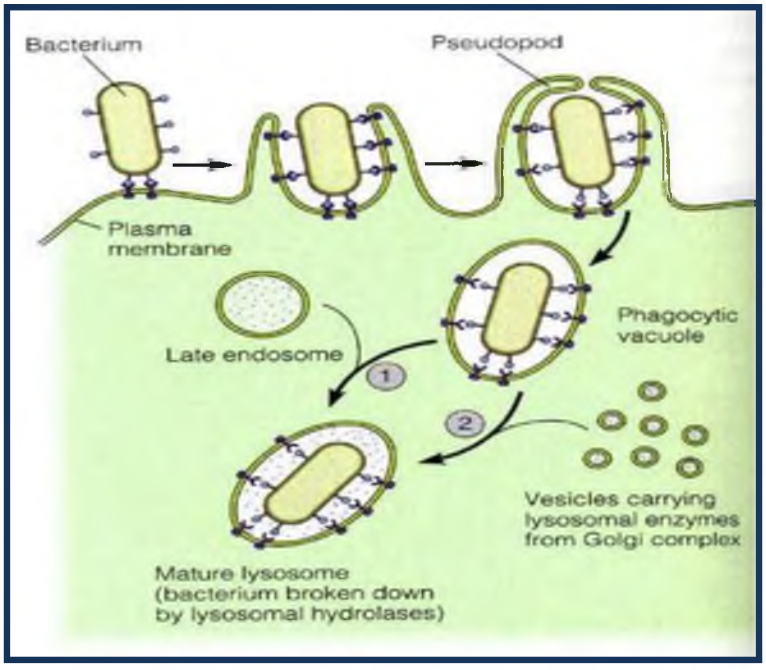

Figure 1: Steps involved during Phagocytosis (Courtesy Wikipedia.)

E. histolytica is a causative agent of amoebiasis. After an establishment of infection to host (human) it starts to phagocytose microorganisms in the large intestine (Bracha et al., 1982), and once it invades the various tissues in host it also ingests the red blood cells (Tsutsumi et al., 1992) and apoptotic immune cells (Huston et al., 2003). Phagocytosis has been one of the most recognized behaviors of *E. histolytica* and erythrophagocytosis has even been used as a diagnostic indicator of invasive *E. histolytica* infection by microscopy (Douglas et al., 2008, Gonzalez-Ruiz et al., 1994). Invasive infection by *E. histolytica* leads to excessive tissue destruction (Jimenez F., 1981, Maltz et al., 1991, Brandt et al.1970, Aikat et al., 1979) including hallmarks of both apoptotic and necrotic host cell death (Berninghausen et al., 1997, Huston et al., 2000., Ragland et al., 1994).

Phagocytosis plays an essential role in growth and constitutes one of the key virulence determinants of *E. histolytica* (Bracha et al., 1984). Amoeba mutants defective in phagocytosis have a growth defect on bacterial lawns (Peracino et al., 1998) and are also defective in the destruction of tissue-cultured mammalian cells *in vitro* and in the formation of hepatic abscesses *in vivo* (Orozco et al., 1983). The virulence potential of *E. histolytica* isolates could be directly correlated with their ability to phagocytose red blood cells (Tsutsumi et al., 1992). How *E. histolytica* kills host cell or phagocytose is poorly understood. Among the few molecules/receptors, which are involved in host cells recognition and attachment to the host or bacterial surface prior to initiation of phagocytosis is the Gal/GalNAc-specific lectin (Petri et al., 2002).

One of the host cell killing and phagocytosis sequential models was shown (Figure 2). This model involves (i) Adherence to the host cell surface, (ii) Contact-dependent cell killing, (iii) Initiation of phagocytosis, and (iv) Engulfment (Figure 2). In this model, host cell killing and phagocytosis requires various kind of proteins which includes cysteine protease adhesin (CPADH), transmembrane kinase (TMK), serine-threonine-isoleucine rich protein (STIRP), reactive oxygen species (ROS), serine-rich *E. histolytica* protein (SREHP), cysteine protease 5 (CP5), and phosphatidylserine (PS).

A large number of molecules and their kinetics of association and dissociation during phagosome biogenesis remain largely unknown. A proteomic approach was used to identify the number of molecules which are part of phagosomes of *E. histolytica* (Okada et al., 2005). The numbers of protein molecules which are identified in phagosome formed by *E.histolytica* are listed in table1.

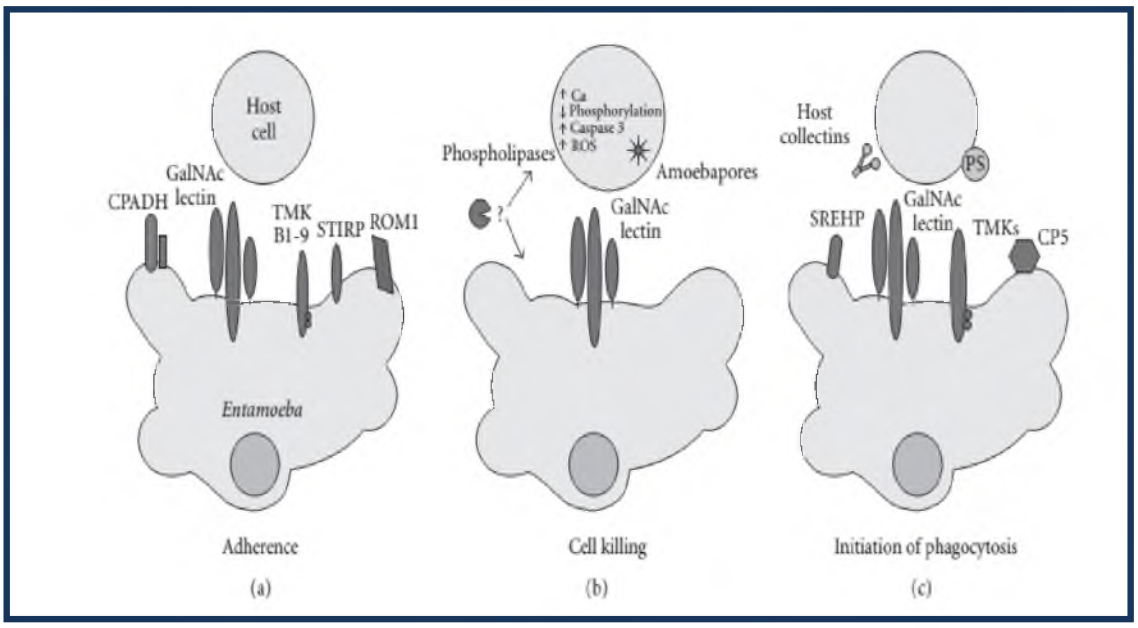

Figure 2: Sequential model of cell killing and phagocytosis by *Entamoeba histolytica*. Adherence, cell killing, and initiation of phagocytosis leading to engulfment of host cells are depicted from left to right. Abbreviations: cysteine protease adhesin (CPADH), transmembrane kinase (TMK), serine-threonine-isoleucine rich protein (STIRP), reactive oxygen species (ROS), serine-rich *E. histolytica* protein (SREHP), cysteine protease 5 (CP5), and phosphatidylserine (PS). (Adapted from Sateriale et al., 2010)

Table 1:

The numbers of protein molecules which are identified in phagosome formed by *Entamoeba histolytica* Adapted from Okada et al., 2005.

Protein group and TIGR ID no. (EH)	NCBI accession no.	Protein name (organism source)	Identity (%)	Frequency (%)[a]
Lectins and surface proteins				
6468		Adhesin p30[b] (*Mycoplasma pneumoniae*)	54	0.53 ± 0.19
879		Aminophospholipid translocase 2b (*Homo sapiens*)[b]	44	0.32 ± 0.08
4887		Gal/GalNAc lectin Hgl1-5[b,d]	100	3.0 ± 0.26
1189/175		Gal/GalNAc lectin Igl1, 2[d]	100	1.7 ± 0.64
385/4767/3450		Gal/GalNAc lectin Lgl1-5[d]	100	3.2 ± 1.6
2521	X55028	Immunodominant variable surface antigen	100	0.29 ± 0.15
2178		Multidrug resistance protein 2 (*Canis familiaris*)	31	3.4 ± 1.0
Vesicular trafficking, other small GTPases, and effectors				
4532	AB054578	Rab1	100	0.34 ± 0.08
289	AF218311	Rab7A[b,c]	100	0.26 ± 0.18
540	AB186363	Rab7B[b,c]	100	0.35 ± 0.31
3974	AB186364	Rab7C[b,c]	100	0.32 ± 0.08
2190	AB186365	Rab7D[b,c]	100	0.29 ± 0.41
1867	AB186366	Rab7E[b,c]	100	1.9 ± 0.82
3360	AB197095	RabX17	100	0.24 ± 0.34
1740	AB054579	RabC1	100	0.24 ± 0.34
1174		Clathrin coat assembly protein ap50[b] (*Dictyostelium discoideum*)	48	0.39 ± 0.01
443		Receptor-mediated endocytosis protein 1[b] (*Caenorhabditis elegans*)	44	0.23 ± 0.05
2883	U29720	RacA	100	0.58 ± 0.26
1250	U29722	RacC	100	0.28 ± 0.01
830	AF055340	RacG	100	0.87 ± 0.28
1948	U01052	Rap2	100	1.2 ± 0.59
Hydrolytic enzymes and degradative proteins				
4000		Acid phosphatase[b,f] (*Homo sapiens*)	25	0.38 ± 0.54
5274		Acid phosphatase[b,f] (*Homo sapiens*)	26	0.86 ± 0.27
4360		α-Amylase (*Paenibacillus polymyxa*)	27	0.23 ± 0.05
1563	AJ417748	β-Hexosaminidase B	100	3.1 ± 0.07
683	Q01957	Cysteine protease 1	100	0.46 ± 0.11
4021	Q01958	Cysteine protease 2	100	2.1 ± 0.57
2010	CAA62833	Cysteine protease 4	100	0.23 ± 0.05
881	CAA62835	Cysteine protease 5	100	4.1 ± 2.1
5015	AF059278	Dipeptidylaminopeptidase	100	1.2 ± 0.13
2776	X87610	Lysozyme 1[b]	100	0.23 ± 0.05
1921		Phospholipase A_2	100	0.54 ± 0.36
5686		Phospholipase B[b,g] (*Dictyostelium discoideum*)	31	0.68 ± 0.16
6042		Phospholipase B[g] (*Dictyostelium discoideum*)	27	2.0 ± 0.17
790		Serine protease[h] (*Caenorhabditis elegans*)	31	0.82 ± 0.35
3172		Serine protease[h] (*Caenorhabditis elegans*)	30	1.1 ± 0.95
Calcium and proton pump				
5038		Calcium-transporting ATPase[i]	100	0.63 ± 0.33
6704	U20321	Calcium-transporting ATPase[i]	100	0.53 ± 0.19
1033		V-ATPase V_o domain subunit a[b] (*Homo sapiens*)	33	0.32 ± 0.08
Other proteins				
2856/5741	U13421	Pyridine nucleotide transhydrogenase[j]	100	8.6 ± 1.5
432	M16339	Actin	100	1.1 ± 0.01
4065		Talin (*Dictyostelium discoideum*)	25	0.98 ± 0.3
3098	X98567	Ubiquitin	100	0.32 ± 0.08
135		p21-activated protein kinase (*Dictyostelium discoideum*)	46	0.46 ± 0.11
4681	AF017993	Cyclophilin	100	0.50 ± 0.04
645	M92073	Elongation factor 1α	100	1.1 ± 0.22

Apart from these proteins various other proteins are also reported in phagocytosis by this organism. And it has been shown that the overproduction of myosin IB led to an increase in cytoplasm viscosity resulting in a delay in the early steps of phagocytosis (Marion et al., 2004, Voigt et al., 1999,). It was also shown that rearrangement of the actin cytoskeleton plays an important role in phagocytosis of mammalian cells (Gordon et al., 1987, 1990). Amoebapores, cysteine protease 2 (CP2), and CP3 were shown to be recruited to phagosomes and involved in permeation and degradation of ingested bacteria (Andra et al., 2003, Que et al., 2002). Involvement of calcium binding proteins was reported and it was found that, EhCaBP1 a cytoplasmic protein is transiently linked with phagocytic cup formation in calcium dependent manner (Jain et al., 2008). Involvement of a novel protein kinase EhC2PK, a C2-domain-containing protein kinase was found to be associated in phagocytosis along with the calcium and actin binding protein EhCaBP1. Down regulation of EhC2PK expression and overexpression of a mutant form reveals its role in the initiation of phagocytic cups. EhC2PK binds phosphatidylserine in the presence of calcium and thereby recruits EhCaBP1 and actin to the membrane during process of phagocytosis (Somlata et al., 2011). Later it was found that involvement of EhC2PK in phagocytosis is linked with autophosphorylation of protein kinase (EhC2PK) at Ser^{428} is critical for initiation of phagocytosis (Somlata et al., 2012). Recently calmodulin-like calcium binding protein EhCaBP3 of *E. histolytica* was shown to be involved in pathogenesis as it involved in cytoskeleton dynamics and scission machinery during erythrophagocytosis. EhCaBP3 was found in phagocytic cups and newly formed phagosomes along with actin and myosin IB. EhCaBP3 directly binds actin, and affect its polymerization and bundling activity. Moreover, it also binds myosin IB and this interaction was found to be calcium dependent, as calcium defective mutant of EhCaBP3 was unable to form phagosome (Saima et al., 2012). Crystal structure of EhCaBP5 is similar to myosin light chain. So we hypothesized that EhCaBP5 could serve as myosin light chain and it may interact with *E.histolytica* myosin. To check whether EhCaBP5 interacts *E. histolytica* myosin, we performed SPR assay using Myosin IQ-motif peptide, colocalization and pull down assay. Our result confirm our hypothesis, as out of two myosins of *E.histolytica* (Myosin IB and Myosin II), EhCaBP5 was found to be interacting with myosin IB and our confocal microscopy data suggest that EhCaBP5 is an important molecule which involved in phagocytosis. Both the molecules (EhCaBP5 & Myosin IB) was found to be colocalized at the time of phagosome formation, moreover actin and EhCaBP1 also found to be colocalized during the phagosome formation however we

could not get direct interaction of EhCaBP5 with actin. Identification of novel proteins in phagocytosis could be an important step towards amoebic biology and these molecules could be the important targets for developing novel therapies against amoebiasis.

Materials and Methods

3.3.1 Surface plasmon resonance (SPR)

E. histolytica genome codes for two myosins, Myosin I and Myosin II. Myosin I has one IQ motif and Myosin II has two IQ motifs. To check the interaction between EhCaBP5 and myosin IQ motifs, we obtained peptides of IQ motif of unconventional myosin (IQKAWKGYRNRKRS) and second IQ motif of myosin II (LQACARAFAARKHFS), which is expected to bind to ELC. For binding study we have used Autolab SPR using Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University, New Delhi, India. The surface (self-assembled monolayer of 11-mercaptoundecanoic acid [MUA] on a gold surface; Autolab) was first activated with N-hydroxysuccinimide (NHS; 0.05 M)/N-ethyl-N-(diethylaminopropyl) and carbodiimide (EDC; 0.2 M). Both the IQ motif peptide was immobilized separately on two different chips at concentration of 2 mg/ml in 10 mM sodium acetate buffer (pH 5.0) as immobilization buffer. After peptide immobilization, surface was blocked with 100 mM ethanolamine at pH 8.5, followed by regeneration using 50 mM NaOH. The running buffer composition was used (10 mM HEPES pH 7.4, 150 mM NaCl and 0.2 mM $CaCl_2$). The association kinetics for IQ motif peptide was monitored for 260 seconds and dissociation was monitored for the next 260 seconds. The different concentrations of EhCaBP5 were prepared 125, 250, 500, 750, 1000, 2000 nM and these solutions were made in running buffer and injected at the rate of 20 µL/min. The surface was regenerated with buffer consisting of 50 mM NaOH. The data were recorded at 25°C and data analysis was performed using Autolab SPR Kinetics evaluation software.

Peptides of Myosin were obtained from the Gene PRro Biotech for the SPR experiments.

3.3.2 Immunofluorescence labeling

Amoebic cells were labeled as described before (Saima et al., 2012). Cells grown at 37^0C for 48h were first washed with PBS and then with incomplete TYI-S-33 medium. The cells were then resuspended in the same medium and were allowed to grow on coverslips at 37^0C for 10 min followed by fixation with 3.7% formaldehyde for 30 min, washed with warm 1X PBS and permeabilized with 0.1% Triton X-100 for 5 min. Additional treatment using chilled methanol (-20^0C) for 3 min was carried out for staining myosin IB. Permeabilized cells were then washed with PBS and quenched with 50 mM NH_4Cl for 30 min at 37^0C, followed by blocking with 1% BSA-PBS for 1h. The cells were then stained with primary antibody for 1h

followed by Alexa Fluor 488 conjugated or TRITC conjugated anti-mouse secondary antibodies.

While F-actin was labeled with phalloidin, similar protocol as above was used except the methanol step was omitted. Antibody dilutions used were: EhCaBP5 at 1:200, EhCaBP1 at 1:200, phalloidin (Sigma; 1 mg/ml) at 1:250, myosin IB at 1:150, anti-rabbit or mice Alexa 488 (Molecular Probes, Catalogue No. A-11008 or A-11001) at 1:200, anti-rabbit or mice Alexa 555 (Molecular Probes, Cat. No. A-21428 or A-21422) at 1:300. The preparations were further washed with PBS and mounted on a glass slide using DABCO [1, 4-diazbicyclo (2, 2, 2) octane (Sigma) 10 mg/ml in 80% glycerol]. The edges of the coverslips were sealed with nail-paint to avoid drying. Confocal images were visualized by using an Olympus Fluoview FV1000 laser scanning microscope.

3.3.3 Immunoprecipitation

Immunoprecipitation was carried out as described before (Aslam et al., 2012). Briefly CNBr-activated Sepharose-4B beads (1 g, Pharmacia) were conjugated with anti-EhCaBP5 antibody. Crude immunoglobulins were collected from the immunized serum using 40% ammonium sulfate and subsequently dialyzed in coupling buffer (bicarbonate buffer). Usually, 10 mg immunoglobulins protein was added per gram of CNBr-activated Sepharose-4B beads. The resin was mixed gently for 18 h at 4 °C. The conjugated Sepharose beads were incubated with *E. histolytica* lysate for 6 h at 4 °C. The beads were then washed thrice with wash buffer (10 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM imidazole, 1 mM magnesium acetate, 2 mM β-ME and protease inhibitor cocktail). Calcium and EGTA were maintained throughout the process as required. After incubation the beads were washed sequentially with 60 mM Tris-Cl (pH 6.8), 100 mM NaCl and with 60 mM Tris-Cl (pH 6.8). The pellet was suspended in 2X SDS polyacrylamide gel electrophoresis (PAGE) buffer and boiled for 5 min followed by centrifugation for 5 min. The proteins were then analysed by western blotting.

3.3.4 Transfer of proteins (Western Blotting)

Polyacrylamide gel to be transferred was incubated in Towbain buffer (For 500 ml: 1.51 g Tris base, 7.2 g Glycine, 100 ml Methanol, pH 8.3) for 15 min. The treated gel was placed on two sheets of Whattman 3 MM paper cut to the size of the gel, saturated with Towbain buffer. A sheet of nitrocellulose presoaked in buffer was placed on the gel taking care that no

air bubble(s) were trapped in between the membrane and the gel. In case of PVDF membrane, it is required to dip it in methanol for 2 min, rinsed with water followed by soaking in towbain buffer. Two sheets of whatman 3MM paper were placed above the membrane. The transfer was set at constant mA, depending on the size of the membrane (0.8 times the area of the membrane) for 1-1.5 h. The membrane was then stained with Ponceau S and was blocked overnight at 4°C with 5% skimmed milk powder in PBS-T (PBS containing 0.05 % Tween 20). Primary antibody followed by secondary antibody incubation was done in 3% milk powder in PBS-T with shaking at RT for 1 h and 30 min, respectively. Blots were washed thoroughly with PBS-T after incubation with antibody. The secondary antibody used was horse radish peroxidase conjugated IgG. Band detection was done using ECL kit (Amersham). Antibody dilutions used: 1:4000, polyclonal anti-EhCaBP5 raised in rabbit/mice; 1:10,000, polyclonal anti-Myosin IB (polyclonal, rabbit); 1:10000, Anti-Rabbit-HRPO/ Anti-Mouse-HRPO (Amersham).

Results

3.4.1 EhCaBP5 interacts with IQ motif peptides of Myosin IB

Structurally EhCaBP5 resembled ELC (essential light chain) of myosin, therefore it raised a question whether the EhCaBP5 interacts with any of the *E. histolytica* myosin? *E. histolytica* genome codes for two myosins, Myosin I (Myosin IB) and Myosin II (Heavy Chain Myosin). Myosin I has one IQ motif and Myosin II has two IQ motifs. We got peptides of IQ motif of myosin I and second IQ motif of myosin II, which is expected to bind to ELC. Surface plasmon resonance results shows that the myosin II IQ motif did not show any binding (data not shown here), while IQ motif from myosin IB shows interaction with EhCaBP5. We performed the kinetics with various concentrations of analyte (EhCaBP5). Kinetic studies indicated that the EhCaBP5 interacts with myosin IB, IQ motif peptide and dissociation constant (K_d) were calculated 240 nM (Figure 3). The binding of protein to peptide was strong, as it is also indicated through K_d value. Dissociation of CaBP5 was found to be very slow during kinetics assay. We expect this protein-peptide interaction is governed by hydrophobic interaction. This result confirms our hypothesis as EhCaBP5-IQ motif is interacting in SPR assay.

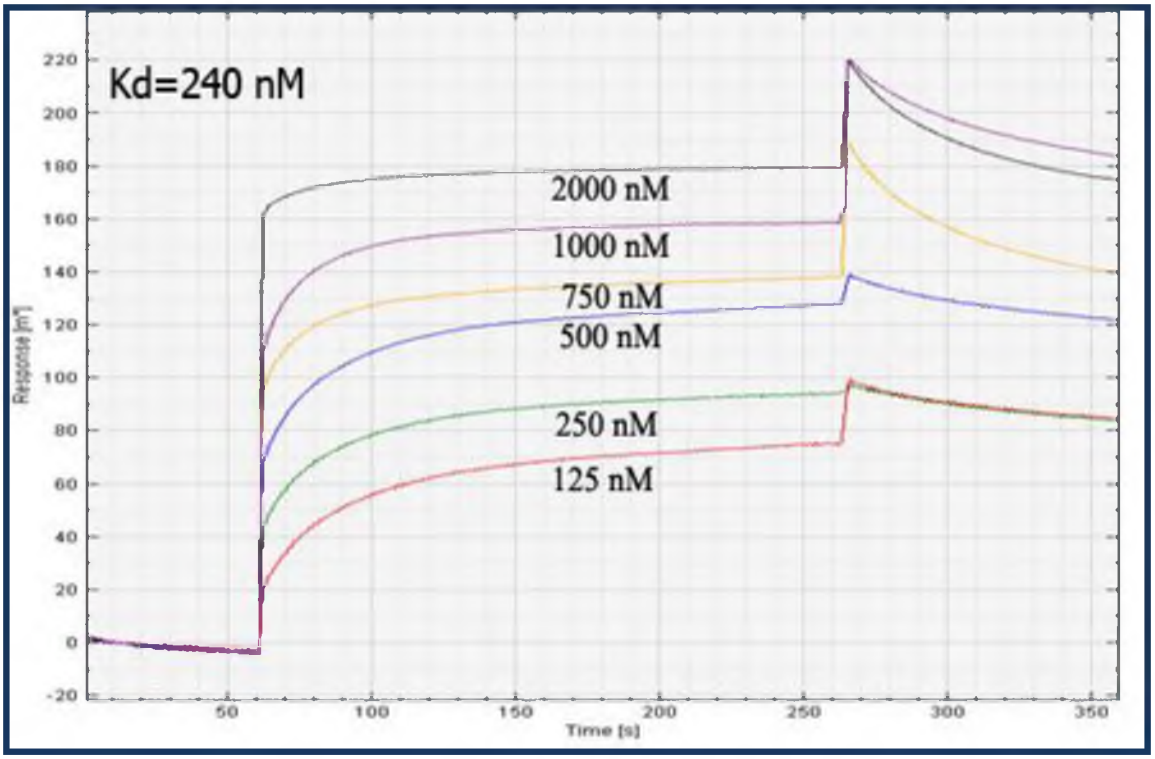

Figure 3: SPR Sensogram: Sensogram showing the interaction between Myosin IB-IQ motif peptide (IQKAWKGYRNRKRS) and EhCaBP5. Different concentrations of CaBP5 were injected onto the sensor chip to calculate the dissociation constant (K_d).

3.4.2 Localization of EhCaBP5 in *Entamoeba histolytica*

To check further if the EhCaBP5 interacts with myosin IB *in vivo*, the localization of EhCaBP5 monitored, in normal trophozoites using anti-EhCaBP5 antibody and Hoechst staining (DNA stain). Confocal microscopy revealed the presence of EhCaBP5 in the cytoplasm as there was no fluorescence signal seen in the nucleus (Figure 4). Since myosin was found to be involved in erythrophagocytosis (Voigt et al., 1999), and our SPR data also confirms the interaction of both proteins. Therefore to investigate whether EhCaBP5 is colocalized with myosin IB and if it so, does EhCaBP5 also involved in erythrophagocytosis? To answer this question, the subcellular localization of EhCaBP5 was checked during RBC uptake by immunostaining with specific anti-EhCaBP5 antibody. The results are shown in (Figure 5A) Fluorescence signals clearly showed the enrichment of EhCaBP5 in the phagocytic cups as has been observed for myosin IB (Voigt et al., 1999). Enrichment of actin was also observed in the phagocytic cup as expected and the superimposition of both CaBP5 and actin suggested that both proteins are colocalized in the phagocytic cups. Since fluorescence data clearly shows the colocation of actin along with EhCaBP5 (Figure 5B). To check whether these proteins interact with each other *in vitro*, we performed co-sedimentation assay of actin with EhCaBP5. We could not get any direct interaction (result not shown), so we expect the involvement of myosin IB which helping them to co-localized EhCaBP5 and actin.

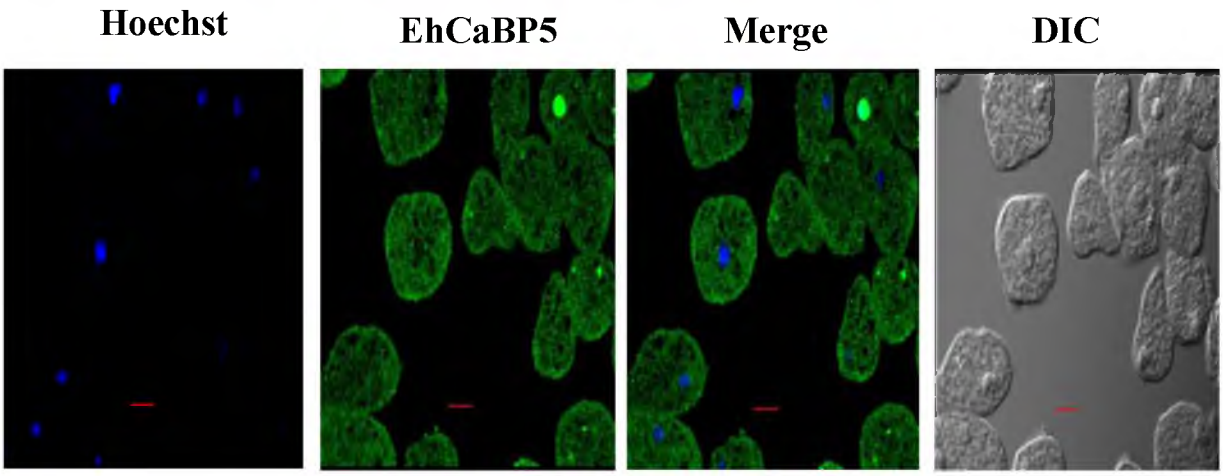

Figure 4: Immunolocalization of EhCaBP5 in *Entamoeba histolytica*: Trophozoites grown for 48 were transferred to pre-warmed coverslips for 10 min at 37^{0}C. The cells were then fixed with 3.7 % paraformaldehyde/PBS, permeabilized with 0.1% triton X-100/PBS and then stained with anti-EhCaBP5 antibody followed by secondary antibody Alexa 488. Hoechst was used to stain the nuclei. Bar represents 10 µm. Magnification 60X.

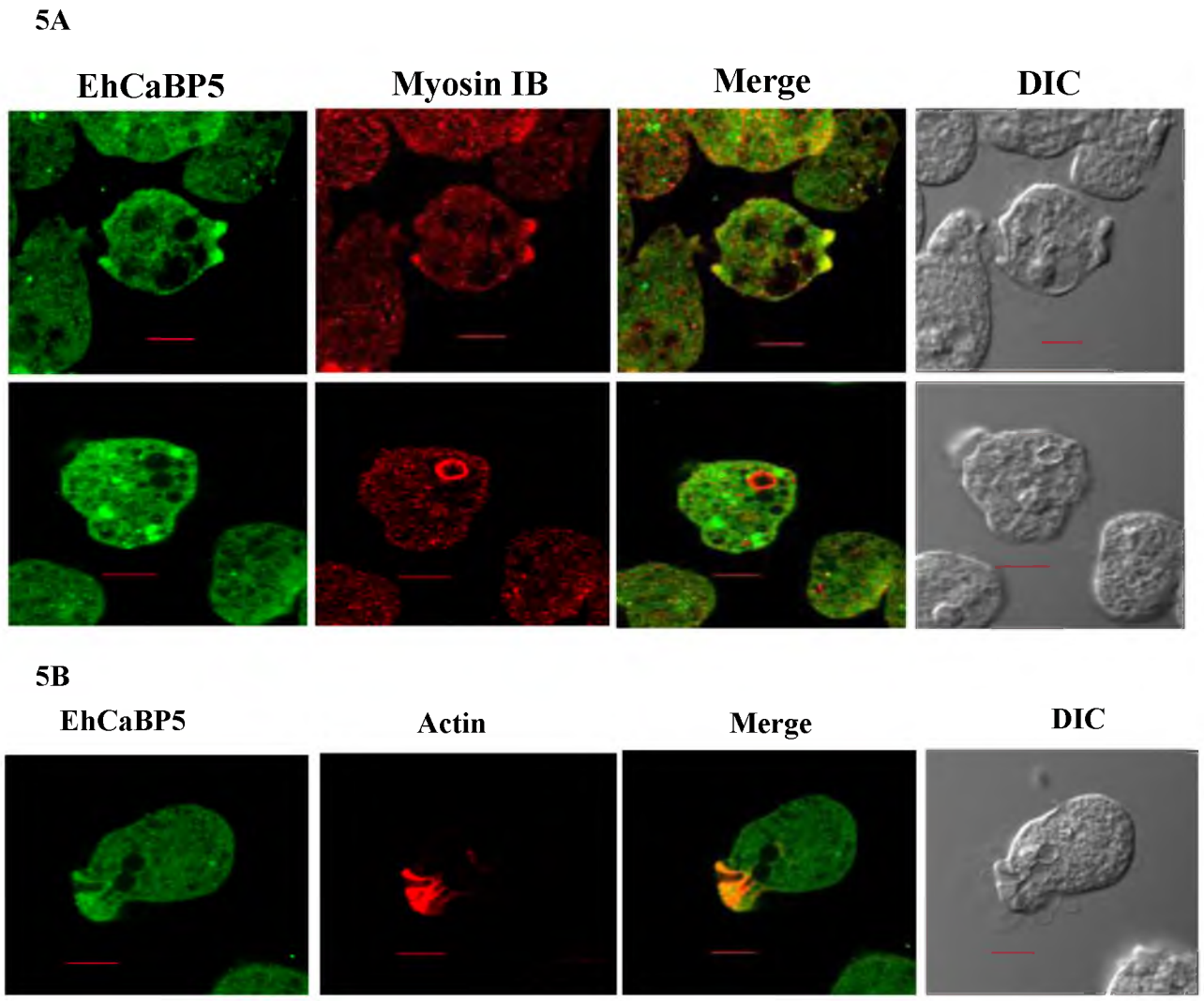

Figure 5: Distribution of EhCaBP5, Myosin IB and actin in *Entamoeba histolytica* during erythrophagocytosis: (A) Co- localization of EhCaBP5 and Myosin IB in *E. histolytica* cells during erythrophagocytosis. Cells were grown for 48h and incubated with RBCs for 10 min at 37^{0}C. The cells were then fixed and immunostained with anti- EhCaBP5 and anti-Myosin IB antibodies followed by Alexa-488 (green) and Alexa-555 (red) secondary antibodies. Arrow head depicts the co- localization of CaBP5 and Myosin IB in the phagocytic cup (upper panel) and an asterisk mark shows the absence of EhCaBP5 in the phagosome. (B) Co-localization of EhCaBP5 with F-actin. Trophozoites were stained with anti-CaBP5 antibody and TRITC-phalloidin (red) was used to stain the F-actin. The secondary antibody used for EhCaBP5 was Alexa-488(green). Bar represents 10μm. (DIC, differential interference contrast).

3.4.3 Colocalization of EhCaBP5 with EhCaBP1

EhCaBP1 is a 14.7 kDa (134 amino acid residues) protein, which shares low (29%) sequence identity with the well-studied eukaryotic EF-hand containing protein, Calmodulin (CaM) (Prasad et al., 1992). EhCaBP1 was found to be localized in cytoplasm and transiently associated in phagocytic cup formation in calcium independent manner (Jain, 2008). As EhCaBP5 is found to be localized in cytoplasm and involved in erythrophagocytosis, to check whether these proteins (EhCaBP1 and EhCaBP5) are colocalized during process of erythrophagocytosis, we performed colocalization experiment. Our result suggests that both EhCaBP1 and EhCaBP5 are colocalized at the time of phagocytic cup formation during process of erythrophagocytosis (Figure 6). Though we could not check the direct interaction of both these proteins invitro but we can speculate that colocalization of both protein EhCaBP1 and EhCaBP5 may be playing some regulatory function during process of phagocytosis or phagosome formation as EhCaBP1 transiently remains in mature phagocytic cup, whereas EhCaBP5 leaves phagosome once it fully mature, suggesting that EhCaBP5 may have role in phagocytic cup formation along with EhCaBP1.

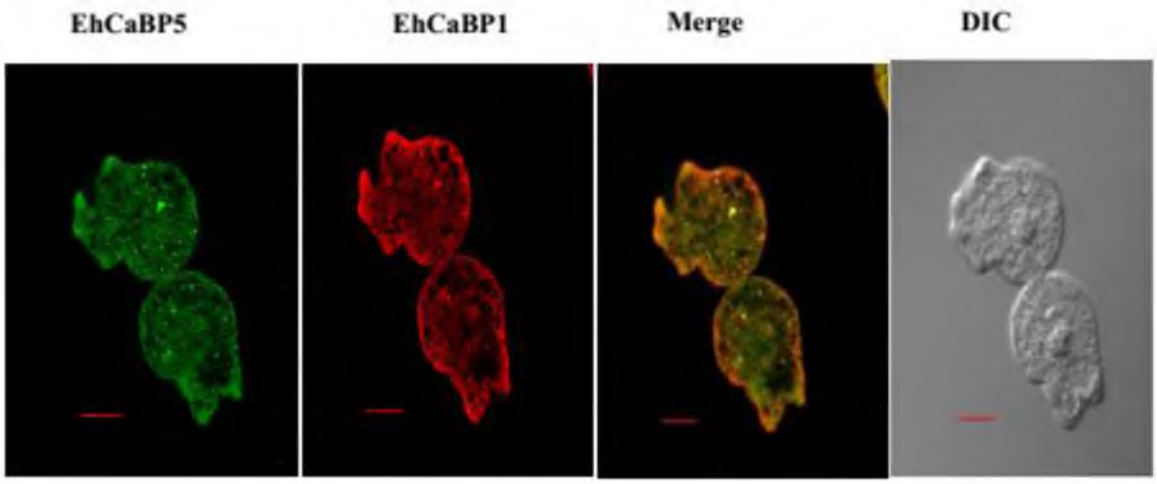

Figure 6: Localization of EhCaBP5 and EhCaBP1 in trophozoites during erythrophagocytosis: Cells were incubated with erythrocytes for 10 min and then stained with anti-CaBP5 (green) and anti- CaBP1 (red) antibodies. The secondary antibodies used were Alexa-488 and Alexa-555 and viewed using confocal microscope. Arrow heads denote the phagocytic cups. Scale bar represents 10 µm.

3.4.4 Identification of EhCaBP5 as an interacting partner of Myosin IB

Myosin IB is the known molecule to be localized and enriched in the phagocytic cups and may be involved in the scission of vesicles along with CaBP3 (Voigt et al., 1999, Aslam et al., 2012). In order to check whether EhCaBP5 also co-localizes with myosin IB, asynchronized amoebic cells were incubated with RBCs for 10 min so as to capture all stages of phagocytosis using anti-CaBP5 and anti-myosin IB antibodies. Confocal microscopy clearly showed the co-localization of both CaBP5 and myosin IB at the phagocytic cups (denoted by arrow head) but once phagocytic cup pinched away from membrane to form phagosomes the CaBP5 was not observed in them (denoted by star) (Figure 4), suggesting that EhCaBP5 was involved in the initiation process of phagocytosis like EhCaBP1(Jain et al., 2008), while EhCaBP1 still remains with phagosomes but EhCaBP5 leaves once phagosomes are formed clearly indicating the important role in phagosomes formation.

In order to further confirm this interaction, we have done the co-immunoprecipitation using immobilized anti-CaBP5 antibody and total cell lysate. Anti-EhCaBP5 antibody precipitated myosin IB along with CaBP5 in a calcium independent manner as myosin IB was also observed in the presence of EGTA, suggesting that calcium is not required for the interaction of these two proteins (Figure 7).

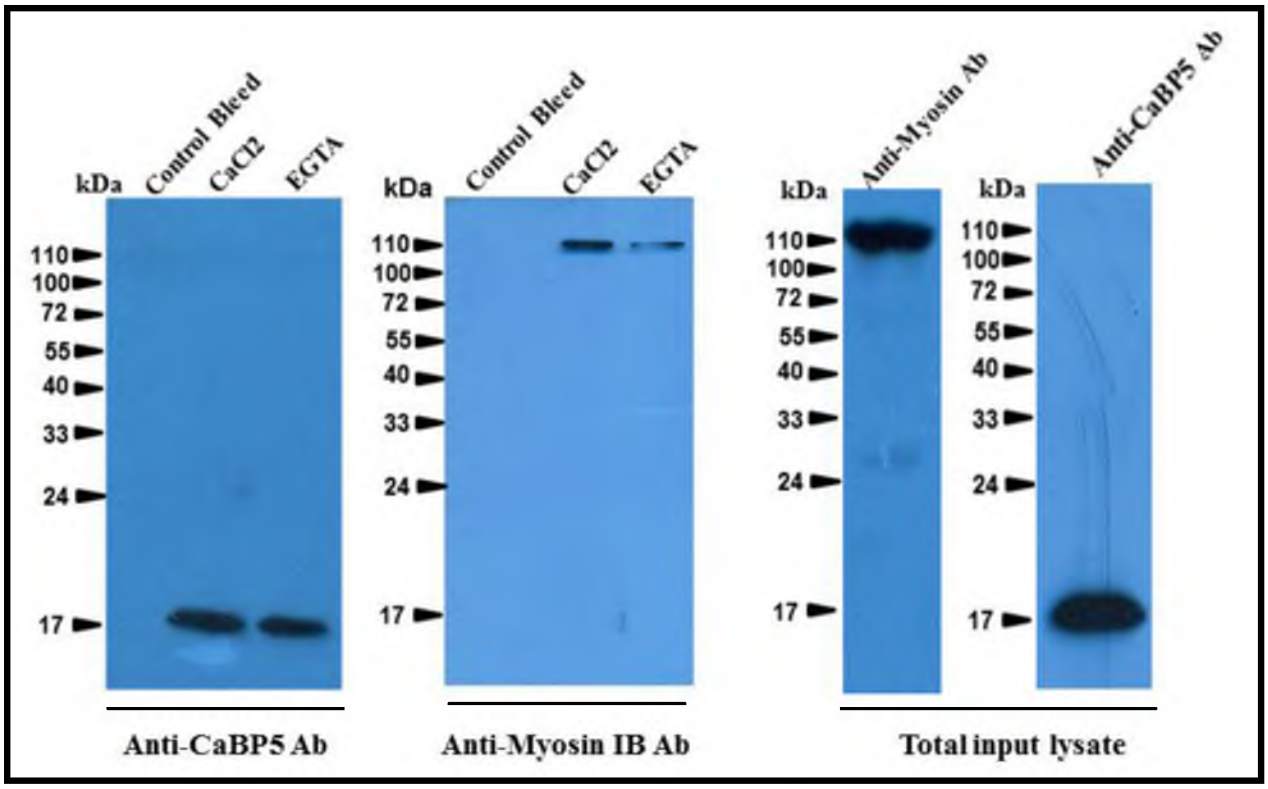

Figure 7: Pull down assay: Interaction of EhCaBP5 with Myosin IB: Total (800 µg) of *E. histolytica* lysate was incubated with Sepharose-anti-CaBP5 antibody conjugate for 6 h at 4°C with shaking. The beads were then washed as mentioned in Materials and methods. The bound material was then eluted and analysed by western blotting and immunostained with anti-myosin IB antibody raised in rabbit. The blot was reprobed with anti-CaBP5 antibody raised in mice. The total input lysate was also probed for the presence of EhCaBP5 and myosin IB by their respective antibodies.

3.5 Discussion

After analyzing the crystal structure of EhCaBP5 in the chapter first, we hypothesize that the molecule EhCaBP5 may interact to myosin. Here our result of surface plasmon resonance with myosin IB-IQ motif peptide, cell co-localization of EhCaBP5 with Myosin IB and pull down assay strengthen our hypothesis and we found that both the molecule (EhCaBP5 and Myosin IB) interact *in-vitro* as well as *in-vivo*.

Our surface plasmon resonance data suggest, EhCaBP5 interacts with myosin IB-IQ motif peptide. Further to check this interaction *in vivo*, we performed cellular localization of Myosin IB and EhCaBP5. Our immunostaining result suggest that EhCaBP5 is colocalized with myosin IB, this result is well validated by SPR assay as the IQ-motif peptide of myosin IB showing strong interaction. To further validate this interaction we performed pull down assay, result confirms that EhCaBP5 pulls the myosin IB from total cell lysate. We also performed pull down assay by using EGTA to confirm whether EhCaBP5-Myosin IB interaction is calcium dependent or not. As the EhCaBP5 pulls myosin IB in presence of EGTA also, we expect that this interaction is calcium independent. Also the crystal structure of EhCaBP5 cross validates our result as EhCaBP5 crystal structure shows that calcium binding does not bring any conformation changes. As the EF-hand motif of EhCaBP5 remains in closed conformation though calcium is bind to it. EhCaBP5 also colocalized with actin at the time of phagosome formation (Figure 5B) suggesting that it may involve in cytoskeleton dynamics but we could not get any direct interaction of EhCaBP5 with actin. Therefore, we expect that the colocalization of these two molecules in cell at the time of phagosome formation is regulated by some other molecule. Another calcium binding proteins EhCaBP1 is associated with phagocytic cup formation (Ruchi Jain., 2008), was found to be colocalized with EhCaBP5. Their colocalization suggests that these two molecules may also play some critical role together in formation of phagosome.

So the interaction of EhCaBP5 with myosin IB and the cellular co-localization of EhCaBP5 with myosin IB, actin and EhCaBP1confirms that all these molecule are playing critical role in phagosome formation along with EhCaBP5, and thus playing an important role in pathogenesis of *E.histolytica*.