Chapter 2

Materials & Methods
2.1. SOURCES OF MATERIALS AND CHEMICALS

_E. coli_ strains DH5α and DH10β were obtained from Bethesda Research Labs (B.R.L., U. S. A.) and used for all recombinant DNA work. Restriction enzymes and other molecular biological reagents were purchased from New England Biolabs (NEB, USA), Roche Biochemicals (Germany), Amersham Pharmacia (USA), Promega (USA), Sigma (USA), MBI Fermentas (Canada) and Qualigens (India). Random priming kit for labeling DNA was obtained from NEB; PCR purification kit was from Amersham; Oligonucleotide primers were synthesized by Microsynth (Switzerland) and Sigma (U. S. A.); ³²P-γ ATP and ³²P-γ GTP (specific activity ~5000Ci/m mol) were obtained from Bhaba Atomic Research Centre (BARC, India) and ⁴⁵Ca was obtained from Perkin Elmer. Adult Bovine Serum was purchased from PAA Laboratories (Austria) and _E. histolytica_ media components were obtained from Amersham Biosciences and DIFCO (U.S.A.). Diamond’s Vitamin mix for _Entamoeba_ culture was purchased from Sigma-Aldrich (U.S.A). X-Ray films were from Konica and charged nylon membranes (GeneScreen plus) was obtained from New England Nuclear (N.E.N, USA) and nitrocellulose papers were obtained from Schleicher and Schuell (Germany).

(All concentrations indicated in percentage are in (w/v) basis unless stated otherwise. All solutions were prepared in double distilled water unless stated otherwise. Autoclaving was done at a pressure of 15lbs per square inch for 20 min.)

2.2. ORGANISMS AND GROWTH CONDITIONS

_E. coli_ DH5α has the genotype: _SupE44 lacU169 (φ80 lacZ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1_. Cells from an agar stab or frozen glycerol stock were first streaked on an LB plate (containing the appropriate antibiotic wherever necessary) and allowed to grow overnight at 37°C. Liquid cultures in LB medium were initiated from a single colony and were grown with constant shaking at 225 rpm at 37°C. The cells grown overnight, were used as inoculum for further growth by diluting 100 fold in fresh LB medium and grown with aeration at 37°C for 3-4 h to obtain log phase cultures.

_E. histolytica_ strain HM-1:IMSS clone 6 was obtained from Dr. William A Petri (University of Virginia, USA); all experiments were done with _E. histolytica_ strain HM-1: IMSS clone 6. The cells were maintained and grown in TY1-33 medium
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complemented with 15% adult bovine serum, 2% Diamond’s vitamin mix and antibiotic (125 μl of 250 U/ml Benzyl Penicillin and 0.25 mg/ml Streptomycin per 90 ml of medium). Hygromycin (Sigma) or Neomycin was added at 10 μg/ml for maintaining transgenic cell-lines.

2.3. MEASUREMENT OF CELL NUMBER

Cell viability was determined by microscopy in presence of trypan blue. Growth of trophozoites was measured by cell counting in triplicates using a hemocytometer (Neubauer, Marienfeld, Germany).

2.4. CULTURE MEDIA

2.4.1. LB Medium

Bacterial Cells were grown in Luria Broth (LB). It was prepared by dissolving 25 gm of LB powder (Amersham) in 1 liter of distilled water and pH adjusted to 7.0 using 2 N NaOH. The medium was sterilized by autoclaving.

2.4.2. LB Agar

LB agar was prepared by adding 1.5 % (w/v) of Bacto-Agar to LB medium and sterilized by autoclaving. Ampicillin 100 μg/ml or kanamycin 60 μg/ml to a final concentration was added (when required) after cooling the LB agar to around 55°C and plates poured under aseptic conditions.

2.4.3. TYI-S-33 medium Composition per 900 ml (10 units) (Diamond et al., 1978)

Potassium phosphate, dibasic 1.0 g
Potassium phosphate, monobasic 0.6 g
Biosafe peptone 30.0 g
Dextrose 10.0 g
Sodium chloride 2.0 g
L-Cysteine hydrochloride 1.0 g
Ascorbic acid 1.0 g
Ferric ammonium citrate 22.8 mg

To these components 700ml of double distilled water was added and pH adjusted to 6.8 using 2 (N) NaOH. The volume was made up to 900 ml and filtered using
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Whatmann #1 filter paper, aliquoted and autoclaved. Incomplete medium was stored at -20°C. Medium was completed by adding 15% heat inactivated adult bovine serum, 2% Diamond’s vitamin mix and 125 µl of antibiotic (250 U/ml Benzyl Penicillin and 0.25 mg/ml Streptomycin). This media is used to grow E. histolytica.

2.4.4. Heat Inactivation of Serum

Adult bovine serum was stored frozen at -20°C. Before heat inactivation, the serum was thawed at room temperature (RT) and incubated in a water-bath at 37°C for 30 min with intermittent shaking. The serum was transferred to 55°C for 45 min with intermittent shaking for complement inactivation and stored at 4°C.

2.5. PREPARATION OF PLASMID DNA FROM E. coli TRANSFORMANTS

2.5.1. Mini-preparation of plasmid DNA (Alkaline lysis method)

A single colony harboring the desired plasmid was inoculated in 2 ml of LB medium containing appropriate antibiotic and grown overnight at 37°C. The cells were pelleted at 6000 rpm for 5 min and the supernatant was aspirated out. The pellet was suspended in 100 µl of Solution I (50mM Glucose, 25mM Tris-Cl pH 7.5 and 10mM EDTA pH 8.0). To the tube 200 µl of freshly prepared Solution II (0.2N NaOH and 1% SDS) was added, mixed gently by inverting and incubated at RT for 5 min, 150 µl of chilled Solution III (3M Potassium acetate, pH 5.2) was then added and the contents were mixed gently by inverting the tube and kept on ice for 10 min. The mixture was centrifuged at 14000 rpm for 10 min at 4°C. The supernatant was transferred to a fresh tube and 0.7 volumes of isopropanol was added and centrifuged at 14000 rpm for 10 min. The pellet was washed with 70% ethanol by centrifugation at 14000 rpm for 5 min at RT. The supernatant was discarded and the pellet was air-dried. The dried pellet was suspended in autoclaved Milli-Q or TE-RNAse.

2.5.2. Midi-preparation of plasmid DNA (Alkaline lysis method)

Bacterial cells were grown overnight in 50 ml LB medium with the appropriate antibiotic and collected by centrifugation (6000 rpm at 4°C for 5 min). The cell pellet was resuspended in 1.5 ml lysis buffer (25 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0, 15% Sucrose) containing 2 mg/ml lysozyme, and kept on ice for 8 min. To the lysed
cells, 3 ml of denaturing solution (freshly prepared solution containing 0.2 N NaOH and 1% SDS) was added and mixed gently by inverting and incubated on ice for 10 min. To the tube, 1.8 ml of 3 M sodium acetate (pH 4.6) was added and incubated on ice for 20 min followed by centrifugation at 12000 rpm for 30 min at 4°C. RNA was removed by incubating the supernatant with 10 μl of 10 mg/ml RNAse A at 37°C for 45 min. The supernatant was extracted twice with phenol:chloroform:isoamyl alcohol [25:24:1 (v/v/v)] and once with equal volume of chloroform:isoamyl alcohol [24:1 (v/v)]. The upper aqueous layer was transferred to an oak-ridge tube and the DNA precipitated by addition of 2.5 volumes of chilled ethanol and left overnight at -20°C or 45 min at -80°C. The DNA was precipitated by centrifugation at 12000 rpm for 20 min at 4°C. The pellet was resuspended in 0.4 ml of nuclease free water and to this was added 120 μl of 4 M NaCl and 0.5 ml 13% PEG 8000. The tube was incubated on ice for 1 h and centrifuged at RT at 12000 rpm for 15 min. The pellet was washed with 200 μl of 70% ethanol, dried in a 37°C incubator and resuspended in 50 μl of T10E1.

2.5.3. Agarose gel electrophoresis
DNA fragments of size > 400 bp were resolved on 1% agarose gel, while those in the range of 250 – 500 bp were resolved on 1.2% agarose gel. The gels were electrophoresed in 0.5 X TBE buffer containing 0.5 μg/ml ethidium bromide.

2.5.4. Elution of DNA from agarose gel
The agarose slice containing the band of interest was cut out from the gel and trimmed. DNA from gel band was isolated using GFX kit following manufacturer’s protocol (Amersham). Eluted DNA was checked by agarose gel electrophoresis.

2.6. ISOLATION OF GENOMIC DNA FROM *E. histolytica* TROPHOZOITES
(Bhattacharya S et al., 1988)
*Entamoeba* cells (approximately 4 X 10^7 cells) were harvested by chilling on ice for 10 min and centrifuged at 280 x g at 4°C for 7 min and washed once with PBS # 8 [0.37% K2HPO4, 0.11% KH2PO4 and 0.95% NaCl, pH 7.2]. The cell pellet was resuspended in 4 ml of ice-cold NET 1 buffer [10 mM Tris-Cl pH 8.0, 100 mM NaCl, 10 mM EDTA pH 8.0]. To this was added 10% SDS to a final concentration of 0.25%,
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gently mixed by inverting and transferred on to ice. After 2-3 min when the cell suspension became viscous, equal volume of phenol was added and mixed thoroughly by gentle inversion. The phases were separated by centrifugation at 6000 rpm for 20 min at 4°C. The upper aqueous layer was transferred to a fresh tube and extracted once with phenol, once with phenol: chloroform: isoamyl alcohol [25:24:1 (v/v/v)] and once with chloroform: isoamyl alcohol [24:1 (v/v)]. All the centrifugation steps were done at 6000 rpm, 4°C, 10 min. The DNA was precipitated with 1/10th volume of 4 M NaCl and 2.5 volumes of ethanol and incubated overnight at -20°C. The DNA was pelleted down by centrifugation at 12000 rpm for 20 min at 4°C. The pellet was resuspended in 4 ml of NET 2 buffer [10 mM Tris-Cl pH 8.0, 10 mM NaCl, 10 mM EDTA pH 8.0] and digested with RNAse A (100 µg/ml) at 37°C for 30 min followed by Proteinase K (100 µg/ml) at 55°C for 45 min. The DNA was extracted with phenol:chloroform:isoamyl alcohol as described above but the centrifugation was carried out at RT. The DNA was precipitated with NaCl and ethanol as mentioned above and the pellet was washed with 70% ethanol. The pellet was dried and dissolved in 100 µl of T10E1.

2.7. DNA MANIPULATIONS FOR CLONING PURPOSES

2.7.1. Polymerase Chain Reaction (PCR)
Forward and reverse oligonucleotide primers flanking the desired region of interest were used for PCR. The oligonucleotides used for various PCR reactions and their sequences are given in table 2.2. All PCR reactions were performed using either Taq polymerase or Taq/Pfu mix. A typical amplification reaction contained 50 ng (plasmid) or 150 ng (Genomic DNA) of template DNA, 1X Taq polymerase buffer, 200 µM dNTPs, and 20 pmoles each of forward and reverse primers, 2.5 mM MgCl2 and 0.5 U of Taq or Taq/Pfu mix (Taq and Pfu DNA polymerase were mixed in a ratio of 3:1 and used for amplicons greater than 1 kb) in a reaction volume of 50 µl.
The PCR cycle comprised of denaturation at 94°C for 30 s followed by annealing at 5-10 degrees below the Tm of the primers used for 1 min, extension at 72°C for 1-2 min (1 min / 1000 bp). The last extension step at 72°C was done for an additional 10 min. The amplification reaction was carried out in a DNA thermal cycler (Techne, USA) after overlaying the samples with mineral oil to prevent evaporation. The size
and integrity of the products were checked by electrophoresing 10 μl of the sample on a 0.8-1.2% agarose gel at 4 V/cm for an appropriate time period.

2.7.2. Restriction enzyme digestion of DNA
All restriction digestions were carried out according to the manufacturer’s recommendations. The digestions were carried out in water bath set at the recommended temperature. For analytical purpose, the reactions were set up in a volume of 20 μl. For preparative purposes, the digestions were set up in a volume of 50 –100 μl. After incubation the reaction mixtures were loaded with 1X gel loading buffer (GLB) (2.5 % Ficoll type-400, 0.04% bromophenol blue, 0.04% xylene cyanol FF) onto an agarose gel.

2.7.3. Dephosphorylation of DNA termini
Removal of 5' phosphate groups from DNA fragments was carried out by using antartic phosphatase (AP). The digested vector DNA (1μg/μl) was resuspended in a 1X AP reaction buffer and incubated with AP at 37°C for 15min. The enzyme was then heat inactivated at 65°C for 5 min and DNA was purified by gel purification using GFX kit.

2.7.4. Ligation of DNA termini
Ligation reactions were carried out in a volume of 10 μl at 16°C for 16 h. The reaction mixture contained about 100ng of the digested vector DNA, insert DNA fragment at 1:3 or 1:5 (vector: insert) molar concentrations and 1X ligase buffer containing 1 mM ATP and 20 U of T4 DNA ligase. The ligation reaction product was transformed in E. coli competent cells and transformants were selected on LB agar plates supplemented with the required antibiotic.

2.7.5. Preparation of competent cells and transformation
Competent cells of E. coli (BL21, DH10β and DH5α) were prepared by the method described by Hanahan D. Single colony from LB agar plate was inoculated in 5 ml LB medium and grown overnight at 37°C. 1% of overnight culture was added to 50 ml LB and grown at 37°C to an OD600 of 0.38-0.42. The culture was then centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was decanted and the pellet was
suspended gently in 10 ml of prechilled 0.1 M CaCl₂. The cells were incubated for 1 h on ice and then centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was re-suspended in 2 ml of 0.1 M CaCl₂. The competent cells were stored as 15% glycerol stocks in 100 μl aliquots at -70°C.

2.7.6. Transformation of competent cells

100 μl competent cells were thawed on ice and 5-10 ng of plasmid DNA was added. The cells were incubated on ice for 30 min. Cells were then given a heat shock at 42°C for 90 s and incubated on ice for 2 min. 0.9 ml of LB was added to the cells and the cells were grown at 37°C for 1 h at 200 rpm. Transformants were plated on LB agar plates with appropriate antibiotic and incubated at 37°C for 14-16 h.

**Table 2.1.** Details of the clonings done in the present study

<table>
<thead>
<tr>
<th>Name of the clone</th>
<th>Insert</th>
<th>Size of insert (kb)</th>
<th>Vector</th>
<th>Size of vector (kb)</th>
<th>Cloning Sites</th>
<th>Primer Pair</th>
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<tbody>
<tr>
<td>GC1</td>
<td>CaBP1</td>
<td>0.405</td>
<td>pEh-NEO-GFP*</td>
<td>6.7</td>
<td>BamH I</td>
<td>CGF and CGR</td>
</tr>
<tr>
<td>EhCaBP2-GFP</td>
<td>CaBP2</td>
<td>0.405</td>
<td>pEh-NEO-GFP*</td>
<td>6.7</td>
<td>BamH I</td>
<td>C2GF and C2GR</td>
</tr>
<tr>
<td>GFP-cter</td>
<td>Cter-CaBP1</td>
<td>0.210</td>
<td>pEh-NEO-GFP*</td>
<td>6.7</td>
<td>BamH I</td>
<td>Cterm-C1 (BamH I) and CGR</td>
</tr>
<tr>
<td>GFP-DLP</td>
<td>DLP</td>
<td>2.049</td>
<td>pEh-NEO-GFP*</td>
<td>6.7</td>
<td>KpnI</td>
<td>dsF and dasR</td>
</tr>
<tr>
<td>Cter-pet</td>
<td>Cter-CaBP1</td>
<td>0.210</td>
<td>pET 3(a)</td>
<td>4.6</td>
<td>Nde I / BamH I</td>
<td>Cterm Cl F and Cterm Cl R</td>
</tr>
<tr>
<td>DLP-pGEMT</td>
<td>DLP</td>
<td>2.049</td>
<td>pGEMT easy (Promega)</td>
<td>3.0</td>
<td>TA cloning</td>
<td>DyF and DyR</td>
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<tr>
<td>DLP-pet</td>
<td>DLP</td>
<td>2.049</td>
<td>pET 30(a)</td>
<td>5.4</td>
<td>Nco I / Sal I</td>
<td>Subcloned from DLP-pGEMT clone</td>
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<td>$^5$ pEhCaBP2-S</td>
<td>CaBP2</td>
<td>0.405</td>
<td>pEh-HYG-Tet-O-CAT (TOC)</td>
<td>8.7</td>
<td>Kpn I / BamH I</td>
<td>F2 and R2</td>
</tr>
<tr>
<td>$^5$ pEhCaBP2-AS</td>
<td>CaBP2</td>
<td>0.405</td>
<td>pEh-HYG-Tet-O-CAT</td>
<td>8.7</td>
<td>Kpn I / BamH I</td>
<td>ANSF2 and ANSR2</td>
</tr>
</tbody>
</table>

* The vector has been previously constructed (N. Guillen, unpublished) by cloning the GFP mut3 allele of GFP (Cormack et al., 1996) in the Kpn I / BamH I site of the pExEhNeo plasmid (Hamann et al., 1995).

$^5$ The target fragment was cloned in the Kpn I / BamH I site of TOC in the place of CAT gene.
### Table 2.2. List of Oligonucleotides used in the study

<table>
<thead>
<tr>
<th>NAME OF THE OLIGO</th>
<th>SEQUENCE (5'- 3')</th>
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<tbody>
<tr>
<td>DyF</td>
<td>GACTATGAAAAAGTCTTATTCCAGTT</td>
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<tr>
<td>DyR</td>
<td>GACGTTAATTAACCTTGATTGTAAC</td>
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<tr>
<td>F2</td>
<td>GCGGTACCAGTATAAAATTCATTACAAAAGATT</td>
</tr>
<tr>
<td>R2</td>
<td>GCGGATCCTTTATAATCTTCTTCTGAAATTAATTGTTCA AGATTTGAATGCAAGAA</td>
</tr>
<tr>
<td>ANSF2</td>
<td>GGGGGATCCTCATTACAAAAAGATT</td>
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<tr>
<td>ANSR2</td>
<td>GGGGGGTACCTTAAAGATTGAATGC</td>
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<tr>
<td>CGF</td>
<td>GGGGGATCCCATATGGGCTGAAGCAGC</td>
</tr>
<tr>
<td>CGR</td>
<td>GGGGGATCCAGTTAGAGTGAAAACT</td>
</tr>
<tr>
<td>dsF</td>
<td>CCCCGTACCATGAAAAGTCTTTATTCCA</td>
</tr>
<tr>
<td>dasR</td>
<td>GGGGGTCATTTAATTAACCTTAGTT</td>
</tr>
<tr>
<td>Cterm C1 F</td>
<td>GCCGATATGGGACAGATCTTTCTGATG</td>
</tr>
<tr>
<td>Cterm C1 R</td>
<td>GGGGGATCCGAAGAAAACTCAAGG</td>
</tr>
<tr>
<td>Cterm-C1(BamHI)</td>
<td>GGGGGATCCCAAGAACAGATCTTTCTGATG</td>
</tr>
<tr>
<td>C2GF</td>
<td>GGGGGATCCATGGAAGCTGATTAATT</td>
</tr>
<tr>
<td>C2GR</td>
<td>GGGGTCTCTAAAGATTGAATGCAAG</td>
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**OLIGOS USED FOR SITE DIRECTED MUTAGENESIS**

<table>
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<th>NAME OF THE OLIGO</th>
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<tr>
<td>DYNFm</td>
<td>GGGTCTCAGAGTGTGCTGGCATCATACTGTATTAGGAAAG</td>
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<tr>
<td>DYNRm</td>
<td>CTTTCTAATACAGATGAATGCAACCAGCACTTTGAGACCC</td>
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<tr>
<td>EF1F</td>
<td>CTTTTTAAAGAAATGGAGATTGGAAGATGGGAG</td>
</tr>
<tr>
<td>EF1R</td>
<td>CTCCATCTCCATTACTGCAATCTTCTTTAAGA</td>
</tr>
<tr>
<td>EF2F</td>
<td>CAAATCTATTGCAGTGTGATGGAA</td>
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<td>EF2R</td>
<td>TTCCCCATCAGCTGCAATAGATTG</td>
</tr>
<tr>
<td>EF3F</td>
<td>CATAAACTTATGCGATTTGAGATGGG</td>
</tr>
<tr>
<td>EF3R</td>
<td>CCATCTCCATCAACTGCGATAAGTTTATAG</td>
</tr>
<tr>
<td>EF4F</td>
<td>GTTATGAAAGCTGCTAGCTAATGGTGATG</td>
</tr>
<tr>
<td>EF4R</td>
<td>CATCAACCTTATGCGATTTTCATAC</td>
</tr>
<tr>
<td>St-dyF</td>
<td>GTTACAATCAAAGGTAATGCGATCTGCTAAAGG</td>
</tr>
<tr>
<td>St-dyR</td>
<td>CTTTTAGACATGGTACCTGCAATCTTTGATTGTAAC</td>
</tr>
<tr>
<td>DGstopA F</td>
<td>CATATGGATCAATTCAATACAAAGATCTTTCTGATG</td>
</tr>
<tr>
<td>DGstopA R</td>
<td>CATGCAAAAGATTTGGATTGACATCCATAG</td>
</tr>
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2.8. TRANSFECTION OF *E. histolytica* TROPHOZOITES BY ELECTROPORATION

Tissue culture tubes containing trophozoites were harvested in log phase and detached by chilling on ice for 10 min, mixed by inverting the tubes several times and then pelleted by centrifugation at 280 x g for 7 min at 4°C. The medium was decanted and the cell pellet was washed twice in cold PBS # 8. The cell pellet was thereafter washed once in 2 ml of cytomix buffer [10 mM K2HPO4/KH2PO4 (pH 7.6), 120 mM KCl, 0.15 mM CaCl2, 25 mM HEPES, 2 mM EGTA, 5 mM MgCl2]. For electroporation, the pellet was resuspended in 0.8 ml of complete cytomix buffer (cytomix buffer containing 4 mM Adenosine Triphosphate, 10 mM Glutathione) and was placed in a cuvette (0.4 cm gap, BIO RAD) along with 200 µg of plasmid DNA (for stable transfection). For “no DNA” control, 10 mM Tris buffer was added. For stable transfection, two consecutive pulses were applied under conditions of 3000 V/cm (1.2 kV) and 25 µF. Electroporated trophozoites were transferred into tissue culture tubes containing 12 ml of TYI-S-33 and were incubated in a slanted position at 35.5°C. Drug selection was started after 2 days of transfection at a concentration of 10 µg/ml. Frequent change of medium was necessary along with appropriate antibiotic selection to eliminate dead cells and cellular debris. The transfected cell lines were stably maintained in the presence of hygromycin or neomycin.

In the present study different cell lines have been generated and maintained. These were GFP, GFP-CaBP1, GFP-CaBP1ΔEF, GFP-nter, GFP-cter, GFP-DLP, GFP-K38A, TOC, EhCaBP2-S and EhCaBP2-AS. Of these, TOC, EhCaBP2-S and EhCaBP2-AS were maintained under the selection of hygromycin and all others were maintained under neomycin (G418).

2.9. ISOLATION OF TOTAL RNA FROM *E. histolytica*

One million trophozoites (from 50ml culture) growing in log phase were harvested at 280 x g for 7 min at 4°C and the cell pellet washed with ice chilled PBS # 8 was resuspended in 1ml of Tripure reagent (Roche). The cells were completely lysed by repeated pipetting. RNA isolation was carried out according to manufacturer’s protocol (Roche). Briefly, the lysed cells were incubated at RT for 10-15 min. To it, 200 µl of chloroform was added and mixture was shaken vigorously for 15-30 s followed by incubation at RT for 10-15 min. The tubes were centrifuged at 12000 x g
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for 15 min at 4°C for complete phase separation. The upper aqueous phase containing RNA was transferred to a fresh microfuge tube and RNA was precipitated with 500μL of isopropanol at RT for 10 min. RNA pellet was collected by centrifugation at 12,000 x g for 10 min at 4°C. Pellet was washed with 1 mL of chilled 70% ethanol in DEPC treated water (freshly prepared) at 7,500 x g for 5 min at 4°C. The pellet was dried at 37°C for 15 min and resuspended in 50 μL of DEPC treated water, aliquoted and stored at −80°C.

2.9.1. Analysis of RNA
RNA samples were run either on 1.2% denaturing agarose gels containing 2.2 M formaldehyde and were prepared in 1X MOPS buffer (20 mM MOPS, 2 mM sodium acetate and 1 mM EDTA). Glassware used for RNA isolation and analysis were treated with 0.1% (v/v) DEPC (Diethyl pyrocarbonate solution in water) for 10-16 h at 37°C followed by baking at 180°C for 8 h as described by Sambrook et al. 1989. Electrophoresis chamber used for RNA samples was treated with 3% (w/v) hydrogen peroxide solution for 10 min in dark and washed extensively with DEPC treated water.

2.9.2. Fractionation of RNA by agarose gel electrophoresis
RNA (10 - 20 μg) was denatured by incubating with 0.5 X MOPS buffer, 2.2 M formaldehyde and formamide at 55°C for 15 min followed by chilling on ice. Samples were centrifuged briefly and mixed with gel loading buffer [50% glycerol, 10 mM EDTA, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol FF] and then loaded on to denatured agarose gel containing 2.2 M formaldehyde and 1 X MOPS buffer. Electrophoresis was carried out at 4V/cm. The gel was then washed extensively with DEPC treated water to remove the formaldehyde. The gel was then sequentially treated for 20 min each with a denaturing (0.05N NaOH and 1.5M NaCl) and neutralizing solution (0.5M Tris-HCl pH 7.5 and 1.5M NaCl) followed by 20 min equilibration in 20 X SSC. The transfer membrane was pre-equilibrated with 20 X SSC and the transfer was carried out using the standard protocols. After transfer, blot was stained with methylene blue to check equal loading and detect the size of molecular marker.
2.10. HYBRIDIZATION OF RADIOLABELED PROBES TO IMMOBILIZED NUCLEIC ACIDS

2.10.1. Preparation of radiolabeled DNA by random priming method
About 50-100 ng of linear DNA was denatured by heating in a boiling water-bath for 10 min and immediately chilled on ice. To the tube containing denatured DNA, 2 µl each of 0.5 mM dGTP, dCTP and dTTP, 5µl of hexanucleotide labeling mixture [containing random hexamers and reaction buffer at 10X concentration (2 M HEPES pH 6.6, 2 mM Tris-Cl pH 7.0, 0.1mM EDTA and 4 mg/ml BSA), 30-50 µCi [α-32P]dATP and nuclease free water was added to make up the volume to 50 µl. The reaction was initiated by adding 5U of Klenow enzyme. All the components used were from Random priming kit, NEB, USA. Incubation was carried out for 2-3 h at 37°C before stopping the reaction by addition of EDTA to a final concentration of 20 mM. Unincorporated dNTPs were removed by ethanol precipitation in the presence of 50 µg of carrier DNA (salmon sperm DNA) and 2.5 M ammonium acetate.

2.10.2. Hybridization
DNA blots were first incubated in prehybridization solution (1% SDS and 1 M NaCl, 0.3-0.4 ml per square cm of membrane) at 65°C in hybridization bottles. After 3 h, heat-denatured radiolabeled probe (2 x 10^5 dpm/ml) and 100 µg/ml denatured salmon sperm DNA were added to the prehybridization mix and hybridization was carried out for 16 h at 65°C. The membranes were washed sequentially to remove non-specifically bound probe using the following protocol: twice with 2 X SSC at RT for 5 min, twice with 1 X SSC and 1% SDS at 65°C for 30 min and finally twice with 0.1 X SSC at RT for 30 min each.

2.10.3. Removal of probe from nylon membrane for rehybridization
The probe was stripped off the membrane by incubating the membrane in a boiling solution of 0.1 X SSC and 0.1 % SDS for 20-30 min. Efficiency of the removal of probe was monitored by exposing the blot to an X-ray film before hybridization.
2.10.4. Autoradiography
After hybridization and washing, the blots were wrapped in saran wrap and mounted. Autoradiography was performed with an X-ray film and intensifying screens at -70°C for appropriate amount of time.

2.11. REVERSE TRANSCRIPTION PCR (RT-PCR)
5μg of total RNA (after DNase I treatment) was taken in a microfuge tube and to this 100 pmole of oligo dT was added. The mixture was incubated at 65°C for 10 min followed by quick chill on ice. To it 10μl of 5X RT buffer (50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM MgCl₂ and 10mM DTT), 1μl of RNA guard (40 U/μl, USB) and 1μl of MuLV reverse transcriptase (200 U/μl, USB) were added in a total reaction volume of 50 μl. The reaction mixture was incubated at 37°C for 1 h and reverse transcriptase was inactivated by incubation at 95°C for 5 min. Ten μl of this mix was used for a regular PCR reaction with gene specific primers.

2.12. SUBCELLULAR FRACTIONATION OF AMOEBCIC EXTRACT

2.12.1. Total Cell lysate preparation
One million trophozoites growing in log phase were harvested at 280 x g for 7 min at 4°C. The pellets were washed with cold PBS # 8. The washed pellet was then resuspended in 10 mM Tris-Cl, pH 7.5, 1% SDS containing 2 mM PHMB, 2 mM PMSF, 0.01 mM Leupeptin, 10 mM NEM. The lysate was mixed well and then boiled for 3 min, followed by immediate chilling on ice. Quantification was done by BCA assay before aliquoting to store at -80°C or analysis by SDS-PAGE.

2.12.2. Sub-cellular fractionation
To separate membrane proteins from cytoplasmic fraction, the cell extract was prepared by resuspending the cell pellet (~ 10⁷, washed with PBS # 8) in 1 ml of 100 mM Na₂HPO₄ buffer containing protease inhibitors (10 mM NEM, 2 mM PMSF, 0.01 mM leupeptin and 2 mM PHMB). The suspension was then subjected to three cycles of freeze-thawing followed by centrifugation at 100,000 x g for 30 min at 4°C. The resulting supernatant was labeled as the cytoplasmic fraction and the pellet as the membrane fraction. The pellet was washed twice with the above buffer and resuspended in the same buffer containing 1% Triton X-100 and re-centrifuged at
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100,000 x g for 20 min at 4°C, to separate the triton soluble fraction from triton insoluble fraction. The protein content of each fraction was estimated by BCA assay.

To separate the nuclear fraction from the cytoplasm and the membrane fraction, the protocol described by Dey et al., (Dey et al., 2003) was followed. Briefly, 10^7 cells growing in log phase were harvested at 280 x g for 7 min at 4°C and the cell pellet was washed with PBS # 8. The washed pellet was resuspended in 2 ml lysis buffer (10 mM Hepes pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 % Nonidet P-40 detergent and protease inhibitors) and incubated on ice for 15 min, followed by centrifugation at 3000 x g for 10 min at 4°C. The pellet, thus obtained contains the nuclear fraction and the supernatant was further ultra-centrifuged at 100,000 x g for 30 min at 4°C to obtain the cytoplasmic and membrane fractions. Nuclei pellet was resuspended in 50 µl of the lysis buffer and the protein content of each fraction was estimated by BCA assay.

2.12.3. Preparation of sub-cortical fraction enriched extract

To isolate the sub-cortical enriched fraction from the Entamoeba lysate the protocol given by Rahim Z et al., was followed (Rahim et al., 1993). Briefly, trophozoites (~ 10^7) grown for 48 h were harvested by centrifugation at 280 x g for 7 min at 4°C. After being washed with cold PBS # 8, the pellet was mixed with 400 µl of extraction buffer (0.3 M NaCl, 0.1 M NaH2PO4, 0.05 M Na2HPO4, 0.01 M Na4P2O7.10H2O, 1 mM MgCl2, 0.01 M EDTA, 0.014 M β-mercaptoethanol) containing 2 mM PHMB, 2 mM PMSF, 4 µM leupeptin and 10 mM NEM and incubated on ice for 1 h. The extract was centrifuged at 10,000 x g for 30 min, and the protein content of the supernatant (sub-cortical fraction) was measured by using the BCA assay with bovine serum albumin as a standard.

2.13. METABOLIC LABELING OF E. histolytica

E. histolytica trophozoites were labeled using [³H] lysine as described earlier (Bhattacharya et al, 1990). Cells (2.5 X 10⁶) were washed once with TYI-S-33 medium and then resuspended in the same medium at 5 X 10⁵ cells/ml in 4ml screw-capped glass vials. The incubation was carried out for 1h at 36°C followed by the addition of [³H] lysine (~ 100 µCi/ ml). After 6h of incubation, cells were harvested and washed twice with chilled PBS # 8. The radio labeled cells were then resuspended
in 7.5 % TCA and incubated on ice for 45 min followed by boiling for 7 min and quickly chilling. The lysate thus prepared was filtered through a GFX-glass filter after giving a brief spin. The filter was washed with 5 % TCA (10 ml) and then with absolute ethanol (5 ml). The filter was dried and transferred to a scintillation vial containing 5 ml of Cocktail ‘O’ and incubated in dark for 30 min. Finally the incorporated counts were measured by a scintillation counter.

2.14. PROTEIN INTERACTION STUDIES

2.14.1. Immunoprecipitation

The cell lysate (~ 500 µg) was spun to remove cellular debris and was then preabsorbed on Protein A-Sepharose beads (Preclearing). The antibody to be used for immunoprecipitation was also conjugated with the Protein A-Sepharose beads simultaneously. The antibody coupled to Protein A-Sepharose beads was used to pull down the antigen and the proteins binding to the antigen by incubating it with the precleared cell lysate for 2 h at 4°C in a reaction volume of 200 µl. Immune complexes were centrifuged at 3000 x g for 5 min followed by three washes each with buffer 1 (10 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.1% ovalbumin (w/v), 0.1% Triton X-100 (w/v), 0.05% sodium azide (w/v)) followed by buffer 2 (10 mM Tris-Cl pH 7.5, 150 mM NaCl) and buffer 3 (0.06 M Tris-Cl, pH 6.8). The pellet was resuspended in 50 µl of SDS-PAGE buffer (125 mM Tris-HCl 6.8, 2% SDS, 0.1 M DTT, 30% glycerol, 5% β-mercaptoethanol, and bromophenol blue), boiled for 5 min. The bound proteins were separated from beads by centrifugation for 5 min and the supernatant was analyzed by SDS-PAGE.

2.14.2. Co-sedimentation assay

Co-sedimentation assay was carried out following published conditions (Sahoo et al., 2004). Briefly, 5µM of rabbit muscle G-actin (Sigma) was polymerized for 60 min in polymerization buffer containing 100 mM KCl and 2 mM MgCl₂ at RT. After polymerization, actin was mixed with 1 mM ATP and appropriate target protein (5 µM) in a total volume of 150 µl of G-buffer (10 mM Tris-Cl, pH 7.5, 2 mM CaCl₂, 2.5 mM β-Mercaptoethanol, 0.5 M KCl, 10 mM MgCl₂) and incubated for 2 h at RT. The samples were centrifuged at 100,000 x g for 45 min at 4°C. The supernatant (one
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fourth of total) and pellet fractions (total) were analyzed by 15% SDS-PAGE followed by Coomassie blue staining.

2.14.3. Solid Phase Assay
The wells of a 96-well plate were coated with 5 μM G-actin in PBS overnight at 4°C and were blocked with 3% BSA in PBS for an additional 24 h. After washing with PBS-T, EhCaBP1 (positive control) and target protein were added to the wells in duplicates at varying concentrations. Bound protein was detected with anti-EhCaBP1 antibody followed by HRPO-linked anti-rabbit IgG using the colorimetric substrate TMB (Sigma). The reaction was stopped with 2N H2SO4 and absorbance was monitored at 405 nm with a microplate reader (Bio-Rad, USA).

2.15. SITE DIRECTED MUTAGENESIS
Site directed mutagenesis of the various residues in the present study was carried out as described by QuickChange™ XL Site-Directed Mutagenesis Kit (Stratagene). EhCaBP1 gene in pET 3(c) or pEh-NEO-GFP vector and EhDLP in pET 30(a) or pEh-NEO-GFP vector as the case may be, were used as the template to generate point mutants in EhCaBP1 or EhDLP respectively. Two internal primers carrying the desired mutations were used to replace the desired nucleotides. The plasmid DNA template was isolated from dam+ E. coli strains. Two complimentary oligonucleotides containing the desired mutation (Mutagenic Primers) were used.

Sample reactions were setup as follows
- 5 μl of 10X reaction Buffer
- 10 ng of dsDNA template
- 125 ng of oligonucleotide primer # F
- 125 ng of oligonucleotide primer # R
- 1 μl of dNTP mix
- Sterile double distilled H2O to a final volume of 50 μl

Finally 1 μl of Pfu Turbo DNA polymerase (2.5 U/μl) is added. The above mixture was then subjected to PCR under the following cycling conditions. Denaturation at 95°C for 30 s was followed by annealing at 55°C for 1 min and finally the extension at 68°C for 2 min/kb of plasmid length. For a single point mutation, 16 such cycles were carried out.
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*Dpn* I (1 μl) restriction enzyme (10 U/μl) was added to each amplification reaction and incubated at 37°C for 1 h to digest the parental strand (which is methylated) supercoiled dsDNA. 2-5 μl of the *Dpn* I treated DNA was transformed in *E. coli* competent cells. The colonies, thus obtained are screened by sequencing for the clone having the desired mutation.

In case of EhCaBP1 mutants, the first aspartate (D) residue of each EF hand was mutated to alanine (A) residue. The mutations were done in a sequential order one residue at a time. Each mutant thus obtained was named EF I, EF II, EF III and EF IV after the EF hand number mutated. The modified amplicon was used as a template for the next round of mutation to get a mutant having all the four D mutated to A (CaBP1ΔEF). In case of EhDLP mutant, lysine (K) residue at position 38 was mutated to alanine (A) and the mutant was named K38A. The mutations were confirmed by nucleotide sequencing. The list of mutations made along with the primers used is given in Table 2.3.

**Table 2.3:** Oligos used for site directed mutagenesis

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Mutation incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF1F and EF1R</td>
<td>Aspartate at position 10 mutated to alanine in EhCaBP1</td>
</tr>
<tr>
<td>EF2F and EF2R</td>
<td>Aspartate at position 46 mutated to alanine in EhCaBP1</td>
</tr>
<tr>
<td>EF3F and EF3R</td>
<td>Aspartate at position 85 mutated to alanine in EhCaBP1</td>
</tr>
<tr>
<td>EF4F and EF4R</td>
<td>Aspartate at position 117 mutated to alanine EhCaBP1</td>
</tr>
<tr>
<td>St-dyF and St-dyR</td>
<td>Mutate stop codon in EhDLP to alanine in GFP-DLP</td>
</tr>
<tr>
<td>DGstopA F and DGstopA R</td>
<td>Glycine at position 67 mutated to stop codon in EhCaBP1 to make nter clone</td>
</tr>
<tr>
<td>DYNFm and DYNRm</td>
<td>Lysine at position 38 mutated to alanine in EhDLP</td>
</tr>
</tbody>
</table>

2.16. RECOMBINANT PROTEIN PURIFICATION

2.16.1. EhCaBP1 protein purification

A single isolated colony of EhCaBP1 clone in pET 3(c) was inoculated in a 5ml culture (LB + 100 μg/ml ampicillin) in a 50 ml glass tube (Borosil) and incubated overnight at 37°C at 225 rpm. A 50 ml culture (LB + 100 μg/ml ampicillin) in 250 ml
conical flask was inoculated with 1% of the above culture and grown at 37°C/225 rpm till O.D<sub>600</sub> reached 0.5. The bacterial culture was induced with 1mM IPTG and incubated for 3-4 h at 37°C/225 rpm. The induced bacteria was harvested at 6000 rpm at 4°C for 5 min and washed once with Wash buffer (25 mM Tris.Cl pH 7.5 and 100 mM NaCl). The cell pellet was suspended in 1/25<sup>th</sup> volume of the original culture in Buffer A (50 mM Tris-Cl pH 7.5 and 2 mM EGTA). The cells were lysed by freeze thawing thrice followed by sonication (3 X 30 s, full burst, with 1min interval) on ice. The cell lysate was kept in a boiling water bath for 3 min with constant shaking. The heat-treated lysate was quickly chilled in an ice ethanol bath for 5 min. This was done to coagulate and later precipitate the <i>E. coli</i> cellular proteins; EhCaBP1, being heat stable, will not get affected. Heat precipitated materials in the lysate were spun down at 12000 rpm for 30 min at 4°C and the supernatant i.e. the heat stable fraction was loaded on to a packed DE-52 column, pre-equilibrated with Buffer A. The column was washed with Buffer A and the bound protein was eluted with Buffer B (50 mM Tris-Cl pH 7.5 and 10 mM CaCl<sub>2</sub>). The eluants containing the pure protein were pooled and dialyzed against 100 volumes of autoclaved Milli Q at 4°C with minimum two changes.

### 2.16.2. EhCaBP1 mutants purification
EhCaBP1 mutants prepared in the present study are EF I, EF II, EF III, EF IV, CaBP1ΔEF, Nter and Cter. In general, all the mutants were purified as described above for the WT EhCaBP1. Few changes were made for Cter and Nter. In case of Cter, CaCl<sub>2</sub> composition in Buffer B was changed from 10 mM CaCl<sub>2</sub> to 5 mM CaCl<sub>2</sub>. For the growth of recombinant Nter protein, Terrific Broth was used instead of Luria Broth due to very less induction in the latter and the clone was transformed in C41 strain of <i>E. coli</i> cells instead of BL 21 (DE3). Briefly, 2% of the primary culture (overnight grown culture) was used as an inoculum for the secondary culture. The culture was induced with 1mM IPTG for 5-6 h after it attains an O.D of 1 (normally takes 3-4 h) at 37°C. The purification was further followed as done for WT EhCaBP1. The purified protein was finally dialyzed against MilliQ and concentrated using Amicon with a cut off of 3 kDa.
2.16.3. EhDLP and EhDLP K38A purification

EhDLP and EhDLP K38A cloned in the pET-30(a) vector were purified using Ni²⁺-NTA affinity chromatography as the recombinant proteins contains a His Tag at the amino terminus. 1 % of inoculum from primary culture was given to the secondary culture (containing 30 µg/ml kanamycin) and grown at 37°C/ 225 rpm for 3 h. The culture was then induced with IPTG (0.1 mM for EhDLP and 0.4 mM for EhDLP K38A) for 3 h at 37°C/ 225 rpm. The induced bacterial cells were harvested at 6000 rpm for 5 min at 4°C. The pellet was then resuspended in lysis buffer (1X PBS + 20 mM Imidazole) containing 200 µg/ml lysozyme, 1 mM DTT and protease inhibitor cocktail. Fifty µl of lysis buffer was added per ml of the cell culture suspension and incubated on ice for 30 min followed by sonication (6 X 30 s pulse with a one min gap). It was centrifuged at 12000 rpm for 20 min at 4°C. Both the WT and mutant EhDLP were found to be associated with the pellet.

In order to purify active EhDLP, the pellet was solubilized in 0.2 % sarkosyl solution [0.2 % N-lauryl sarcosine solution containing 25 mM Triethanolamine, 20 mM Imidazole and 1 mM EDTA (pH-8.0)] supplemented with 0.1% each of 0.1 M CaCl₂ and Triton X-100 for 1 h at 4°C with shaking followed by centrifugation at 15000 rpm for 20 min. The supernatant containing the desired protein was used for protein purification using Ni²⁺-NTA affinity chromatography.

The solubilized protein (EhDLP or the mutant) was loaded onto Ni²⁺-NTA beads (pre-equilibrated in 1X PBS and 20 mM Imidazole). The flow through was passed through the packed column thrice to allow maximum binding and the binding was done under a gravity flow. The column was further washed with 50 volumes of Buffer W (1X PBS and 50 mM Imidazole). Finally bound EhDLP was eluted with Buffer E (1X PBS + 200 mM Imidazole + 1 mM DTT) and the mutant with Buffer E' (1X PBS + 100 mM Imidazole + 1 mM DTT) respectively. The fractions were analyzed on 10% SDS-PAGE. Fractions containing purified fusion protein were pooled and dialyzed against HCB 300 (20 mM Hepes pH 7.2, 2 mM EGTA, 1 mM MgCl₂, 1mM DTT, and 300 mM NaCl) containing 10% glycerol. The protein samples obtained were further concentrated with Amicon concentrator (30,000 MW cut-off) and stored at -80°C.
2.17. BIOCHEMICAL STUDIES WITH EhCaBP1 AND MUTANTS

2.17.1. Ca$^{2+}$ BINDING STUDIES

2.17.1.1. Overlay assay with $^{45}$Calcium
The ability of WT or mutant EhCaBP1 to bind Ca$^{2+}$ was tested by radioactive Ca$^{2+}$ overlay assay. Briefly, 2 μg of purified protein was run on a SDS-PAGE and blotted to a PVDF or NC membrane. The blot was first washed with 10 mM Imidazole and 2 mM EGTA for 10 min, followed by two washes with chelex treated Milli Q each for 5 min. It was further incubated in Buffer D (10 mM Imidazole pH 6.8, 60 mM KCl, 5 mM MgCl$_2$) for 15-20 min at RT and then 1 μCi $[^{45}Ca]$ was added to 15 ml of Buffer D and the incubation was continued for another 1 h with constant slow shaking. The blot was then given a brief and gentle wash with chelex treated Milli Q for 2 min, followed by wash with 50 % ethanol for 30 s. The blot was finally air dried and exposed for autoradiography.

2.17.1.2. Circular dichroism spectroscopy
CD measurements were performed using a Jasco-815 spectropolarimeter. Each spectrum was measured in the far-UV region (200-260 nm) and was an average of 5 scans. Scans were done at a protein concentration of 33 μM in the buffer containing 50 mM Tris.Cl, pH 7.0 and 100 mM NaCl using a cuvette of path length 1.0 cm in presence of 5 mM CaCl$_2$. Percentage helical content was calculated using the method described by Barrow et al. (Barrow et al., 1992).

2.17.2. Dynamic Light Scattering
DLS experiments were carried out using a Spectroscatter 201 (RiNA NETZWERK RNA-Technologen, Berlin) instrument. All solutions were filtered through 0.2 μm millipore filters. Duplicate runs were made taking 30 scans at 30 s each at RT and analyzed by the CONTIN software. To get proper signal and to deconvolute the data, 5 mg/ml EhCaBP1 and CaBP1ΔEF proteins were used to determine the size and molecular weight of the protein in different solvent conditions. The size of the EhCaBP1 was measured in Tris buffer (pH 7.5), 1 mM CaCl$_2$ or 2 mM EGTA with 150 mM NaCl. Under these conditions EhCaBP1 protein was shown earlier to exist as a trimer (Kumar et al., 2007).
2.17.3. In vitro kinase assay
Total *Entamoeba* cell extract was prepared and the activity of EhCaBP1-dependent kinases was estimated as described previously (Chakrabarty et al., 2004). The reaction mixture contained 30 mM HEPES, pH 7.5, 5 mM MgCl$_2$, 15 µg Histone (Type IIIS, Sigma), 25 µg *E. histolytica* cell extract, protease inhibitor cocktail, 10 µM CaCl$_2$ or 500 µM EGTA. Varying amount of either EhCaBP1 or mutant was also added. The reaction volume was adjusted to 40 µl. The reaction was initiated by adding 100µM (γ-$^{32}$P) rATP (specific activity 5000 Ci/mmol, BRIT) and allowed to proceed at 30°C for 30 min. The reaction was terminated by adding 1X-SDS PAGE buffer and resolved on a 12% SDS PAGE. The gels were dried and exposed to an X-ray film or an imaging plate and densitometry was done.

Alternately, the reactions were by adding 10 % TCA and total protein precipitation carried out at 4°C for 45 min. The reaction mixture was spotted onto a GF/C paper and washed with 5 % TCA (10 ml) followed by wash with ethanol (5 ml). The filter was then air dried and counts were taken in Cocktail O.

2.18. BIOCHEMICAL STUDIES WITH EhDLP

2.18.1. GTP BINDING

2.18.1.1. UV-Crosslinking
To perform the UV cross-linking assay, 2 µg of purified EhDLP or mutant in buffer containing 20 mM HEPES pH 7.0, 2 mM MgCl$_2$, 1 mM dithiothreitol, 10 µCi [$\alpha$-$^{32}$P] GTP, 150 mM NaCl and 10 % glycerol was incubated on ice for 10 min followed by exposure to UV light (254 nm) irradiation at a distance of 5 cm (Stratagene) for 30 min at 4°C. After termination of UV exposure, 0.8 µl of 100 mM dGTP and 20 µg of BSA were added to reaction mixtures. Proteins were precipitated by incubating in 10 % TCA for 45 min at 4°C and washed with acetone. These were separated on SDS–PAGE and the labelled protein was visualized by a PhosphorImager.

2.18.1.2. Filter Binding Assay
EhDLP or the mutant (2 µg) was incubated in buffer containing 20 mM HEPES pH 7.0, 2 mM MgCl$_2$, 1 mM dithiothreitol, 150 mM NaCl and 1 µCi [$\alpha$-$^{32}$P] GTP for 20 min at 4°C. Using a multichannel pipette, samples were applied to a nitrocellulose
membrane (0.45 μm) in a filter dot blot apparatus under vacuum. The filter was rapidly washed once with 250 μl of cold buffer. The dried filter was imaged using a PhosphorImager and quantified using MultiImage Software.

2.18.2. GTPase assay
GTP hydrolysis by EhDLP and the mutant was monitored as the hydrolysis of \([\gamma^{32}P]\)GTP by the purified proteins. One μg of purified proteins were incubated in 1 X GTPase buffer (20 mM HEPES pH 7.4, 2 mM MgCl₂, 150 mM NaCl, 1 mM DTT) containing 1.0 μCi of \([\gamma^{32}P]\)GTP in a 20 μl of reaction volume for 15 min at RT. The reaction was terminated by addition of 1X SDS sample buffer and the reactants were resolved by PEI-TLC using 0.75 M KH₂PO₄ (pH 3.75). The TLC plates were then exposed to a PhosphorImager or an X-Ray film. No protein control was also taken to monitor the hydrolysis of GTP.

2.18.3. Velocity sedimentation
Three μg of purified protein dialyzed against high salt concentration buffer HCB 300 was used per 100 μl total reaction. The final salt concentration was then varied to either 150 or 50 mM NaCl. To achieve the final required salt concentrations, HCB 300 was diluted with HCB (containing no NaCl). 3-5 μg EhDLP or the mutant was added to buffers with different salt concentration in the ultracentrifuge tubes. These tubes were then incubated for 10 min at 20°C and then centrifuged at 100,000 x g for 10 min. The supernatant containing unassembled protein was then carefully collected and transferred to an eppendorf. The ultracentrifuge tubes with the pellet containing the assembled protein oligomers were resuspended in 1 X SDS-PAGE dye. The supernatant was TCA precipitated and resuspended in 1 X SDS-PAGE dye.

2.19. MISCELLANEOUS PROTEIN RELATED TECHNIQUES
2.19.1. Protein Estimation (BCA assay)
The amount of protein in a sample was estimated by the bicinchoninic acid (BCA) assay using BSA as the standard. The working solution was prepared by mixing BCA (Sigma) and 4% copper sulphate in a ratio of 50:1. Equal volumes of the sample and the working solution were mixed in a microtitre plate and incubated at 37°C till a
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purple color develops in the lowest concentration of BSA. The absorbance was taken at 560 nm using a microtiter plate reader (Bio-Rad, USA).

2.19.2. SDS-Polyacrylamide gel electrophoresis
SDS-PAGE was carried out under reducing conditions. The separating gel was prepared using acrylamide (acrylamide:bis-acrylamide = 29:1) in 1.5% Tris-Cl pH 8.8, 0.1% (w/v) SDS, 0.04% (w/v) APS and TEMED. After polymerization of separating gel, stacking gel was poured. The stacking gel contained 4% acrylamide in 0.5% Tris-Cl pH 6.8, 0.1% (w/v) SDS, 0.04% (w/v) APS and TEMED. Samples were mixed with 4 X SDS-PAGE loading dye to a final dye concentration of 1 X. After electrophoresis, proteins were fixed in the gel by incubating in fixing solution (50% methanol, 7.5% acetic acid) and detected by Coomassie Brilliant Blue (0.25% CBB R-250 in fixing solution). The gels were destained in the fixing solution and dried.

2.19.3. 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 Whatman 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 and then soak in towbain buffer. Two sheets of Whatman 3MM paper were placed above the membrane. The transfer was set at constant mAmps, 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 every incubation with antibody. The second antibody used was horse radish peroxidase conjugated IgG. Band detection was done using ECL kit (Amersham). Antibody dilutions used: 1:3000, EhCaBP1 (polyclonal, rabbit); 1:2000, EhCaBP2 (polyclonal, rabbit); 1:1000, EhDLP (polyclonal, rabbit); 1:1000, EhActin (polyclonal, rabbit); 1:2000, GFP (Molecular...
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Probes); 1:500, Myosin II; 1:5000, Anti-His; 1:10000, Anti-Rabbit-HRPO, 1: 10000, Anti-Mouse-HRPO (Amersham).

2.19.4. Enzyme linked Immunoabsorbent Assay (ELISA)

2-5 μg of the Entamoeba total lysate in PBS was used to coat wells of a microtiter plate (Costar, USA) overnight at 4°C. Nonspecific sites were blocked with 3% (w/v) BSA in PBS for 1 h at RT. Primary antibody incubation was carried out for 1 h at RT in 1% BSA/PBS-T. Secondary antibody incubation was done for 30 min at RT with HRPO linked IgG. The wells were washed thoroughly with PBS-T after every incubation. Bound antibodies were detected using the colorimetric substrate TMB (Amersham) and the reaction was stopped with 2 N H2SO4. The final yellow color was measured by monitoring the absorbance at 405 nm with a microtiter plate reader (Bio-Rad, USA).

2.20. CELL BIOLOGICAL TECHNIQUES

2.20.1. Immunostaining

E. histolytica resuspended in warm incomplete TYI-S-33 was transferred to prewarmed, acetone-cleaned coverslips placed in a petri-dish and allowed to adhere for 10 min at 37°C. The culture medium was removed and coverslips were fixed with 3.7% prewarmed paraformaldehyde (PFA) for 30 min. After fixation, the cells were permeabilized with 0.1% Triton PBS for 1 min. Cells were then washed with PBS and quenched for 30 min in PBS containing 50 mM NH4Cl. The cover-slips were blocked with 1% BSA/PBS for 30 min, followed by primary antibody incubation at 37°C for 1h. The coverslips were washed with PBS followed by 1% BSA/PBS before secondary antibody incubation of 30 min at 37°C. Antibody dilutions used were: 1:50, EhCaBP1; 1:500, Phalloidin (Sigma, 1 mg/ml in methanol); 1:50, EhDLP; 1:50, EhCaBP2; 1:200, Anti-GFP; 1:200, Anti-Rabbit Alexa 488; 1:300, Anti-Rabbit Alexa Cy3. To stain DNA, a DNA specific dye Hoescht 33342 was used. The fixed cells were incubated with 200 μg/ml Hoescht 33342 for 10 min at RT. The cells were then washed three times with PBS-T. 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. Sealing of the cover-slip edges was done with nail-paint to avoid drying.
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2.20.2. Confocal Microscopy
Fluorescent samples were examined on LSM 510 confocal laser scanning microscope (CSLM) (Zeiss, Germany) equipped with a 63X objective. Rhodamine-labeled samples were visualized after excitation at 543 nm using He/Ne Laser and Alexa-green labeled samples after excitation at 488 nm using Argon Laser. Focal planes of 0.8 μm sections with a shift of objective by 1 μm, captured for 20-30 planes from the bottom to the top of each cell. Pictures were processed using offline version of LSM 510 software, Zeiss.

2.20.3. Time Lapse Imaging
The cells expressing GFP-CaBP1, GFP-CaBP1ΔEF or GFP alone were plated onto a 35-mm Mat Tek glass bottom culture dish (Mat Tek Corporation) at 37°C. After the cells got settled at the bottom, the medium was removed and the glass chamber was filled with pre-warmed PBS. The dish was kept on a platform with a temperature controller (Tempcontrol 37-2 digital, Zeiss) to maintain temperature at 37°C. High resolution fluorescent time lapse imaging of a moving and phagocytosing amoeba was performed using a high speed spinning disk confocal system (UltraView RS, Perkin Elmer) equipped for axial z-stack sampling throughout the cell volume. This system was attached to an inverted 200M microscope (Zeiss). The images were captured with an Orca D-ER detector (Hamamatsu) and a 40 X objective and 2 X 2 binning. Images of 1.5 μm (along z-axis) at 3 or 4 s interval (as indicated) were captured. This z-spacing was optimized to: 1) monitor the entire depth of amoeba from top to bottom, and 2) accomplish fast capturing of a moving amoeba. The raw images were processed using ImageJ software available freely on the web (http://rsb.info.nih.gov/ij/). For each time point, raw images were three-dimensionally reconstituted before further analysis.

2.20.4. Image Analysis
Raw images obtained from the microscope were processed and analyzed using Image J software (Plugins/PE_raw/Convert files to tiff series) and the three dimensional images were reconstructed (Images/Stacks/Z project) by taking standard deviation of the selected slices and projected as a time series. For quantitative measurements of the
pseudopods, the time point just before the pseudopod protrusion was referred to as t =0. The measurement of gray scale intensity of the images was done as described elsewhere (Aizawa et al., 1997).

2.21. ISOLATION OF ERYTHROCYTE MEMBRANE
Total human blood (stored in heparin) was washed thrice in 10 volumes of PBS at 600 x g for 10 min at 4°C. It is recommended to triturate (repeatedly pipetting up and down keeping the tip immersed) the suspension. The pellet containing only RBC was resuspended in 30 volumes of lysis solution (5mM sodium phosphate buffer pH 8.0, 1mM EDTA and 0.3 mM PMSF) and mixed rapidly and centrifuged at 12000x g for 10 min at 4°C. The same step in repeated until a white colored pellet is obtained. The pellet was finally resuspended in sodium phosphate buffer. To get a protein rich membrane fraction, protocol described by Wessel et al., was followed (Wessel et al., 1984). Briefly, the membrane (isolated above) was mixed with 4 volumes of methanol and centrifuged at 9000 x g for 10 s. One volume of chloroform was then added, mixed and centrifuged at 9000 x g for 10 s. To the above mixture 3 volumes of water was added and mixed vigorously and again centrifuged at 9000 x g for 1 min. The upper phase was removed and 3 volumes of methanol was added to the lower phase, mixed and centrifuged at 9000 x g for 2 min to get the protein rich membrane in the pellet. The protein pellet was resuspended in 100 mM phosphate buffer, pH 8.0 and used for labeling the magnetic beads.

2.22. MAGNETIC BEADS COATING WITH ERYTHROCYTE MEMBRANES
Erythrocyte membranes isolated as described above (section 2.23) were covalently linked to the surface of magnetic beads of 2.8mm on (M-280 tosylactivated, ref:142.04, Dynal) according to the instructions of the manufacturer.

2.23. PHAGOSOME PURIFICATION
Phagosome purification procedure was followed as described by Marion et al. (Marion et al., 2005). Briefly, adherent amoeba (2 X 10^8) were washed in incomplete TYI-S-33 and incubated in warm TYI-33 medium containing coated beads at the ratio 10 beads per cell. Beads were immediately sedimented on the surface of the amoeba’s confluent layer by applying a magnet for 30 s. The cells were then washed twice with
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the medium at 37°C in order to eliminate the unbound beads. Phagocytosis was initiated by removing the magnet and after 5 min chilled PBS# 8 was added to stop the reaction. Detached amoeba were harvested and washed once in cold PBS# 8 and resuspended in 1 ml of lysis buffer (250 mM sucrose, 50 mM Tris pH 7.6 and protease inhibitor cocktail). The cells were mechanically lysed by applying six strokes of a Dounce homogenizer and phagosomes were separated from the lysate by applying a permanent magnet (Dynal, ref:120.20) and the washed five times in lysis buffer containing protease inhibitors.

The purified phagosomes were lysed in Buffer L (2% Triton X-100, 10 mM DTT, 50 mM Tris pH 8 and protease inhibitors) by vortexing for 15 min at 4°C. The lysate was separated from the magnetic beads by applying a permanent magnet (Dynal) and then precipitated in acetone. The precipitated preparation was sent for LC-MS/MS analysis to the ‘Biomolecular Research Facility’ at the Virginia University [contact: Nicholas Sherman, University of Virginia, 1300 Jefferson Park Avenue Jordan Hall, Room 1101, Charlottesville, VA 22908; tel. (+1) 434 924 2356].

2.24. ENDOCYTIC STUDIES

2.24.1. Phagocytosis of red blood cells by trophozoites

To quantify the RBC ingested by amoebae, the colorimetric method of estimation with some modifications was followed (Sahoo et al., 2004). Briefly 10⁷ RBC, washed with PBS and TYI-33, were incubated with 10⁵ amoebae for 10 min or as mentioned at 37°C in 0.2 ml culture medium. The amoebae and erythrocytes were pelleted down, non-engulfed RBC were bursted with cold distill water and recentrifuged at 1000 x g for 2 min. This step was repeated twice, followed by resuspension in 1ml formic acid to burst amoebae containing engulfed RBC. Samples were measured against a formic acid blank with a spectrophotometer at 400 nm.

To quantify the number of beads (RBC membrane coated) ingested per amoeba at a given time point, adherent amoebae were incubated with these beads (ratio 1:10) for indicated time points. The uptake was stopped by addition of 3.7 % PFA and the cells were further incubated with anti-glycophorin (1:10, Caltag Antibodies) antibody coupled to FITC for 30 min at 37°C. Beads were counted under the fluorescence light microscope. Depending on the experiments, amoebae were pre-incubated with cytochalasin D (1 μM, 15 min) or wortmannin (0.1 μM, 45 min) before adding the
magnetic beads. The percentage of cells that have ingested at least one BHS after 30 min is then calculated for 150 amoebae examined per experiment.

2.24.2. Bacterial Uptake Assay
Bacterial uptake by *E. histolytica* transfectants was determined by measuring the number of bacteria ingested (cfu/ml). Briefly, 10^5 amoebae were incubated with 10^7 bacteria (*E. coli*) at 37°C for 15 min in a volume of 200 μl. The mixture was centrifuged at 300 x g for 5 min to pellet only the amoeba and not the non-engulfed bacteria. Amoebae were then washed with PBS three times and then resuspended in PBS containing 0.1 % Triton X-100. The ingested bacteria were pelleted by centrifugation and washed with PBS (thrice) to remove traces of the detergent. The number of bacteria was then determined by plating on LB-Agar plates at different dilutions.

2.24.3. RITC-dextran uptake analysis
The pinocytosis of *E. histolytica* was studied by observing the uptake of RITC-dextran as described before (Sahoo *et al.*, 2004). The mid-log phase cells were harvested, washed and resuspended in fresh medium. The washed cells were then incubated with RITC-dextran (2mg/ml, SIGMA) for 30 min at 36°C followed by harvesting and washing with PBS. The cells were then resuspended in PBS containing 0.1 % Triton X-100. The amount of pinocytosed particles was determined by measurement of total fluorescence using a Cary Fluorescence Spectrophotometer.

2.24.4. Cytopathic Assay
The destruction of monolayer of CHO cells was assayed as described by Bracha and Mirelman (Bracha *et al.*, 1984). Briefly, trophozoites (10^5/ml suspended in DMEM without FCS) were added in triplicate to wells containing a confluent monolayer of CHO cells (10^5/ml) pre-washed with DMEM to remove traces of fetal calf serum and incubated for 60 min at 37°C in an atmosphere of 95 % air and 5 % CO₂. The reaction was stopped by chilling for 10 min and the wells were then washed thrice with cold PBS. The monolayer was fixed with 4 % PFA for 10 min and stained with methylene blue (0.1 % in borate buffer, 0.1 M pH 8.7). The excess stain was washed with 0.01 M borate buffer and the incorporated dye was extracted by adding 1.0 ml of 0.1 M HCl
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at 37°C for 30 min. The color was read in a spectrophotometer at 660 nm after appropriate dilutions with 0.1 M HCl. Destruction of cells was expressed in relation to the amount of dye extracted from the control monolayer of CHO cells.

2.25. INTRACELLULAR Ca$^{2+}$ MEASUREMENTS

Trophozoites (10$^6$) were washed twice with PBS # 8 and loaded with 20 μM FURA 2-AM (Sigma Chemicals) at RT in the dark for 1h in buffer A (20 mM Hepes, pH 7.2, 140 mM NaCl) supplemented with 0.1% BSA. Loaded cells were washed twice with PBS # 8 and resuspended in buffer A. Cells were transferred to a thermostated fluorometric cuvette containing a magnetic stir bar and maintained at 37°C with gentle agitation. BAPTA-AM was added directly to the cuvette. Fluorescence values were registered in a Cary Fluorescence Spectrophotometer, programmed to obtain the emission at 510 nm on excitation at 340 and 380 nm simultaneously. $[\text{Ca}^{2+}]_i$ is represented as the 340 to 380 nm ratio as these are considered proportional to each other (Gryniewicz et al., 1985).

2.26. PRODUCTION OF POLyclONAL PURIFIED ANTIBODY

The purified antigen (rEhDLP or rEhCaBP2) was dialyzed against PBS. One rabbit was immunized subcutaneously at multiple locations four times with 100 μg of protein per injection with an interval of 3 weeks between each injection. The first dose of the protein was emulsified with complete Freund’s adjuvant while the following doses were emulsified with incomplete Freund’s adjuvant. Following the inoculation series, the rabbit was bled to death and the serum was stored in aliquots at -80°C.

To purify EhCaBP2 specific immunoglobulins, the antisera was purified using an EhCaBP1 affinity column. rEhCaBP1 was coupled to CNBr activated sepharose as per manufacturer’s recommendations. EhCaBP2 antisera was loaded onto the column and the flow through was collected. The column was washed with Tris/CaCl$_2$ buffer (20 mM Tris-Cl pH 7.5 and 0.1 mM CaCl$_2$). The fractions were eluted with glycine pH 3.0 and immediately diluted with Tris-Cl pH 9.0. The flow through collected contained EhCaBP2 specific immunoglobulins and used for immunolocalization. To check the titer of the polyclonal antibody, an ELISA followed by western analysis of the E. histolytica lysate was performed.
2.27. BIOINFORMATICS ANALYSIS

All the sequences were retrieved from the *E. histolytica* genome database at The Institute of Genome Research (http://www.tigr.org/tdb/e2k1/eha1) and Pathema (http://pathema.jcvi.org/cgi-bin/Entamoeba/PathemaHomePage.cgi). The identified genes from *E. histolytica* genome database were then further analyzed and confirmed by BLAST. CD search at NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and Pfam (http://www.pfam.wustl.edu/hmmsearch.shtml) were used to identify the domains in EhDLP.

Amino acid composition determination, restriction enzyme sites analysis for a DNA sequence and multiple alignments (CLUSTAL W) were performed using BioEdit sequence alignment editor (Tom Hall, version 7.0).

Secondary structure analysis of the protein sequence was carried out using PSIPRED software available freely on web (http://bioinf.cs.ucl.ac.uk/psipred/).

Phylogenetic analysis of the extracted sequences from different organisms along with *E. histolytica* sequence was done using PHYLIP 3.67 package. The analysis was done with a bootstrap value of 100. UPGMA method was used to draw the phylogenetic tree.