SUMMARY
Entamoeba histolytica is an enteric protozoan parasite and is the etiologic agent of amoebiasis. This is the third leading cause of parasitic death in the world. It can occasionally invade extra-intestinal tissues leading to pathogenesis associated with intestinal lesions and liver abscesses. Calcium plays an important role in the pathogenesis of amoebiasis by modulating the cytopathic properties of the parasite. In several protozoan parasites, Ca\(^{2+}\) is documented to play a significant role in pathogenesis. However, the role of Ca\(^{2+}\) in the pathogenesis of E. histolytica is still poorly understood and need to be studied at a molecular level.

The gene encoding a novel Ca\(^{2+}\)-binding protein (CaBP1) from E. histolytica was cloned and characterized in our laboratory. The protein was overexpressed in E. coli and functionally characterized. Though it shares some of its biophysical properties such as small size, negative charge, heat stability and number of Ca\(^{2+}\)-binding EF hand domains with calmodulins (CaM), it is functionally different from CaM.

The major objective of this study is to further study the function of CaBP1 and related proteins. With this aim, we have cloned and partially characterized a paralog of CaBP1 named CaBP2. To delineate the molecular mechanisms of interaction of CaBP1 and CaBP2 with different targets, we have isolated peptides from a random phage display library. In this study, we describe the interaction of these peptides with CaBP1 and CaBP2 at biochemical and functional levels.

1. CaBP1 and CaBP2 are paralogous proteins:
Comparison of CaBP1 (initially named EhCaBP) and CaBP2 show 82% identity to each other, however, there is only 40% identity between the two proteins in the central linker region at the nucleotide level. The central linker region of CaM and CaM-related proteins play a pivotal role in the binding and activation of target proteins. Differences in the central linker regions of the two proteins suggests that these may be involved in unique and independent Ca\(^{2+}\) signal transduction
pathways. The CaBP2 gene is present in other strains and species of *Entamoeba* implying that CaBP2 indeed is responsible for initiating a parasite-specific novel signal transduction pathway. The CaBP2 sequence has been compared with other EF hand proteins as well as calmodulin sequences and the results show that both the CaBPs are not closely related to any of the CaM-like calcium-binding proteins. An inspection of the nucleotide and amino acid sequences of CaBP1 and CaBP2 revealed the following details. Among the 74 nucleotide differences between CaBP2 and CaBP1, 26 are in the third codon positions and do not cause any change in the amino acid coded. EF hand Domain 4 of is totally devoid of any substitutions while Domains 1, 2 and 3 have a total of 6 amino acid substitutions. The major difference is in the central linker of CaBP2 which has 10 amino acid substitutions out of a total of 29 amino acid residues. Thus there is a non-random distribution of amino acid substitutions in the CaBPs. It is possible that gross changes in the central linker region have resulted in a contextual change that can account for the differential Ca$^{2+}$ binding of the two proteins. Table 4 lists the differences between CaBP1 and CaBP2. From preliminary experiments, it may be inferred that CaBP2 is a single copy gene. Presence of CaBP2 transcript has been demonstrated, thus it can be ruled out that CaBP2 is not a pseudogene.

2. **CaBP1 and CaBP2 are functionally distinct:**

The fact that CaBP2 binds calcium and undergoes conformational changes has been demonstrated by SDS PAGE gel mobility shift and CD/ORD spectroscopic analysis. Calcium-dependent affinity chromatography has demonstrated that CaBP2 interacts with several intracellular proteins of molecular weight 21.5, 24, 30, 35, 42, 50, 60 and three proteins in the range of 70-90 kDa in a calcium-dependent manner. On the other hand, CaBP1 interacts with intracellular proteins of molecular weights 12, 20, 30, 33, 51 and 67kDa (Yadava et al, 1997). Thus, CaBP2 may perform some unique physiological functions. Some of the
target proteins may be common but the level of the activation may differ for each protein. It is also possible that CaBP2 and CaBP1 dependent signal transduction pathways may be unique but may merge at some point.

3. CaBP2 can activate protein kinase(s):
Protein Kinases present in *E. histolytica* extracts were assayed using *E. histolytica* total cell lysate as the source of enzyme. We demonstrate here that CaBP2 regulates a protein kinase activity at 100μM added Ca$^{2+}$ concentration. Interestingly, the protein kinase activated by CaBP1 and CaBP2 seems to phosphorylate a common substrate of 50kDa though calcium requirement of the two proteins are different. In the absence of actual determination of binding parameters, it can be nevertheless speculated that CaBP1 may bind Ca$^{2+}$ with greater affinity and can function under small fluctuations of [Ca]$_i$. On the other hand, CaBP2 may activate a similar cascade at a higher [Ca]$_i$. It is possible that CaBP1 and CaBP2 may supplement each other in the intracellular environment and even be involved in crosstalk. It can be hypothesized that CaBP1 and CaBP2 have functional overlaps; however, their Ca$^{2+}$ maxima being distinctly different, it is possible that these two proteins act as agonists at different intracellular Ca$^{2+}$ concentrations.

4. Isolation of phage clones binding to CaBP1 and CaBP2:
In order to delineate the functions of CaBP1 and CaBP2, it is important to identify the various target proteins to which this molecule can bind to trigger Ca$^{2+}$-dependent signal transduction. Of the many possible approaches one can take to obtain this information, we have used the random peptide display library to select out possible peptide sequences that can bind to CaBP1. Random peptide libraries have been used in a number of applications including identification of
peptide mimics of non-peptide ligands. In order to select CaBP1-binding peptides we used column affinity chromatography to enrich peptides in a Ca\(^{2+}\)-dependent manner. Phages, which can bind to those structural motifs of CaBP1 exposed to the solvent after saturation with Ca\(^{2+}\) (i.e., in vivo functional domains), have been enriched by this study. Twenty-eight phages were sequenced and five of the sequences were identical. Though the sequences of these peptides lacked an obvious motif, certain features like the position of Glutamine and Proline in the peptide and motifs containing tryptophan (SW and WP) were repeated at least six times. Interestingly, CaM affinity chromatography also isolated peptides with WP motifs. Some of the proteins identified in a database search using the peptide sequences are YAK1 which is a Ser/Thr kinase and the *Entamoeba* diaphanous protein. One of the peptides showed homology to PI-3 kinase which has been shown to bind and activate CaM.

5. Functional analysis of the interaction between peptides and CaBP1 and CaBP2:

All the selected phage peptides were examined for their binding to CaBP2. It was found that some of the peptides bind CaBP2 better than CaBP1. The properties of these peptides may reflect their abilities to interact with different subdomains of CaBPs. Two peptides, C61 and C112, were studied in details. In phage ELISA experiments and binding studies by Surface Plasmon Resonance, C61 was found to bind equally well to CaBP1 and CaBP2 whereas C112 was shown to bind to CaBP2 specifically. Interaction of the peptides with CaBP1 and CaBP2 was also studied using CD/ORD spectroscopy. The data confirmed the specificity of binding. Moreover, using a direct binding assay, it was demonstrated that C61 could inhibit the binding of CaBP1- and CaBP2-target proteins to the respective protein. C112 could only specifically inhibit the binding of target proteins to CaBP2. This raises interesting possibilities of homing peptides which would preferentially inhibit the cellular functions of one of a paralogous group of
proteins. The CaBPs, like CaM, activate target proteins after binding. One of the target proteins is a protein kinase. Binding of the peptides led to the inhibition of protein kinase activation. Both C61 and C112 could inhibit the kinase activation. Therefore, we conclude that peptide binding leads to the inactivation of at least some of the CaBP1 and CaBP2 functions.

It has been demonstrated that both CaBP1 and CaBP2 can interact with a variety of cellular proteins, in other words, it can bind to different target sites in the Ca$^{2+}$-bound conformation. An intriguing question is that how after binding calcium can it display such versatility. The determination of the CaBP-peptide complexes would greatly aid to answer this question. This study can potentially address the following issues that will help us in the molecular characterization of CaBP1 and CaBP2:

i. the isolated CaBP-binding sequences may serve as good references for locating CaBP-binding proteins in databases or for mapping the CaBP-binding domains in the target proteins.

ii. these may also serve as useful tools for studying CaBP-isoform specific functions in Entamoeba. For example, C112 is highly specific for CaBP2 and not for CaBP1; therefore, it can be used to probe CaBP2 functionality in vivo.