5. Summary
Entamoeba histolytica, a protozoan parasite, is the causative agent of human amebiasis. Calcium is thought to play a major role in this process. Calcium signals are normally transduced through calcium binding proteins such as calmodulin in almost all eukaryotic cells. We have identified and characterized a calcium binding protein EhCaBP1 from E. histolytica which is different from calmodulin in its biochemical properties (Prasad J et al., 1993).

In this study, we have used regulatable antisense RNA expression strategy to manipulate gene expression of EhCaBP1 and studied the phenotype of a cell line that has reduced EhCaBP1 expression. The overexpression of EhCaBP1 in E. histolytica could also be achieved by conditional expression.

The Ph.D dissertation work is summarized below:

Significant inhibition of expression of EhCaBP1 was found, which is comparable to that obtained with constitutive synthesis of antisense RNA for amoebapore and Gal/GalNac lectin genes or by antisense oligos based on protein nucleic acids (Bracha R et al., 1999, Ankri S et al., 1999 and Stock RP et al., 2001). However, no significant difference was found in the basic functions such as protein and DNA synthesis in the lines expressing antisense RNA as compared to the control cells. Expression of EhCaBP2, a functionally divergent paralogue of EhCaBP1, was also not affected by the gene manipulation of EhCaBP1, suggesting the system to be tightly regulated for gene expression.

Both overexpressing and antisense blocked cells did not show any obvious phenotypic changes in absence tetracycline. However, in presence of tetracycline the EhCaBP1-AS cells were found to be defective in proliferation. Also, the antisense-blocked cells were less motile and lacked directional movement. These impaired functions are indicative of inefficient cytoskeleton machinery.

In addition, the phagocytosis ability of antisense-blocked cells was severely reduced. The cell lines overexpressing EhCaBP1 did not show this defect. Phagocytosis is one of the most important physiological properties of E. histolytica and is thought to be associated with pathogenesis (Orozco E et al., 1983). EhCaBP1 may regulate endocytic transport by modulating an essential component(s) of the membrane traffic machinery.
Pathogenesis studies using *in vitro* assays suggested that these cells have normal cytopathic properties. Parasite adhesion to enterocytes as well as cytotoxic effect was unaffected in antisense cell lines.

Differential gene expression was studied in these expression-blocked cells using mRNA display technique. The results showed that profilin 40S ribosomal protein S26E and rpl29 genes were down regulated. All the genes identified through mRNA display appear to be involved in cellular proliferation directly or indirectly and could in principle explain inhibition of cellular proliferation in absence of EhCaBP1.

Confocal microscopy studies showed the colocalization of EhCaBP1 with actin in pseudopods and phagocytic cups suggesting their involvement in these functions probably by interacting with actin filaments. On incubation of amoebic cells with RBC, phagocytic cups can be visualized prior to engulfment of the red blood cells, which could be due to its early role in changes during RBC uptake. EhCaBP1 was also seen to colocalize with myosin1b and PAK on pseudopods. It is difficult to speculate on the detailed mechanism from these observations, however, it is clear that EhCaBP1 in association with actin and myosin1b may be involved in both motility and endocytosis. Interaction of EhCaBP1 with the cytoskeletal fraction was also demonstrated using cell fractionation and immunoprecipitation. Binding of EhCaBP1 to actin filaments has also been shown by co-sedimentation with actin. The binding of EhCaBP1 to actin was found to be highly specific as there was no binding when a mutated protein and an isoform EhCaBP2 were used in the assay. This interaction is calcium dependent and involves the central-linker of the protein.

The binding of EhCaBP1 to actin was also visualized by atomic force microscopy using an indirect gold-labeled immunological staining. The actin strands could be getting affected by this interaction. With further improvements in the preparation and/or imaging conditions, we should next be able to investigate the dynamic behavior of actin polymers and their molecular interaction with EhCaBP1.

This study suggests that EhCaBP1 might be a novel addition to the already known list of calcium binding proteins involved in cytoskeleton related functions. Further studies on the co-relation between the already characterized factors will help us in understanding the patho-physiology of the organism.