INTRODUCTION
I. INTRODUCTION

*Entamoeba histolytica*, a protozoan parasite, is the causative agent of amoebiasis. It is classified as

- **Family**: Entamoebidae
- **Order**: Amoebida
- **Class**: Lobosea
- **Subphylum**: Sarcodina
- **Phylum**: Sarcomastigophora

It is dispersed widely and is more prevalent in the tropical and subtropical regions of the globe. Reported incidences of *E. histolytica* infection range from less than 1% to greater than 50%. The lower incidences occur in the developed countries located in temperate zone while the developing countries situated in warmer climates show higher endemicity. The global prevalence rate is shown in Table I. The best estimates suggest that about 500 million people are infected with *E. histolytica*, the majority residing in Asia, of which 40 million develop disabling colitis or extraintestinal abscesses leading to 40,000 deaths per year (Walsh, 1985).

There are six parasitic amoebae of the genus *Entamoeba* that are known to infect humans (Clark and Diamond, 1991). *Entamoeba coli*, *Entamoeba moshkovskii* and *Entamoeba hartmanni* have not been found to be pathogenic to humans but *Entamoeba polecki*, *Entamoeba gingivalis* and *E. histolytica* are pathogenic.

I.1 Life cycle of *E. histolytica*

The life cycle of *E. histolytica* was extensively studied by Dobell (1928). There are two stages in the life cycle: the cyst, which is a host-infective stage and the trophozoite, a vegetative host tissue-invasive stage. Following ingestion the quadrinucleate cyst can lodge in the crypts of the intestine where, if it finds a suitable microenvironment it excysts as a trophozoite. From a single cyst containing four nuclei, eight trophozoites emerge after excystment. The trophozoites are capable of invading the host tissues causing the symptoms of amoebiasis. However, only in a fraction of patients, the amoeba is invasive. Most of the time, the trophozoites undergo encystment to produce quadrinucleate cysts which are passed through the faeces to infect the next host. The factors or mechanisms that govern interconversion between the two stages are not known. Thus, in *E. histolytica*, a second host
Table I: Global prevalence and Incidence of Amoebiasis

<table>
<thead>
<tr>
<th>Continent</th>
<th>Infection</th>
<th>Disease (Abscess and colitis)</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>North and South America</td>
<td>95</td>
<td>10</td>
<td>0.01-0.03</td>
</tr>
<tr>
<td>Asia</td>
<td>300</td>
<td>0.02-30</td>
<td>0.02-0.05</td>
</tr>
<tr>
<td>Africa</td>
<td>85</td>
<td>10</td>
<td>0.01-0.03</td>
</tr>
<tr>
<td>Europe</td>
<td>20</td>
<td>0.1</td>
<td>*</td>
</tr>
<tr>
<td>Total</td>
<td>500</td>
<td>0.04-50.1</td>
<td>0.04-0.11</td>
</tr>
</tbody>
</table>

All Figures are given in millions. Adapted from Walsh (1986)
is not required to complete the life cycle and a vector is not needed for
transmission.

I.2 Morphology and Cellular organization of *E. histolytica*

The plasma membrane of *E. histolytica* is similar to that of other euca-
ryotes though it is somewhat thicker. The amoebal surface is enveloped by a
thick coat called glycocalyx which is made up of charged complex carbohydrates
since concanavalin A and ruthenium red were demonstrated to bind to the glyco-
calyx (McLaughlin and Aley, 1985). Recent studies suggest that the glycocalyx
layer may be mainly due to the presence of lipophosphoglycan (LPG) on the
surface (Stanley et al, 1992). The LPG of *E. histolytica* is structurally
similar but not identical to the molecule present on the promastigote of
*Leishmania* (Bhattacharya et al, 1992b). Apart from LPG, the plasma membrane
has about 12-18 distinct proteins (Aley et al, 1980). A number of these pro-
teins have now been structurally and functionally characterized. Some of these
are involved in target cell recognition and are discussed later (section I.5).

The trophozoites vary in size from 12-60 μm with larger forms in tissues
and smaller forms found in asymptomatic carriers. They are uninucleate but
occasionally under in vitro conditions show multiple nuclei. The nucleus
occupies about one fifth the trophozoite volume (Lushbaugh and Miller, 1988).

The cytoplasm consists of the ectoplasm which is devoid of refractile
organelles and is found near the tip of advancing pseudopodia. The endoplasm
consists of the nucleus, vacuoles and other refractory elements. The major
cytoplasmic components are the ingestive and the digestive vacuoles, the
residual bodies, the nucleus, and a number of smaller membranous vesicles.
Alpha glycogen is distributed throughout the trophozoite cytoplasm. Both golgi
apparatus and mitochondria which are typical eukaryotic organelles, are absent
in *E. histolytica*. Rough endoplasmic reticulum is present as bundles and
larger crystalline arrays, forming the chromatoidal bodies. Smooth endoplasmic
reticulum is poorly developed in *E. histolytica* and may be functionally re-
placed by the vacuolar system, a tubular network or exist as loosely organized
structures (Lushbaugh and Miller, 1988).

The vacuolar system of *E. histolytica* consists of structures which help
in the internalization of particles or fluids. The pinocytic channels extend
into the cytoplasm giving rise to small vesicles. It has not yet been estab-
lished as to how hydrolytic enzymes synthesized in the ribosomes are trans-
ported to the vesicles since the golgi complex and lysosomal structures are absent (Gonzales-Robles and Martinez-Palomo, 1983).

Tubulin or tubulin-related proteins have not yet been identified (Solis, 1991). Moreover, the presence of microtubule organizing centers, kinetochores or chromosome movement during mitosis which would suggest the presence of tubulin have not yet been observed in *E. histolytica*.

The trophozoites of *E. histolytica* have a well defined nuclear envelope containing nuclear pores similar to other eukaryotes. However, no connection has yet been described with the cytoplasmic membranous system that suggests communication between the nucleus and the "endoplasmic reticulum" (Lushbaugh and Miller, 1988). The inner surface of the nuclear membrane is lined with dense particles known as "peripheral chromatin", which are nonuniformly distributed. However, the areas immediately adjacent to the pores are free of "peripheral chromatin".

The presence of chromosomes in *E. histolytica* has not yet been reported. The condensed chromatin remains as such throughout interphase and nuclear divisions without formation or separation of metaphasic chromosomes. The nuclear division is independent of the cell division and is amitotic (Orozco et al, 1988). The ploidy level in trophozoites or cysts are still unknown. As yet there is no single study which throws any light on the molecular basis of cell division in *E. histolytica*.

There is only one study indicating the presence of chromatin formed by 10 nm long nucleosomes which is highly sensitive to micrococcal nuclease. The basic nucleosomal repeating unit was found to be 130 bp and not 200 bp as seen in higher eukaryotes. The linkers connecting the nucleosomes were of variable length. (Torres-Guerrero et al, 1991). DNA was found to be associated with six basic proteins similar but not identical to histones. However, another group has shown the presence of a homologue of histone H3 in *E. histolytica* (Fodinger et al, 1993). The gene corresponding to the amoebic histone H3 was isolated as a clone from a cDNA expression library though its in vivo localization was not demonstrated. The presence of other histones are yet to be demonstrated.

I.3 Energy metabolism in *E. histolytica*

The absence of mitochondria in *E. histolytica* suggests the presence of different pathways for energy generation and metabolism compared to most other
eukaryotes (Muller, 1988). The cytosol is the site for all or most steps in metabolism. *E. histolytica* is a microaerophilic organism, i.e. it can tolerate small amounts of oxygen (Lindmark, 1976). Glycolysis proceeds along the ubiquitous Embden-Meyerhoff pathway. Pyruvate, the key intermediate and precursor for most metabolic products formed by glycolysis, is released into the medium. Energetically, there is a net yield of two moles of ATP for every mole of glucose degraded. Anaerobically, the pyruvate is converted to ethanol and carbon dioxide. Interestingly, *E. histolytica* shares an atavistic feature with bacteria as inorganic pyrophosphate (PP$_i$) replaces adenine nucleotides as a high energy compound in several reactions. It also uses PP$_i$-dependent protein kinases instead of ATP-dependent protein kinases (Wood, 1977).

The absence of mitochondria makes the study of transfer of energy through the electron transport chain during metabolism an interesting feature. The presence of ferredoxin, NAD and NADP-dehydrogenase and pyruvate:ferredoxin oxidoreductases have been demonstrated but not characterized (McLaughlin and Aley, 1985). Using a series of inhibitors, a truncated chain of carriers was identified (Weinbach, 1988). The absence of heme proteins in this organism is also significant.

I.4 Biochemistry of *E. histolytica*

Amongst the various proteins present in the cytoskeleton, actin is the only protein which has been purified and characterized. It was found to be similar to muscle actin but distinct in its molecular weight, pI and ability to bind DNase I (Gadasi, 1982; Meza, 1983). The presence of a spindle apparatus, or tubulin has not been demonstrated.

Actively moving *E. histolytica* do not show anisotropic filaments. Actin is organized in different forms and actively participates in motility related processes. *In vivo*, actin appears diffused in the cytoplasm or in the form of aggregates in the leading pseudopods of actively moving amoebas. Bailey et al (1985, 1990) described the induction of actin phagocytic mouths at contact sites by challenging trophozoites by red blood cells. Moreover, cytochalasin inhibition studies have shown that the amoeba cytoskeleton is required for effective parasite attachment and destruction of target cells (Ravdin et al, 1988). Therefore, contact induced actin polymerization may be an essential event in the initiation of target cell destruction process. However, the regulation of actin polymerization or actin binding proteins is unexplored.
The role of Ca\(^{2+}\) in signal transduction has been well documented (Berridge and Irvine, 1989). However, the different signalling pathways and the enzymes involved in them have not yet been characterized in *E. histolytica*. Calmodulin, a highly conserved calcium binding protein, has been shown to be present in *E. histolytica*. The protein has been purified and partially characterized (Munoz et al, 1992). The amoebic protein has the same features, like molecular weight, ability to activate phosphodiesterase and susceptibility to inhibitors as mammalian calmodulin (Munoz et al, 1991). The presence of this protein suggests that *E. histolytica* may have calmodulin-dependent Ca\(^{2+}\) signalling pathway similar to other eukaryotes. The ability of *E. histolytica* to convert \(^{3}\text{H}\)-inositol to inositol triphosphate, the putative mediator for the release of intracellular calcium from membrane stores such as endoplasmic reticulum, has been reported (Long-Kruz and Ravdin, 1988). Whether the inositol signalling pathway as described in mammalian cells is also present in *E. histolytica* remains to be determined.

A Ca\(^{2+}\)-ATPase is one of the enzymes characterized in the plasma membrane of *E. histolytica* (McLaughlin and Muller, 1981). This enzyme displays an absolute and specific requirement for Ca\(^{2+}\) and was suggested to control intracellular levels of Ca\(^{2+}\). The enzyme is thought to be present in the plasma membrane and the lumen of the internal vesicles. It is likely that this enzyme may be involved in the import of nucleosides (Bakker-Grunwald and Parduhn, 1993). This is an interesting observation as the pentose phosphate pathway is absent in *E. histolytica* which is the prime source for synthesis of ribose, the backbone sugar moiety present in nucleotides. The presence of two forms of phospholipase A which differ in the pH optima, Ca\(^{2+}\) requirement and subcellular localization has also been reported (Long-Kruz et al, 1985).

### I.5 Pathogenesis and *E. histolytica*

Brumpt (1925) suggested the presence of two morphologically different species of *E. histolytica*, one pathogenic and the other nonpathogenic. Analysis of zymodeme patterns and clinical histories, including serology, of more than 6,000 individuals infected with *E. histolytica* was reported by Sargeaunt (1987). Their data suggested a distinct difference in zymodeme pattern of *E. histolytica* isolated from invasive (pathogenic) and asymptomatic (nonpathogenic) patients. This led to the belief that the invasive amoebiasis is caused by a genetically distinct organism as originally suggested by Brumpt (1925). This
was further shown by a number of studies showing distinct genetic differences between pathogenic and nonpathogenic strains (Tannich et al, 1989, 1991c; Torian et al, 1990b; Edman et al, 1990), e.g. in the case of cysteine proteases, both Southern and Northern analysis has shown that the pathogenic strains of \textit{E. histolytica} have a larger number of genes and a higher degree of expression (10-100 fold) when compared to nonpathogenic strains (Tannich et al, 1991a). The nonpathogenic strains also showed less expression of collagenase when compared with pathogenic strains (Munoz et al, 1991). These data support the original suggestion of Brumpt (1925) that there are two species of \textit{E. histolytica}, one pathogenic (\textit{E. histolytica}) and the other nonpathogenic (\textit{E. dispar}) and this nomenclature has now been revived (Diamond and Clark, 1993).

Studies on the pathogenic nature of \textit{E. histolytica} have focused on two different pathways. The first considers the contact-dependent and direct killing of target cells and the second involves the export of toxins from the cells. Though a large number of \textit{E. histolytica} adherence receptors have been identified, the 170 kDa galactose specific lectin originally described by Petri et al (1987) has been the most intensively studied (Mann and Petri, 1991). This receptor is thought to be responsible for mediating attachment to colonic mucus and epithelial cells and is present as a heterodimer (170 kDa and 35 kDa; Petri et al, 1989). Although target cell-surface glycan binding to the \textit{E. histolytica} galactose lectin is responsible for attachment of target cells, it is not directly involved in the mechanism of amoeba cytoskeleton activation (Bailey et al, 1990). Both glycoprotein and glycolipid glycans participate in target binding. The transmembrane signals that lead to \textit{E. histolytica} actin polymerization following target contact are triggered, at least in part, by negatively charged lipids of the target plasma membrane. However, phagocytosis and the binding of the lectin to the target cell are two independent events. Some of the other molecules involved in the attachment to target cells including red blood cells, are the 112 kDa surface adhesin (Arroyo and Orozco, 1987), 220 kDa N-acetyl glucosamine-binding lectin (Meza et al, 1987) and 29 kDa adhesin (Vinayak et al, 1990).

It has been postulated that amoeba can secrete pore-forming proteins and produce cytotoxic products on release can cause cytotoxic effect on host cells and tissues (Leippe et al, 1992; Lynch et al, 1982). The pore forming peptide,
porin, is transferred from the trophozoite to the target cell causing a disrup­tion of the transmembrane gradient and subsequent death by colloid-osmosis lysis.

*E.histolytica* may require collagenase and other proteinases to invade host tissue (Bruckner, 1992). Collagenase is present in the cell as electron­dense particles and its secretion is stimulated 9-10 fold in presence of collagen type I and III as substrates (Munoz et al, 1991). It is similar to mammalian collagenase in this respect and has been partially purified from *E. histolytica* extracts. It consists of a single polypeptide of 141 kDa. Cysteine proteinases which are the major proteolytic enzymes, have been correlated with virulence, as they might be involved in invasiveness at intercellular junctions. These processes may be mediated by calmodulin (Munoz et al, 1991).

The *in vitro* cytolytic activity of amoeba is inhibited by calcium channel blockers bepridil and veerampimi, the putative calcium antagonist TMB-8, and the Ca$^{2+}$ specific chelator, EGTA (Ravdin et al, 1982, 1985). In addition, the membrane bound phospholipase A and protein kinase C are also thought to play a major role in the cytolytic event (Ravdin et al, 1988; Weikel et al, 1988). These evidences suggest that the free intracellular Ca$^{2+}$ may serve as a second messenger regulating parasite cytolytic activity. The signal transduction pathway involves the production of diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP$_3$). DAG binds to protein kinase C and a host of other enzymes which play a role as kinases or phosphatases and thereby activating them whereas, IP$_3$ increases the intracellular levels of Ca$^{2+}$ by releasing these from internal/external stores (Berridge, 1987). Ca$^{2+}$ then is bound by effector molecules (e.g. calmodulin) and /or enzymes which are activated. Both these signals thereby regulate gene expression and other metabolic/secertory processes. Measurement of intracellular Ca$^{2+}$ concentrations using fluorescent dyes showed a rapid and substantial rise in the target cell whereas amoebic [Ca$^{2+}$] was not elevated following parasite adherence to target cells (Ravdin et al, 1988).

### I.6 Molecular Biology of *E. histolytica*

Though the presence of condensed chromosomes during the metaphase of mitosis has not been demonstrated, bands were observed in pulse field gel electrophoresis (Valdes et al, 1990). There were 9-11 bands ranging from 0.3 to greater than 2 Mb in size. From this data it is difficult to predict the
number and size of chromosomes of \textit{E. histolytica}. Recent data suggests that different actin genes (discussed later) may be present on different "chromosomes" (Petter et al, 1992).

The rRNA genes of \textit{E. histolytica} are localized in a extrachromosomal 25 kb plasmid (Bhattacharya et al, 1989; Huber et al, 1989). This plasmid has two rRNA transcription units and 200 copies are present in each cell. The transcription units are present as inverted repeats and contain the 18S, 5.8S and the 28S rRNA genes in that order which is typical for eukaryotes (Ramachandran et al, 1993). This extrachromosomal plasmid accounts for approximately 10\% of the total genomic DNA of 5x10^7 bp.

A list of the genes which have been cloned and characterized are shown in Table II. The analysis of 5'-upstream sequences of a few selected genes of \textit{E. histolytica} has indicated the presence of a TATA box-like motifs as the recognition site for RNA polymerase (Edman et al, 1990; Petter et al, 1992). One of the sequence motifs, YATTTAAA, is found to be present at -24 region while the other is found to be at -60 unlike other eukaryotes where it is present at -25 region. A transcription initiation consensus sequence (ATTCA) was also found just downstream of the transcription initiation site. This sequence was found to be present adjacent to the cap site and present only 9-12 bp upstream of the translation start site. These studies suggested that the \textit{E. histolytica} genes have short 5'-untranslated regions present in the transcript. Mapping of the 3'-untranslated regions has shown that these are short (Huber et al, 1987). The analysis of the genomic sequence present downstream from coding region has shown the absence of a typical polyadenylation signal (AAUAAA) in the \textit{E. histolytica} genes. However, a different sequence (ATTTTAA) could act as polyadenylation site as suggested by (Huber et al, 1987). The high A-T content (63-77\%) of the \textit{E. histolytica} genome (Lopez-Revilla and Gomez-Dominguez, 1988) makes these observations still more difficult to interpret and further studies are required to assess the role of these sequences. Introns seem to be absent in all genes of \textit{E histolytica} with one exception (Lohia and Samuelson, 1993a). This can be probably correlated with the small genome size of \textit{E. histolytica} and its requirement to encode a normal repertoire of proteins during its life cycle. The sequence of the genes of \textit{E. histolytica} known to date differ significantly from their eukaryotic counterparts whereas the functional domains are conserved. Though this can be, in
Table II: A selected list of genes reported in *E. histolytica*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Size</th>
<th>Copy No</th>
<th>Remarks</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>376 a.a.</td>
<td>&gt; 5</td>
<td>Does not bind</td>
<td>Huber et al, 1987</td>
</tr>
<tr>
<td>Ferredoxin</td>
<td>250 a.a.</td>
<td>2</td>
<td>resembles Clostridial type</td>
<td>Huber et al, 1988</td>
</tr>
<tr>
<td>Iron containing Superoxide dismutase</td>
<td>25 kDa</td>
<td>1</td>
<td></td>
<td>Tannich et al, 1991c</td>
</tr>
<tr>
<td>NADP⁺-dependent alcohol dehydrogenase</td>
<td>39 kDa</td>
<td>1</td>
<td>Involved in glycolysis</td>
<td>Kumar et al, 1992</td>
</tr>
<tr>
<td>Membrane derived Serine rich protein</td>
<td>52 kDa</td>
<td>-</td>
<td>Expressed only in <em>E. histolytica</em></td>
<td>Stanley et al, 1990</td>
</tr>
<tr>
<td>Cysteine proteinase</td>
<td>27 kDa</td>
<td>1</td>
<td>P strains contain more genes and higher expression than NP</td>
<td>Tannich et al, 1991a</td>
</tr>
<tr>
<td>P-glycoprotein</td>
<td>145 kDa</td>
<td>3</td>
<td>homologue of human mdr1 gene</td>
<td>Descoteaux et al., 1992</td>
</tr>
<tr>
<td>EF-1</td>
<td>49 kDa</td>
<td>-</td>
<td>high homology with EF-1 of <em>S. cerevisiae</em></td>
<td>De Meester et al, 1992</td>
</tr>
<tr>
<td>EF-2</td>
<td>2.52 kb cDNA</td>
<td>-</td>
<td>homology with human analogue</td>
<td>Plaimauer et al, 1992</td>
</tr>
<tr>
<td>70 kDa Heat shock protein</td>
<td>-</td>
<td></td>
<td>Immunoreactive in patients</td>
<td>Ortner et al, 1992</td>
</tr>
<tr>
<td>29 kDa cysteine rich surface protein</td>
<td>-</td>
<td></td>
<td>surface antigen</td>
<td>Torian et al, 1990</td>
</tr>
<tr>
<td>Gene</td>
<td>Size</td>
<td>Copy No</td>
<td>Remarks</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>------------</td>
<td>---------</td>
<td>--------------------------------------------------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>12. 170 kDa N-acetyl-galactosamine inhibitor lectin*</td>
<td>&gt; 1</td>
<td></td>
<td>involved in target cell recognition</td>
<td>Mann et al, 1991</td>
</tr>
<tr>
<td>13. 125 kDa surface antigen (M17)*</td>
<td>3.35 kb cDNA</td>
<td>1</td>
<td>Immunoreactive in patients</td>
<td>Edman et al, 1991b</td>
</tr>
<tr>
<td>14. L21</td>
<td>19 kDa</td>
<td>1</td>
<td>physically linked to actin gene by 2.1 kb intergenic spacer</td>
<td>Petter et al, 1992</td>
</tr>
<tr>
<td>15. Myosin heavy chain</td>
<td>220 kDa</td>
<td>1</td>
<td>homology with myosin II of Dictyostelium</td>
<td>Rahim et al, 1993</td>
</tr>
<tr>
<td>16. Amoeba pore</td>
<td>0.3 kb cDNA</td>
<td>1</td>
<td>amino terminus homologous to mellitin</td>
<td>Leippe et al, 1992</td>
</tr>
<tr>
<td>17. 35 kDa Subunit</td>
<td>-</td>
<td></td>
<td>subunit of 170 kDa lectin</td>
<td>Tannich et al, 1992</td>
</tr>
<tr>
<td>18. Ubiquitin</td>
<td>8-9 kDa</td>
<td>-</td>
<td>has 6 a.a. changes</td>
<td>Wostmann et al, 1992</td>
</tr>
<tr>
<td>19. cdc2</td>
<td>33.8 kDa</td>
<td>&gt; 1</td>
<td>Presence of a 79 bp intron</td>
<td>Lohia and Samuelson, 1993</td>
</tr>
<tr>
<td>20. Histone H3</td>
<td>15 kDa</td>
<td>-</td>
<td>Immunoreactive in patients</td>
<td>Fodinger et al, 1993</td>
</tr>
<tr>
<td>21. Aldehyde dehydrogenase</td>
<td>60 kDa</td>
<td>1</td>
<td>Involved in glycolysis</td>
<td>Samuelson et al, 1992</td>
</tr>
</tbody>
</table>

*= RFLP between pathogenic (P) and nonpathogenic (NP) strains. a.a.= amino acid.
part, attributed to the codon bias in *E. histolytica* (Char and Farthing, 1992), the peculiarities in primary sequences of histone H3, actin, myosin, ferredoxin and elongation factor 1 alpha are yet to be explained functionally.

A brief description of some of the genes of *E. histolytica* characterized so far has been outlined below.

The actin genes were the first to be characterized from the main genome. As discussed earlier, the inability of *E. histolytica* actin to bind DNase I was peculiar. However, sequence analysis revealed the presence of actin-actin, actin-depactin and actin-myosin ATP-ase interaction sites which seemed to be conserved (Huber et al, 1987; Edman et al, 1987). Cloning and sequence analysis of the elongation factor 1 alpha showed the presence of an unusual protein with limited sequence homology but antigenically related to the *S. cerevisiae* EF-1 alpha. It also revealed the presence of a GTP-binding site. Similar analysis of ferredoxin and EF-1 alpha showed a close similarity with their bacterial counterparts.

As shown in Table II, a number of genes show differences in organization and copy number between pathogenic and nonpathogenic strains, e.g. the gene encoding cysteine proteinases. There is a marked difference in both copy and the level of expression of this gene in pathogenic and nonpathogenic strains. These differences have led to reclassification of nonpathogenic strain as *E. dispar* (Diamond and Clark, 1993).

### I.7 Secondary messenger system

Knowledge of phosphoinositides and their role in cell signalling has expanded enormously in recent years (Berridge, 1987). Stimulation of cell-surface receptors initiates hydrolysis of a membrane-bound inositol lipid, which produces at least two second messengers—diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP$_3$). These messengers are generated by three main components: a receptor, a coupling G protein and phosphoinositidase C. DAG acts by stimulating protein kinase C while IP$_3$ releases Ca$^{2+}$ from internal stores.

A variety of tissues have a high affinity stereospecific binding site for IP$_3$ which is linked to the Ca$^{2+}$-channels connected with IP$_3$-sensitive pool (Berridge and Irvine, 1989). The binding is cooperative suggesting the presence of three molecules involved in receptor function. In addition to mobilizing Ca$^{2+}$ through IP$_3$, many agonists can promote an influx of external
calcium. There are five types of calcium channels one of which is an agonist-dependent type while the second one is opened directly through a receptor (receptor-operated channel) and the third opened by an internal diffusible messenger (second-messenger operated channel) (Table III; Tsien and Tsien, 1990). These Ca\(^2+\)-channels differ in the way they are activated and probably, in different Ca\(^2+\)-signals they generate. Voltage-gated Ca\(^2+\)-channels give rapid but brief Ca\(^2+\) pulses when activated by membrane depolarization. Opening of receptor-mediated channels, however, results in rapid and maintained elevation in intracellular Ca\(^2+\) which is dependent on extracellular Ca\(^2+\). The Ca\(^2+\) signal derived from the opening of the voltage-operated channels may be amplified by the mobilization of intracellular Ca\(^2+\) through a Ca\(^2+\)-dependent formation of IP\(_3\). The second messenger derived Ca\(^2+\)-channels are very slow in their ability to increase the levels of Ca\(^2+\) and have not been characterized very well (Berridge and Irvine, 1989).

The presence of an insensitive pool of IP\(_3\) was shown when measurements showed that only 30-50% of the total Ca\(^2+\) was released by IP\(_3\). Thus the presence of a second secondary messenger in the form of cyclic adenosine diphosphate-ribose (cADPR) was not a surprise. cADPR is converted by ADP-ribosyl cyclases from nicotinamide adenine dinucleotide (NAD\(^+\)) and induces the Ca\(^2+\)-release by microsomes derived from the endoplasmic reticulum of sea urchin eggs (Galione, 1991). The identification of glucose induced cADPR as a new Ca\(^2+\)-mobilizing second messenger is further demonstrated by the stimulation of insulin secretion from digitonin permeabilized pancreatic \(\beta\)-cells.

Another pointer towards the universal function of this molecule is its presence in all mammalian cell types. However, the precise mechanism as to how this dual pathway functions in various cell types is yet to be elucidated.

I.8 Calcium-binding Proteins

Calcium controls a variety of cellular and extracellular processes with a high degree of spatial and temporal precision. Thus mechanisms must exist which control Ca\(^2+\) in a spatial and temporal fashion. The concentration of Ca\(^2+\) within the cytosol of all quiescent cells is \(10^{-7}\) M. The sole function of calcium is to transmit information and because free Mg\(^2+\) is present at \(10^{-2.5}\) M, the binding molecule requires very high affinity and selectivity for Ca\(^2+\). The concentration of Ca\(^2+\) rises to \(10^{-5.5}\) M during stimulation, thereby, reducing the Mg\(^2+\)/Ca\(^2+\) ratio from \(10^{4.5}\) to \(10^{3.0}\) (Kretsinger, 1987).
Table III: Diverse Ca\textsuperscript{2+} entry pathways in vertebrate cells: 
a simplified summary

<table>
<thead>
<tr>
<th>Type</th>
<th>Properties</th>
<th>Function and location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Voltage-operated Ca\textsuperscript{2+} channels (VOCs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-type</td>
<td>High-voltage activated</td>
<td>excitation-contraction, etc., endocrine, neurons.</td>
</tr>
<tr>
<td>T-type</td>
<td>Low-voltage activated, slowly deactivating</td>
<td>Pacemaker activity, repetitive firing; heart and neurons</td>
</tr>
<tr>
<td>N-type</td>
<td>High-voltage activated</td>
<td>Transmitter release; neurons</td>
</tr>
<tr>
<td>P-type</td>
<td>Moderately high-voltage activated</td>
<td>Transmitter release, spiking; neurons</td>
</tr>
<tr>
<td>2. Receptor-operated Ca\textsuperscript{2+} channels (ROCs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMDAR</td>
<td>Ligand-gated, indirectly voltage-activated</td>
<td>Neuronal Ca\textsuperscript{2+}-entry</td>
</tr>
<tr>
<td>ATPR</td>
<td>Ligand-gated, without indirectly voltage-activated</td>
<td>Cation and Ca\textsuperscript{2+}-entry; smooth muscle</td>
</tr>
<tr>
<td>3. Second messenger-operated Ca\textsuperscript{2+} channels (SMOCs)</td>
<td></td>
<td>Neutrophils, Lymphocytes, Platelets</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+} or IP\textsubscript{3} activated</td>
<td>Secretion or gene expression</td>
<td></td>
</tr>
<tr>
<td>4. Mechanically operated Ca\textsuperscript{2+} channels (MOCs)</td>
<td></td>
<td>Smooth, skeletal muscle, endothelial cells</td>
</tr>
<tr>
<td>Stretch-activated or inactive enzyme activity</td>
<td>Steady Ca\textsuperscript{2+} influx; sets resting [Ca\textsuperscript{2+}]</td>
<td>Muscle cells</td>
</tr>
</tbody>
</table>
signal is then transduced into an intracellular response, in part by calcium-binding proteins that are thought to be involved in the regulation of many cellular activities (Meyer, 1991). These proteins may be subdivided into four classes based on function and the different ways in which they bind calcium (Van Eldik et al., 1982). These are:

1. **Proteins containing gamma-carboxyglutamic acid**: Proteins which contain gamma-carboxyglutamic acid (due to a Vitamin K-dependent posttranslational modification of Glu) bind calcium in the millimolar range (Suttie et al., 1980). Some proteins in this class are prothrombin, factor X and osteocalcin. The main function of calcium in these proteins appears to be the promotion of protein interaction with extracellular supramolecular structures or surfaces. Calcium is bound due to simple chelation.

2. **Calcium binding lectins**: The role of calcium binding to this class of proteins appears to be mainly to stabilize their structure. Multiple protein ligands are used to bind Ca\(^{2+}\) in lectins. The polyhedron that is formed by wrapping of the peptide is found at the amino terminus of the lectins (Gebauer et al., 1981).

3. **Calcium binding hydrolytic enzymes**: Calcium is involved in substrate binding by stabilization of the enzyme-substrate intermediate and enzymatic catalysis when it participates in the activation of a zymogen. The coordination of calcium occurs by six ligands that form an octahedron with most of the ligands being contributed by the oxygens from peptide carbonyl groups. At least one side chain oxygen, the carboxylate oxygen of an Asp, is also used (Dijkstra et al., 1981).

4. **Calcium-modulated proteins**: These can reversibly bind calcium with dissociation constants in the nanomolar to micromolar range under physiological conditions. As the tertiary structure and activity of these proteins are thought to be modulated by the reversible formation and dissociation of a calcium-protein complex, they are termed as Ca\(^{2+}\)-modulated proteins. Two types of calcium binding structures, both of which utilize a helix-loop-helix arrangement of the peptide chain have been found in this class of proteins (Strynadka and James, 1989). While the Vitamin D-dependent calcium binding proteins mostly utilize carbonyl oxygens from the peptide backbone, the other Ca\(^{2+}\)-modulated proteins utilize the side chain carbonyl oxygens from the peptide backbone. The Ca\(^{2+}\)-binding structure in the
latter type are referred the EF-hand structure (Moews and Kretsinger, 1975) and is discussed in detail here.

I.9 EF-hand calcium binding proteins

The EF-hand family of proteins consist of the helix-loop-helix (HLH) calcium binding proteins that upon binding Ca$^{2+}$, undergo a change in conformation. This signal is subsequently transmitted to the respective target molecules. For this reason, they are often termed as Ca$^{2+}$-modulated. The two most well characterized examples of this family are troponin C and calmodulin, the latter present ubiquitously in all eukaryotes. The features of these proteins are discussed in detail below.

The EF-hand structure was first proposed when the crystal structure of the calcium binding carp parvalbumin was elucidated (Moews and Kretsinger, 1975). It consisted of a calcium binding loop of 12 contiguous amino acids flanked by two alpha-helices. Each loop has the capacity to bind one Ca$^{2+}$-ion. The nomenclature of the Ca$^{2+}$-binding loop derives from the original octahedral description of the Ca$^{2+}$-coordinating ligand symmetry (Moews and Kretsinger, 1975). In that description, six residues in positions 1(X), 3(Y), 5(Z), 7(-Y), 9(-X) and 12(-Z) provide oxygen ligands to the Ca$^{2+}$-ion. In all known functional calcium binding proteins of the EF-hand family, the first amino acid is Asp and the twelfth is Glu, the latter contributing both its side-chain oxygen atoms to the metal ion coordination. The other five residues contribute one oxygen each. Therefore, since there are seven oxygen ligands, the true Ca$^{2+}$-coordination is pentagonal bipyrimidal and not octahedral as proposed before. The -Y ligand is probably not contributed by the amino acid side chain at this position in each loop, but rather is supplied by the alpha-carboxyl oxygen of the adjacent polypeptide backbone (Strynadka and James, 1989). The potential ligand residues are interspersed with small side-chain amino acids. This is particularly important at the apex of the loop, where Gly is an invariant residue, presumably to allow a sharp bend. Each loop structure is flanked on either side by 8-10 residues of alternating hydrophilic and hydrophobic character, an arrangement favourable to formation of the outer and inner surfaces of an alpha-helical cylinder.

These HLH proteins are characterized by the relatively high percentages of acidic residues (troponin C, 29%; calmodulin, 25%; intestinal calcium binding protein, 23%; parvalbumin, 18%) (Klee and Vanaman, 1982). Calmodulin
is considered to be the prototype for the EF-hand family of proteins. This is a small 148 amino acid protein which upon binding Ca\(^{2+}\), undergoes a change in conformation. The change in the conformation results in an increase in alpha-helicity by 8-10% (Klee and Vanaman, 1982). In contrast, the smaller changes elicited by other divalent cations like Mn\(^{2+}\) or Mg\(^{2+}\), the Ca\(^{2+}\)-induced are observed in the presence or absence of Na\(^+\) or K\(^+\).

The electrophoretic mobilities of all known EF-hand family of calcium binding proteins are altered by Ca\(^{2+}\). The effect varies depending on the protein and the electrophoretic conditions employed. With nondenaturing polyacrylamide gels run in alkaline buffer system, the mobility of calmodulin is substantially slower in the presence of Ca\(^{2+}\) than in the presence of EGTA. All EF-hand calcium binding proteins tested have greater mobility on SDS gels in the presence of Ca\(^{2+}\), although the increased migration is much larger for calmodulin.

The ability of calmodulin to function as a Ca\(^{2+}\)-dependent regulator of enzyme activity is a consequence of changes that occur upon its binding of Ca\(^{2+}\). Since Ca\(^{2+}\)-binding alters neither the molecular weight nor the gross hydrodynamic properties of calmodulin, it was assumed that this binding results in a conformational change. The target proteins which interact with calmodulin and are regulated by its postulated Ca\(^{2+}\)-binding, are protein kinases, NAD kinase, phosphodiesterase, calcium pumps and proteins involved in cellular mobility. The mechanisms by which calmodulin recognizes such a diverse set of proteins is thought to be mediated by the stretch of basic amphipathic alpha-helical structures (baa helices) present in the target proteins (O'neil and Degrado, 1990).

Crystal structure analysis of calmodulin had shown the presence of two globular domains, each of which have two Ca\(^{2+}\)-binding motifs composed of two helices with a Ca\(^{2+}\)-binding loop in between. Both domains have large hydrophobic patches flanked by regions of highly negative electrostatic potential which are excellent candidates as binding sites for positively charged amphipathic helices.

Though the sequences of calmodulin and troponin C are 75-80% homologous in the region of overlap (which excludes the first NH\(_2\)-terminal helix of troponin C), the major differences are a three amino acid insertion present in the linker region connecting the second and the third Ca\(^{2+}\)-binding domains.
and an extra 18-19 amino acids in troponin C. The linker region, encompassing residues 65-92, is important as it is able to impart flexibility to the molecule. In vitro experiments had suggested that calmodulin was able to activate target enzymes in a differential manner despite having deletions in the linker region.

All the studies mentioned above were performed under in vitro conditions. The first few studies to show that the calmodulin gene is essential for cell survival was carried out in Saccharomyces cerevisiae (Davis et al, 1986). Subsequent experiments showed that the vertebrate calmodulins, sharing only 60% homology with yeast calmodulins, can complement yeast calmodulin mutants (Davis and Thorner, 1989; Pereschini et al, 1991).

The presence of a trimethyllysine at position 115 in most calmodulins has been demonstrated. Studies with calmodulin in the fission yeast Schizosaccharomyces pombe have shown that the Arg at position 116 (this the same as position 115 of calmodulin in higher systems as S. pombe has an extra amino acid at the NH$_2$-terminus) is required as a mutation in this position results in a sporulation-deficient phenotype. Two other mutations at this residue resulted in leakier phenotypes (Takeda et al, 1989).

Calmodulin mutants of Paramecium which show abnormal response to certain stimuli due to loss of either a Ca$^{2+}$-dependent Na$^+$ current or a Ca$^{2+}$-dependent K$^+$ current indicate functional differences between the COOH-terminal and NH$_2$-terminal lobes. Sequence analysis of the mutants showed single or double amino acid substitutions placing them in two groups (Kink et al, 1990). Members of the first group which are behaviourally overreactors had mutations in the COOH-terminus while the second group of mutants which are underreactors had mutations present in the NH$_2$-terminus. Further analysis has shown one of the mutants to interact specifically with the membrane bound enzyme, Ca$^{2+}$-Mg$^{2+}$-ATPase. Injection of wild-type calmodulin gene to the nucleus of mutant cells reverted them to the wild type (Preston, 1990; Kanabrocki et al, 1991; Hinrichsen et al, 1992).

The working model for action of calmodulin involve interaction of the Ca$^{2+}$-calmodulin complex with one or a number of enzymes leading to activation. However, the inactivation of all the Ca$^{2+}$-binding sites in yeast calmodulin did not abolish the ability of the protein to support growth. These results raised the possibility that calmodulin does not act as a Ca$^{2+}$ receptor during
growth, but instead performs its essential function without binding Ca\(^{2+}\) (Geiser et al, 1991).

I.10 Role of calcium in eukaryotic microbes

The release of lysosomal enzymes from Tetrahymena e.g. acid phosphatase and \(\beta\)-hexoaminidase, is a calcium dependent event. Other processes include mucocyst exocystosis and food vacuole egestion (Tiedtke et al, 1990). The presence of calmodulin has already been discussed earlier (section I.9). The presence of two other calcium binding proteins of molecular weights 23 and 25 kDa which regulate ciliary beat patterns in the cilia, demonstrated the existence of three closely related calcium binding proteins in close proximity. These may be expected to produce markedly different effects on transient increase in Ca\(^{2+}\) levels.

The aquatic fungus Allomyces arbuscula possesses a calpain-like Ca\(^{2+}\)-activated neutral protease (CaNP). This is activated by binding to the plasma membrane in a Ca\(^{2+}\)-dependent manner and can be either present on the membrane or be released into the cytoplasm. CaNP of A. arbuscula is similar to the mammalian CaNP and acts on protein kinase C and signal transduction components like phospholipase C (Ojha, 1989).

Interestingly two different calmodulins, Cam-1 and Cam-2 have been described in Naegleria. While one is localized in the flagella, the other is found in the cell body. This ameboflagellate, like E. histolytica, uses an actin-based Ca\(^{2+}\)-dependent mechanochemical system in its amoeboid stage. A low molecular weight factor, \(u\), which has been purified and partially characterized, has been suggested to regulate the intracellular Ca\(^{2+}\) release (Fulton, 1986).

Despite a wealth of evidence, fundamental questions about calcium-regulatory pathways remain to be addressed. Functions need to be assigned to the different calcium binding proteins found in different systems (Van Eldik et al, 1982; Heizmann and Hunziker, 1991). At present, no known pathways have been identified that are activated by a calcium signal, though a large number of calcium-binding proteins have been cloned and characterized and implicated in the transduction of signal and/or secondary effects of the signal on regulation of gene expression.