Functional Characterization of *E. histolytica* MAPK
Chapter V  
Functional characterization of E. histolytica MAPK

INTRODUCTION

1. Mitogen Activated Protein Kinases (MAPKs)

Mitogen activated protein kinases compose a family of protein kinases whose function and regulation has been conserved during evolution from unicellular organisms such as brewer’s yeast to complex organisms including human [1]. MAPKs phosphorylate specific serine and threonine residues within a variety of substrates and thereby regulate cellular activities ranging from gene expression, mitosis, movement, metabolism and even programmed cell death [2]. Among the various MAPK substrates, are included other protein kinases, phospholipases, transcription factors and cytoskeletal proteins. MAPKs are part of a phosphorelay system consisted of three sequentially activated kinases and like their substrates MAPKs are also regulated by phosphorylation [3]. MAPKs are activated by the phosphorylation of a threonine (T) and a tyrosine (Y) residue within the TXY activation motif of the protein. MAPK phosphatases however reverse the phosphorylation event leading to the inactivation of MAPKs. MAPK kinase kinases (MAPKKK) are the upstream activators of MKKs and also mediate activation through phosphorylation. MAPKKKs have distinct motifs in their sequences that selectively confer their activation in response to different stimuli. Cells receive different stimuli from their environment that influence their metabolic rate, interaction with other cells, survival and proliferative potential and other cellular processes involved in homeostasis and health of the organism. One outcome of having many different MAPKKKs is that they can be matched with specific MKK-MAPK cassettes such that cells can respond to different stimuli with the activation of a specific MAPK pathway [2]. There are at least five distinct MAPK subgroups each containing several protein homologues. These five subgroups of MAPKs are the extracellular signal regulated kinase protein homologues 1 and 2 (ERK 1/2), the big MAPK 1 also known as ERK 5, stress activated protein kinases 1 (SAPK 1) also known as c-Jun N-terminal kinase homologues 1, 2 and 3 (JNK 1/2/3), the SAPK-2 homologues α, β and δ (P38 α/ β/ δ) and finally ERK 6 also known as P38 γ [4, 5]. Figure 1 below gives an outline of the various MAPK cascades operative within the mammalian cells.
Chapter V  Functional characterization of *E. histolytica* MAPK

**Fig 1.** MAPK phosphorelay systems. The modules shown are representatives of the pathway connections for the respective MAPK phosphorelay systems.


2. The structural characteristics of MAPKs

MAPKs share a common structural topology that is a derivation of the typical protein kinase fold first described in the crystal structure of the protein kinase A (PKA) [6]. The protein kinase fold comprises two distinct globular lobes, a small N-terminal one mainly composed of β strands and a large C-terminal lobe that is predominantly α helical. Based on the crystal structure of both the inactive and active/phosphorylated forms of MAPKs it is suggested that upon phosphorylation of the TXY loop MAPKs undergo a conformational arrangement of the N- and the C-terminal lobes in a way that the catalytic
Chapter V  
Functional characterization of E. histolytica MAPK

cleft can attain optimal kinase activity [4]. The MAPKs possess well conserved docking sites for their interactions with upstream activator kinases (MKKs), macromolecular scaffolds, inactivating phosphatases and different substrates. The best characterized docking site is the so called docking groove located in the convex side of the MAPK globular structure [7]. Two features make MAPKs different from other protein kinases. First the MAPK insert is a distinctive structural segment of the MAPK family, when compared with PKA and other protein kinases. This is a flexible extension of about 50 amino acids located in the C-terminal lobe [8, 9]. Although the functional implications of the MAPK insert and the C-terminal extension is not well understood yet, based on hydrogen mass spectrometry it has been presumed that they represent protein binding interfaces [10].

3. The extracellular signal regulated kinases

ERKs were originally identified as protein kinases that phosphorylate microtubule associated protein 2 in response to activation of receptor protein tyrosine kinases (RPTKs) by insulin and other polypeptide growth factors [11]. Moreover the extracellular factors which can act through G protein coupled receptors [12], tyrosine kinase receptors [13] and ion channels [14] can initiate a variety of intracellular signalling responses that also result in the activation of the ERK cascade. The activation of ERKs however requires adaptor proteins which are linked to guanine nucleotide exchange factors (GEFs) of small GTP binding proteins. Upon stimulation the adaptor protein GEF complex is recruited to the plasma membrane where it induces the activation of the small GTP binding protein itself (eg. Ras, Rap) which further transmits the signal to the MAP3K level of the cascade that comprises of Raf-1, A-Raf, B-Raf and TPL2 [15]. The MAP3Ks in turn phosphorylate the serine residues within the Ser-Xaa-Ala-Xaa-Ser/Thr motif in the activation loop of MEKs thereby leading to their activation. The activated MEKs however are dual specificity kinases which demonstrate a unique selectivity towards ERKs at the MAPK level [16]. Two main ERK genes are known, ERK 1/2 which encode two main proteins p44 and p42 [17] and few alternatively spliced forms such as rodent ERK 1b [18] and primate ERK 1c [19]. Activation of ERKs is mediated by the phosphorylation of the threonine and the tyrosine residues within the Thr-Glu-Tyr motif.
within the activation loop which occurs exclusively by the MEKs. Although ERK 1 and ERK 2 are considered to be functionally redundant some differences in their substrate specificities have been reported [20]. The ERKs are however proline directed ser/thr kinases and phosphorylate the serine or the threonine residues that are at the proximity of the proline residues within their substrates. Like for instance the most stringent consensus sequence for the substrate recognition by the ERKs is Pro-Leu-Ser/Thr-Pro [21]. The main substrates of the kinase identified thus far include the downstream kinases RSK, MNK and MSK, transcription factors such as Elk1, the cytosolic PLAP₂, a few cytoskeletal elements and other factors [22].

Structure function relationships of ERKs

An important regulatory domain in ERKs is their activation loop which has been shown previously to be essential not only for the activation of ERKs but also for the detachment from MEKs [23]. However besides the activation loop there are several other structural features of ERK that mediate its association with MEKs. These include the residues in the subdomain III [24], multiple regions in the N- and the C-termini [25], amino acids 19-25 of ERK2 [26] and residues 312-320 that constitute the cytosolic retention sequence [27]. In addition to these structural regions of ERKs certain scaffold proteins like MP1 or KSR have also been postulated to play significant role in the MEK ERK interaction [28]. Moreover the cytosolic retention sequence (CRS) of the protein has been found to be involved in its interaction with a wide variety of different substrates, activators and even inhibitors like MEKs, MAPK phosphatases [29], protein tyrosine phosphatases [30], Elk1, RSK [31].

Regulation of ERK cascade

There are several different mechanisms through which the activity of the ERK cascade is regulated. Among these the primary regulation is at the level of the ERK itself which is mostly mediated by the MAPK phosphatases (MKP). Since the dual phosphorylation at the Threonine-X-Tyrosine motif is essential for ERK activity both threonine phosphatases and the tyrosine phosphatases can efficiently inactivate ERKs. The MKPs are widely distributed within the cell. Like for instance MKP1, MKP2, PAC1 and B23 are localized within the nucleus while MKP3 is localized within the cytosol and the localization of MKP3/6 has been shown to change between the nucleus and the cytosol.
depending upon the cell type. All the MKPs known till date are inducible proteins and their expressions are regulated in response to growth factors and cellular stresses [32]. For example nerve growth factor (NGF) stimulation can initiate MKP3 expression in PC12 cells whereas serum stimulation can only weakly induce this expression [33]. Besides MKPs certain protein serine/threonine phosphatases (PP) and protein tyrosine phosphatases (PTP) take part in MAPK inactivation. The cascade is also regulated through the differential localization of the component proteins within various intracellular compartments. In most resting cells the components of the MAPK cascades are apparently localized primarily in the cell cytosol. However in response to extracellular signals this localization changes that aid in the transmission of the signal further. For instance in ERK cascade extracellular stimulus induces Raf-1 recruitment to the plasma membrane [34] and translocation of MEKs, ERKs and RSKs into the nucleus [35, 36]. Certain functions of ERKs and RSKs are completely dependent on their appropriate subcellular localization. For example expression of some genes requires the activity of ERKs and hence their translocation to the nucleus, the prevention of which causes inhibition of the respective gene expression. Similarly RSK2 activity in the nucleus was found to be necessary for EGF induced transcription of c-Fos gene [37]. Besides being tightly regulated the components of the ERK cascade are specific for their substrates, activators and inhibitors. The strength of protein protein interaction varies significantly among different components. For instance in PC12 cell line stimulation by nerve growth factor (NGF) results in sustained activation of ERKs that leads to differentiation into cells containing developed neurites while stimulation by epidermal growth factor (EGF) results in transient activation of ERKs that is essential for subsequent proliferation of the cells [38]. Thus the duration and the strength of the signal also aid in determining the specificity of the extracellular signals mediated by the ERK cascade.

**Physiological roles of ERK cascade**

The ERK cascade possesses a wide variety of different functions within the cell. It is known to be involved in diverse cellular processes like development, stress response, learning and even morphology determination. The rapid activation of MEKs and ERKs in response to mitogens in various cell lines has implicated these kinases in the control of cell proliferation. Moreover, as soon as the ERK cascade had been elucidated, it was
noticed that it may participate in the transmission of many mitogenic and oncogenic signals that lead to the accelerated proliferation observed with malignant transformation. Among the approx 200 oncogenes that are known to date, many have been proven to encode proteins that participate in the cascade of events by which growth factors stimulate normal cell division [39, 40]. Moreover the sustained activation of ERKs had been shown to be required by many cells to pass the G1 restriction point and to enter the S phase of the cell cycle [41]. In most cell types and conditions ERK cascade also plays an antiapoptotic effect and a reduction of its activity is essential for the induction of apoptosis. Thus in serum starved PC12 cells it was shown that ERK cascade is inhibited in correlation to cell death [42]. Similarly activation of ERK cascade protects NIH3T3 cells from doxorubicin induced cell death [43]. In contrast to prevention of cell death in some cases ERK cascade has been found to be associated with the induction of apoptosis as well. Like for instance de novo synthesized ceramide induces apoptosis in astrocytes through ERK cascade [44] and an involvement of the ERK cascade in taxol induced apoptosis has also been noticed [45]. Thus ERKs play a central role in the regulation of most of the important cellular processes.

**ERK7 and ERK8**

ERK7 is a 61 kDa protein having a C-terminal extension of 195 amino acids. ERK 7 is ~47% homologous to ERK1/2 in its kinase domain and contains T-E-Y activation motif. It is not activated through upstream MEKs. The potential substrates of ERK7 include c-Fos and c-Myc but not Elk-1, c-Jun and ATF2. ERK7 C-terminus contains a putative nuclear localization signal and two SH3 domain binding motifs. ERK7 is targeted to the nucleus in both active as well as inactive states and can function as a negative regulator of growth independent of its kinase activity. ERK7 has an appreciable kinase activity in serum starved cells. Autophosphorylation is sufficient for its activation and its C-terminal domain regulates its activity. Moreover ERK7 is also rapidly ubiquitinated and degraded by proteasome. ERK7 may play cytostatic or cytotoxic roles and the loss of ERK7 expression correlates with the progression of breast cancer [46, 47, 48].

ERK8 is a 60 kDa protein and ~69% similar to ERK7. The C-terminal tail of ERK8 contains two SH3 domain binding motifs. Src associates with and activates ERK8.
Chapter V  Functional characterization of E. histolytica MAPK

The expression of ERK8 is high in lung and kidney. ERK8 seems to function in longer term responses to mitogenic factors [46, 49]. ERK8 activity is largely determined by the phosphorylation of threonine within the T-E-Y activation motif and it depends upon the rate of ERK8 auto- and dephosphorylation. Like ERK7 it is also not activated by MEK1/2/5. ERK8 activity could be increased by exposure to \( \text{H}_2\text{O}_2 \), the Ser/thr phosphatase inhibitor okadeic acid or more weakly by osmotic shock. Moreover the major residue in myelin basic protein that is phosphorylated by ERK8 is different from that of ERK2 [48].

4. The p38 MAPK cascade

The p38 kinases were first defined in a screen for drugs inhibiting TNF\( \alpha \) mediated inflammatory responses [50]. Since then four distinct isoforms of p38 MAPK with more than 60% overall sequence homology and more than 90% identity within the kinase domains have been described in human tissues [51]. Despite their high sequence homologies these isoforms have notable differences in their tissue expressions, upstream activators and downstream effectors. Like all other MAPKs p38 MAPKs are also activated through the phosphorylation of the threonine and the tyrosine residues within the Thr-X-Tyr activation motif (the X being Glycine in case of p38) in response to a variety of external stimuli. Unlike ERKs p38 MAPKs however have been found to be involved in a number of different cytokine dependent inflammatory diseases like rheumatoid arthritis, Crohn’s disease, psoriasis and asthma. Besides these activation of p38 MAPKs has been implicated in both detrimental and protective processes in the myocardium and the development of the cardiovascular diseases [52].

Regulation of p38 signalling pathway

Activation of p38 isoforms has been found to be controlled through different regulators and co-activated by various combinations of upstream regulators [53, 54]. Although two different classes of MAPKK namely MKK3 and MKK6 are actually involved in the activation of p38 MAPK they possess differential activities towards different p38 isoforms. For instance while MKK6 is a potent activator of p38\( \beta \), MKK3 is completely unable to effectively activate the same [55]. Besides MKK4, an upstream kinase of JNK has also been shown to aid in the activation of p38\( \alpha \) and p38\( \delta \) in specific
Chapter V  
Functional characterization of E. histolytica MAPK

cell types [56]. However in addition to MAPKK mediated activation of p38 MAPK there is also a MAPKK independent activation mechanism operative within the cell. It includes the participation of TAB1 (transforming growth factor-β-activated protein kinase 1 (TAK1)-binding protein). The activation of p38 in this pathway is achieved by the autophosphorylation of p38α after interaction with TAB1 [57]. The activity is however terminated through the dephosphorylation mediated by many dual specificity phosphatases which act on both the threonine as well as the tyrosine residues within the activation motifs. In addition, other phosphatases like serine/threonine protein phosphatase type 2C (PP2C) has been shown to have a role in downregulating the MAPK HOG1 pathway as well as negatively regulating human MKK6 and MKK4 levels in vitro and in vivo [58, 59].

Substrates of p38 MAPKs

P38 MAPKs possess a wide range of substrates including protein kinases and transcription factors. The first p38 MAPK substrate identified was the MAP kinase-activated protein kinase 2 (MAPKAPK2 or MK2) [60]. Later on, several other substrates including different transcription factors encompassing a broad range of actions were identified. The substrates of p38 MAPK include ATF-1/2/6, SRF accessory protein (Sap1), CHOP (growth arrest and DNA damage inducible gene 153 or GADD153), p53, C/EBPβ, myocyte enhance factor 2C (MEF2C) and high mobility group-box protein 1 (HBP1) [61-64].

5. The c-Jun N-Terminal Kinase (JNK) Cascade

As their name suggests the JNKs have been characterized by their ability to associate with and phosphorylate regulatory sites in the N-terminus of the transcription factor c-Jun [65]. In mammals there are three JNK genes (jnk1, jnk2 and jnk3) each on a different chromosome [66]. Each mammalian JNK gene however is subjected to alternative splicing such that the three genes are predicted to give rise to at least 10 different JNK proteins [67]. These 10 different JNK isoforms differentially recognize and activate their transcription factor substrates [67]. JNKs are strongly activated in response to cytokines, UV radiation, growth factor deprivation, DNA-damaging agents, environmental stress and to a lesser extent some G-protein coupled receptors, serum and
Chapter V  

Functional characterization of E. histolytica MAPK

growth factors. c-Jun, one of the well known substrates of JNKs represses the expression of several MAPK phosphatases and thus regulates the efficiency of signaling by ERK, p38 and JNK. Among several other transcription factors phosphorylated by JNKs are ATF-2, NF-ATc1, HSF-1, Elk-1, p53, c-Myc and STAT3.

6. Protozoal MAPKs

In the recent years a lot of study has been done to investigate the molecular and functional characteristics of the protozoal MAPKs. Several studies have revealed some prime functions of the kinase in the protozoal life cycle. All these informations contribute towards the understanding of the parasite pathobiology and posses the potential to be exploited for therapeutic interventions.

Dictyostelium discoideum MAPK

D. discoideum expresses two ERKs, ERK1 is developmentally regulated and essential for vegetative growth and multicellular development while ERK2 is essential for receptor mediated activation of adenyl cyclase and also for differentiation, pattern formation and amoeboid chemotaxis. D. discoideum grows vegetatively as single amoeba but on starvation, a multicellular developmental program is initiated in which aggregation is coordinated by pulsatile emissions of cAMP which acts as a chemoattractant for surrounding cells. Binding of extracellular cAMP to GPCR transiently stimulates phosphorylation, activation and nuclear translocation of DdERK2. Thus a self regulating system controls the chemotactic signaling mechanism of Dictyostelium, where pulses of cAMP are generated with a periodicity of 7 minutes and spontaneous oscillations in activation of ERK2 occurred in phase. ERK2 modulated cAMP levels through the phosphodiesterase RegA. The chemoattractants cAMP and folic acid activate DdERK2 which then coordinates the formation of cell projections [68-72].

Giardia intestinalis MAPK

ERK1 and ERK2 of G. intestinalis play a critical role in the trophozoite differentiation into cysts, having different subcellular localization and activation in trophozoites and encysting cells. The localization of ERK1 into basal bodies presumably reflects its potential association with microtubule associated proteins. The search of the Giardia genome sequencing database revealed the presence of putative homologues of
Chapter V  

Functional characterization of *E. histolytica* MAPK

Giardial MEK and MEKK suggesting the presence of the three kinase module of the MAPK pathway in *Giardia* [73].

*Leishmania mexicana* MAPK

Nine MAPK homologues have been genetically characterized in *L. Mexicana*. The mRNA levels of the MAPK homologues varied significantly in the pro and the amastigote life stages of the parasite [74, 75]. *LmxMPK1* is required for amastigote survival and proliferation in macrophages. Its deletion mutant was able to infect host cells and transform to amastigotes but was unable to proliferate. *LmxMPK1* has been portrayed as a promising drug target [76, 77]. *LmxMPK2* however is involved in the regulation of the adenylyl cyclase activity affecting differentiation [74].

*Trypanosoma brucei* MAPK

The first ERK homologue of *T. brucei* was named KFR1 (KSS1- and FUS3-related kinase 1). Its activity was found to be enhanced in the bloodstream form than in the insect (procyclic) form of the parasite. The activity of the bloodstream form was however found to be drastically reduced by serum starvation that arrested cell growth. Interferon-γ strongly enhanced the KFR1 activity thus suggesting its involvement in mediating the interferon-γ induced proliferation of *T. brucei* in mammalian hosts [78-80]. *TbMAPK2* is homologous to *LmxMPK4* and bloodstream forms of its null mutant when triggered to differentiate developed into the procyclic form with markedly delayed kinetics and underwent cell cycle arrest. *TbMAPK5* is involved in the growth and differentiation of the bloodstream forms. *TbMAPK2* and *TbMAPK5* might be counterparts of the control of proliferation of procyclic and bloodstream forms respectively [78, 79].

*Toxoplasma gondii* MAPK

*T. gondii* is an apicomplexan parasite that possesses 3 MAPKs. *TgMAPK1* is 58 kDa and displays 45% identity with human p38α and 35-45% with ERKs. *TgMAPK2* is 72 kDa and shares 64% identity with human ERK8. The third MAPK has recently been annotated possessing TGH motif. *TgMAPK1* transcript accumulation increased in trophozoites subjected to osmotic stress and upon conversion of tachyzoites to the latent bradyzoite form in vitro. Further p38 MAPK inhibitors blocked *T. gondii* replication,
Chapter V Functional characterization of E. histolytica MAPK

induced cyst like structures, upregulate bradyzoite specific molecules and downregulated tachyzoite specific molecules and also blocked TgMAPK1 autophosphorylation [81-83].

*Plasmodium falciparum* MAPK

The human malaria parasite *P. falciparum* possesses two distinct MAPK homologues Pfmap-1 and Pfmap-2. Pfmap-1 is expressed during erythrocytic schizogony and in gametocytes while Pfmap-2 is detectable only in the later stages. Pfmap-2 possesses the atypical TSH motif with both the T and the H residues important for its kinase activity. Further the insertions located in the immediate vicinity of this site also suggest a divergent mode of activation/regulation compared with that of mammalian MAPKs [84-86].
RESULTS

1. Purification of recombinant EhMAPK and subsequent characterization of anti EhMAPK antibody

The transformed *E. coli* BL21 DE3 cells containing the EhMAPK clone in pET30 Ek/LIC vector, when induced with 1 mM IPTG for 3 h at 37°C produced the recombinant EhMAPK protein with the 6X His tag. This was further confirmed by western blot analysis using anti 6X His antibody which showed the unambiguous presence of a band migrating at ~ 47 kDa in the induced sample only (fig 2a). The observed molecular weight also conforms to that theoretically calculated (~40 kDa + ~7 kDa vector encoded tag). The subsequent purification of the protein from the inclusion body on a Ni-NTA agarose column yielded about 95% pure recombinant EhMAPK protein (fig 2b). Antibody against the purified recombinant EhMAPK was then raised in rabbit. The specificity of the antisera against EhMAPK was analysed by western blot with *E. histolytica* total cell lysate and purified recombinant EhMAPK with both the pre and the post immune sera. The blots indicated a clear recognition of the endogenous EhMAPK as well as the purified recombinant EhMAPK displaying bands at the expected molecular weights of ~ 40 kDa and ~ 47 kDa respectively by the post immune sera but not by the pre immune sera (fig 2c).
Chapter V  Functional characterization of E. histolytica MAPK

Fig 2. Expression, purification and antibody production of recombinant EhMAPK. (a) Western blot analysis with mouse anti-His antibody to check the expression of recombinant His tagged EhMAPK. Lane 1: uninduced E. coli BL21DE3 cell lysate, Lane 2: Cell lysate from E. coli BL21DE3 induced with 1 mM IPTG at 37°C for 3 h. (b) Purification of recombinant His tagged EhMAPK protein. Lane 1: Protein molecular weight marker (Fermentas), Lane 2: Uninduced transformed E. coli BL21DE3 cell lysate, Lane 3: Cell lysate from transformed E. coli BL21DE3 induced with 1 mM IPTG at 37°C for 3 h, Lane 4: Inclusion body solubilised in 50 mM Tris pH 8, 300 mM NaCl, 8 M urea and 10 mM imidazole, Lane 5: Purified recombinant EhMAPK. (c) Western immunoblotting with post and pre immune sera from rabbits immunized with purified recombinant EhMAPK. Lane 1-3: 1 µg of purified recombinant EhMAPK, 20 µg and 40 µg of E. histolytica total cell lysate (TCL) respectively probed with post immune rabbit sera, Lane 4-6: 1 µg of purified recombinant EhMAPK, 20 µg and 40 µg of E. histolytica TCL respectively probed with pre immune rabbit sera.

2. Characterization of the kinase activity of the refolded recombinant EhMAPK.

Kinase assay with the purified recombinant refolded EhMAPK indicated Mg$^{2+}$ dependent kinase activity of the protein. As is evident from the fig 3a, EhMAPK undergoes autophosphorylation besides being able to phosphorylate MBP, a typical MAPK substrate. Although no significant change in the kinase activity of the protein was observed when Mg$^{2+}$ concentration was varied from 10 mM to 30 mM, the activity was completely abolished in the absence of the divalent cation. So, 10 mM Mg$^{2+}$ was used in all further kinase assay experiments. In order to further characterize the autophosphorylation activity of recombinant EhMAPK kinase assays were performed in the absence of MBP and both in the presence and absence of ATP. The resulting products were probed with anti-phospho tyrosine, anti-phospho serine/threonine, anti-phospho ERK1/2 and anti-EhMAPK antibodies in several western blots. Fig 3b shows that recombinant EhMAPK can get autophosphorylated on both serine/threonine as well as tyrosine residues. However to confirm that the phosphorylated threonine and tyrosine are actually the one from the TXY activation motif of the protein the autophosphorylated EhMAPK was probed with anti phospho ERK1/2 antibody which is raised against the phosphorylated TXY motif of human p44/42. The recognition of the phosphorylated EhMAPK by the anti-phospho ERK1/2 antibody strongly suggests the autophosphorylation occurs on threonine and tyrosine residues within the TXY activation motif.
motif of the protein. Unlike other antibodies, antibody raised against recombinant EhMAPK as expected recognized the recombinant protein in both the kinase assay samples (in the presence and in the absence of ATP). The next question that we tried to address was whether the heterologous anti-phospho ERK1/2 antibody could recognize the singly phosphorylated TXY motif in EhMAPK. Although the activation site sequence of EhMAPK is highly homologous with other mammalian MAPKs (fig 3c) a considerable deviation in the activation lip containing TXY motif has already been noticed in the atomic model of the protein built on rat ERK2 as template [87]. So, to investigate whether this deviation has any effect on the recognition of the singly phosphorylated forms of the protein by the antibody we carried out kinase assay with the recombinant protein followed by incubation with either PTP1B or PP2A. Being a tyrosine specific phosphatase the PTP1B treatment will in effect generate threonine phosphorylated form of recombinant EhMAPK. Similarly treatment of the dually phosphorylated recombinant EhMAPK with serine/threonine specific phosphatase PP2A will generate tyrosine phosphorylated form of the protein. A subsequent western blot analysis of the treated samples with antiphospho ERK1/2 will then indicate the specificity of the antibody. Thus the resulting products were probed with one of the four antibodies mentioned above in different western blots. As evident from fig 3c unlike untreated kinase assay samples neither of the PTP1B treated samples nor the PP2A treated samples were recognized by anti-phospho ERK1/2 antibody indicating the specificity of the antibody to only the dually phosphorylated TXY motif of EhMAPK. On the contrary the anti EhMAPK antibody recognized both the treated as well as the untreated samples, anti phospho tyrosine recognized the untreated and the PP2A treated samples and anti phospho serine/threonine antibodies recognized the untreated and the PTP1B treated samples as previously expected thereby serving as proper controls of the experiment. To critically evaluate the kinase activity of EhMAPK that is singly phosphorylated at its TXY motif, radioactive kinase assays were performed in the presence of either of the phosphatases. Although in the absence of the phosphatases a significant kinase activity was found both with respect to the EhMAPK itself as well as MBP, in the presence of either of the phosphatases no kinase activity of the protein was found towards its substrate. However the autophosphorylation was still found albeit slightly lower compared to the untreated
samples (fig 3d). Although this experiment gives a preliminary idea about the relative inactivity of the protein in its singly phosphorylated forms further experiments are needed to establish the fact. Moreover significant signals obtained from the EhMAPK bands of the kinase assays with either of the two phosphatase treated samples indicate that despite the presence of one of the phosphatases, the phosphorylation event at the unaffected residue within the activation motif was occurring. The intensities when compared to that of the phosphatase untreated samples were lower which further confirms the dephosphorylation at either threonine or tyrosine residue depending on whichever phosphatase is present in the reaction mixture. But when the intensities of the substrate MBP was compared almost no signal was obtained from the phosphatase treated samples. Although the data from this experiment is good enough to negate the kinase activity of threonine phosphorylated EhMAPK towards MBP, no conclusion can be drawn for tyrosine phosphorylated form of the protein since a kinase assay in the presence of PP2A will lead to the dephosphorylation of the substrate MBP at serine/threonine residues in addition to the dephosphorylation at threonine residue within the TXY activation motif of EhMAPK. Thus to further evaluate the activities of the singly phosphorylated forms of the protein, kinase assays were performed with both the purified T178A and the Y180A mutant EhMAPK proteins. As expected none of the mutant proteins showed any activity towards MBP when compared with the wild type and the autophosphorylation was also found to be significantly lower than that of the wild type protein (fig 3e). This observation further raised the question regarding the autophosphorylation event at the phosphorylatable residues within the TXY motif of the mutant EhMAPK proteins. A non radioactive kinase assay with the recombinant EhMAPKs in the absence of MBP followed by western blot analysis with either anti-phospho serine/threonine, anti-phospho tyrosine, anti-phospho ERK1/2 or anti EhMAPK antibodies revealed that despite the substitution of one of the phosphorylatable residues within the TXY motif of the mutant EhMAPKs the phosphorylation at the other residue was still taking place (fig 2f). Like for instance following kinase assay, T178A mutant although not recognized by anti-phospho threonine was clearly recognized by anti-phoshtyrosine indicating a phosphorylation event has taken place at tyrosine residue. Similar results were obtained in case of Y180A mutant the phosphorylated form of which was not recognized by anti-
Chapter V  Functional characterization of E. histolytica MAPK

phospho tyrosine but distinctly by anti-phospho serine/threonine antibody. In addition to these neither of the phosphorylated mutant EhMAPKs was recognized by anti-phosphoERK1/2 which again confirmed the specificity of this heterologous antibody towards the dually phosphorylated form of EhMAPK.
Fig 3. Kinase assay with the recombinant refolded EhMAPK. (a) Mg$^{2+}$ dependence of the kinase activity of EhMAPK, lane 1-4: 0.0 mM to 30.0 mM Mg$^{2+}$, lane 5: Protein blank. Both the EhMAPK (top) and the MBP (bottom) have been shown. In each figure lower panel represents coomassie staining of the respective proteins and upper panel represents the autoradiograph of the corresponding dried gel. (b) Western blot of the EhMAPK kinase assay samples with anti-phospho tyrosine, anti-phospho serine/threonine, anti-phospho ERK1/2 and anti-EhMAPK antibodies respectively from top to bottom, either in the presence (lane 1) or in the absence (lane 2) of ATP. (c) Sequence alignment of EhMAPK and mammalian MAPKs showing the conservation of the active site sequence; the highlighted region indicate the TXY activation motifs; Hs, Homo sapiens ERK8 (AAH40897); Rn, Rattus norvegicus MAPK15 (NP_775453); Mm, Mus musculus MAPK1 (NP_001033752). Western blot of purified EhMAPK kinase assay samples after treatment with either tyrosine phosphatase, PTP1B or serine/threonine phosphatase, PP2A. The blots were probed with anti-phospho tyrosine, anti-phospho serine/threonine, anti-phospho ERK1/2 and anti-EhMAPK antibodies. Lane 1: in the absence of both PTP1B and PP2A but in the presence of ATP, lane 2: In the presence of PTP1B and ATP but in the absence of PP2A, lane 3: in the presence of PP2A and ATP but in the absence of PTP1B, lane 4: In the absence of ATP, PTP1B and PP2A. (d) Kinase assay in the presence of phosphatases. Both the EhMAPK (top) and MBP (bottom) have been shown. In each figure lower panel represents coomassie staining of the respective proteins and the upper panel represents autoradiograph of the corresponding dried gels. Lane 1: In the absence of either of the phosphatases, lane 2: in the presence of PTP1B, lane 3: in the presence of PP2A, lane 4: EhMAPK Blank. (e) Kinase assay with the wild type and mutant EhMAPKs. Both the EhMAPK (top) and MBP (bottom) has been shown. In each figure lower panel represents coomassie staining of the respective proteins and upper panel represents the autoradiographs of the corresponding dried gels. Lane 1: wild type, lane 2: T178A mutant EhMAPK, lane 3: Y180A mutant EhMAPK. (f) Western blot analysis of the kinase assay samples from wild type and mutant EhMAPKs. The blots were probed with anti-phospho serine/threonine, anti-phospho tyrosine, anti-phospho ERK1/2 and anti EhMAPK (top to bottom), lane 1: wild type, lane 2: T178A mutant EhMAPK, lane 3: Y180A mutant EhMAPK.

3. Effect of heat shock and H$_2$O$_2$ treatment on the transcript levels of EhMAPK gene

The mRNA levels of *E. histolytica* MAPK gene was found to remain unaltered in response to either heat shock or H$_2$O$_2$ treatment. When compared to control, the transcript levels from the trophozoites exposed to heat shock for up to 60 min at 43°C did not show any significant change as evident from fig 4a. Similarly no change in the transcript levels of EhMAPK was noticed in the trophozoites exposed to either 0.5 mM H$_2$O$_2$ for different time periods up to 90 min or 2.0 mM H$_2$O$_2$ for up to 30 min (fig 4b). We have previously
Chapter V  

Functional characterization of E. histolytica MAPK

studied the effect of different concentrations of \( H_2O_2 \) on the viability of \( E. \ histolytica \) trophozoites and we found that 2.0 mM \( H_2O_2 \) can drastically reduce the viability of the trophozoites to about 5% over a time period of 6 h but 0.5 mM \( H_2O_2 \) cannot [88]. In the present study the trypan blue viability assay for control, heat shocked and 0.5 mM \( H_2O_2 \) treated cells showed approximately the same viability over a time course of 3 h. But upon treatment with 2.0 mM \( H_2O_2 \) the viability remained comparable with the other sets for only until 30 min of incubation after which the viability started reducing drastically (fig 5). Under these conditions the viability falls to about 30% when incubated for about 3 h. Hence the 30 min time point was chosen as the optimum time of incubation for further experiments with the 2.0 mM \( H_2O_2 \) treated samples. Moreover the heat shocked trophozoites as well as the 0.5 mM \( H_2O_2 \) treated trophozoites showed no change in viability over a period of 3 h. So in the present study we treated the trophozoites with both the lethal 2.0 mM \( H_2O_2 \) for 10, 20 and 30 mins as well as 0.5 mM \( H_2O_2 \) for 15, 30, 45, 60 and 90 min. However in all the cases no change in mRNA levels of EhMAPK was evident when compared to control untreated trophozoites (fig 4b).
Chapter V  Functional characterization of E. histolytica MAPK

Fig 4. Semi-quantitative RT-PCR showing the transcript levels of EhMAPK gene in H2O2 treated, heat shocked and untreated E. histolytica trophozoites. (a) EhMAPK mRNA levels of E. histolytica trophozoites subjected to heat shock at 43°C for different time periods. Lane 1: untreated control trophozoites, Lane 2-5: heat shock at 43°C for 15, 30, 45 and 60 minutes respectively. (b) EhMAPK mRNA levels of E. histolytica trophozoites exposed to 0.5 mM or 2.0 mM H2O2 for different time periods. Lane 1: untreated control trophozoites, Lane 2-6: 0.5 mM H2O2 treatment for 15, 30, 45, 60 and 90 minutes respectively, Lane 7-9: 2.0 mM H2O2 treatment for 10, 20 and 30 minutes respectively.

Fig 5. Effect of heat shock and H2O2 on the viability of E. histolytica trophozoites. The vital dye trypan blue was used to discriminate between live and the dead trophozoites. As compared to the untreated control trophozoites (▼) which shows a stable viability curve, 2.0 mM H2O2 treated trophozoites (▲) however are associated with a gradual loss of viability being about 30% at the end of 3 h incubation period. The viability curve for 0.5 mM H2O2 treated trophozoites (●) is more or less similar to that of control. Similar is the case with heat shock which also shows a stable viability curve. The viabilities are represented as percentage of the viable cells out of the total population.
4. Heat shock at 43°C and 0.5 mM H₂O₂ treatment induces increased phosphorylation whereas 2.0 mM H₂O₂ treatment induces dephosphorylation of endogenous EhMAPK

The expression profile of EhMAPK at the endogenous protein level matched with that of the mRNA content among untreated, heat shocked and H₂O₂ treated samples in all cases of dose and time. But a significant difference was obtained in the phosphorylation status of the protein in response to different stresses. For instance, probing the immunoblots with anti-phospho ERK1/2 antibody revealed that in response to heat shock at 43°C, the levels of phosphorylated EhMAPK increased as early as 15 min of exposure and became saturated at 60 min exposure (fig 6a). Similar was the observation with 0.5 mM H₂O₂ where an increase in the phosphorylated EhMAPK was evident within 45 min to 90 min of incubation (fig 6b). Moreover the most interesting part was the dephosphorylation of the protein at a lethal dose of 2.0 mM H₂O₂. As shown in fig 6b an exposure to 2.0 mM H₂O₂ induces dephosphorylation of EhMAPK as early as 10 min of exposure and the effect lasts for even about 30 min of exposure. The densitometric analysis of the bands corresponding to the endogenous phosphorylated MAPK, total MAPK and actin from the respective western blots gives an estimate of the relative changes of the proportions of phosphorylated MAPK to total MAPK in control untreated trophozoites and those treated with either heat shock at 43°C or 0.5 mM H₂O₂ or 2.0 mM H₂O₂ for different time points. The phosphorylated MAPK to total MAPK ratio in the trophozoites exposed to heat shock at 43°C for 60 min is ~ 2.8 times that of the control untreated trophozoites (fig 6c). However in case of H₂O₂ treatment an exposure to 0.5 mM H₂O₂ for 90 min leads to ~ 1.8 times higher and an exposure to 2.0 mM H₂O₂ for 30 min leads to 4 times lower intracellular phosphorylated MAPK to total MAPK ratio when compared to the untreated control trophozoites (fig 6d). Moreover in order to confirm the specificity of the heterologous anti-phospho ERK 1/2 antibody towards the phosphorylated form of EhMAPK in vivo, a λ phosphatase assay was carried out. Since the aim of the experiment was to simply check the phosphospecificity of the antibody a single sample was considered. Both λ phosphatase-treated and λ phosphatase untreated TCL from control trophozoites and those exposed to heat shock at 43C for 60 min were subjected to western blot analysis with anti phospho-ERK1/2, anti-EhMAPK and anti-
Chapter V  Functional characterization of E. histolytica MAPK

actin antibodies (fig 7). The absence of bands corresponding to phosphoMAPK in λ phosphatase-treated samples confirmed the specificity of the heterologous anti-phospho ERK 1/2 antibody to the phosphorylated form of the endogenous E. histolytica MAPK.

Fig 6. Western immunoblotting of total cell lysates from untreated and treated E. histolytica trophozoites. Upper panel: anti phospho ERK1/2, Middle panel: anti EhMAPK, Lower panel: anti actin. (a) Differential phosphorylation of intracellular EhMAPK in response to heat shock. Lane 1: untreated, Lane 2-5: heat shock at 43°C for 15, 30, 45 and 60 min respectively. (b) Differential phosphorylation of intracellular EhMAPK in response to H2O2 treatment. Lane 1: untreated, Lane 2-6: 0.5 mM H2O2 for 15, 30, 45, 60 and 90 min respectively, Lane 7-9: 2.0 mM H2O2 for 10, 20 and 30 min respectively. (c) Densitometric analysis representing the proportions of phosphorylated MAPK to total MAPK and total MAPK to actin in E. histolytica trophozoites subjected to heat shock at 43°C for different time periods as a relative change over that of the control untreated trophozoites. (d) Densitometric analysis representing the proportions of phosphorylated MAPK to total MAPK and total MAPK to actin in E. histolytica trophozoites treated with either 0.5 mM H2O2 or 2.0 mM H2O2 for different time periods as a relative change over that of the control untreated trophozoites. Data represent mean ± S.D. of three independent experiments.
Fig 7. Western immunoblotting of either lambda phosphatase treated or untreated total cell lysate from *E. histolytica* trophozoites. Upper panel: anti-phospho ERK1/2, Middle panel: anti-EhMAPK, Lower panel: anti-actin. Lane 1&2: Untreated and heat shocked at 43°C for 60 min *E. histolytica* total cell lysate respectively without lambda phosphatase treatment, Lane 3&4: Untreated and heat shocked at 43°C for 60 min *E. histolytica* total cell lysate respectively with lambda phosphatase treatment.

5. Differential kinase activities of the endogenous EhMAPK in response to heat shock and H₂O₂ treatment

In the mammalian cells it is well established that the kinase activity of MAPK is associated with the phosphorylation of threonine (T) and tyrosine (Y) residues in the TXY motif of the activation loop [89, 90]. In *E. histolytica* the kinase assays with the purified recombinant EhMAPK also revealed a significant kinase activity associated with only the dually phosphorylated form of the protein which is also recognized by anti-phospho ERK1/2 antibody. So, to investigate the kinase activity of EhMAPK in vivo, kinase assay was performed with the phosphorylated EhMAPK which was immunoprecipitated with anti-phospho ERK1/2 antibody and its ability to phosphorylate MBP was evaluated. As expected a significant kinase activity was found to be associated with the phosphorylated EhMAPK. However when the kinase activities of the phospho EhMAPKs from the treated samples were compared to the untreated control samples significant changes were noticed that correlated well with their differential endogenous levels as revealed by immunoblots in fig 6. For instance the increase in the kinase activity of the phospho EhMAPK is clearly evident in case of heat shock at 43°C for 60 min (fig 8a) and 0.5 mM H₂O₂ treatment for 90 min (fig 8b) whereas a decrease in the kinase activity is observed in case of 2.0 mM H₂O₂ treatment for 30 min (fig 8b). A
densitometric analysis of the MBP bands in the autoradiographs and coomassie stained gels represents ~2.6 times increase in the phosphorylated MBP to total MBP in case of *E. histolytica* trophozoites treated with heat shock at 43°C for 60 min (fig 8c), ~1.8 times increase in case of 0.5 mM H₂O₂ treatment for 90 min and ~4 times decrease in case of 2.0 mM H₂O₂ treatment for 30 min (fig 8d) when compared to untreated control samples. Moreover the western blot analysis of the immunoprecipitated phospho EhMAPK with anti-EhMAPK antibody yielded similar results with a higher proportion of dually phosphorylated MAPK in case of heat shock treatment for 60 min (fig 9a) and 0.5 mM H₂O₂ treatment for 90 min and a lower proportion in case of 2.0 mM H₂O₂ treatment for 30 min (fig 9b). However the change in the amount of phosphorylated EhMAPK in response to different treatments when compared to the untreated control could be due to either the change in the amount of the protein getting dually phosphorylated or dephosphorylated or due to the change in the amount of phosphorylation on individual protein molecules if there exist some singly phosphorylated EhMAPK population within *E. histolytica* trophozoites. Although within the scope of the present study we could not discriminate between these two possibilities a clear evidence of activity associated with the dually phosphorylated and not the singly phosphorylated protein towards its substrate MBP was obtained. So, our data is therefore indicative of the increase in the dually phosphorylated EhMAPK in response to heat shock and 0.5 mM H₂O₂ treatment and a decrease in response to 2.0 mM H₂O₂ treatment with corresponding increase or decrease in the activity towards its substrate.
Chapter V  Functional characterization of E. histolytica MAPK

Fig 8. Kinase assay with phosphorylated EhMAPK immunoprecipitated with anti-phospho ERK 1/2 antibody from E. histolytica TCL. Lower panel: Coomassie stained gel showing MBP, Upper panel: corresponding autoradiograph. (a) Assay with heat shock samples. Lane 1: untreated, Lane 2: Heat shock at 43°C for 60 min, Lane 3: IP blank (kinase assay in the absence of immunoprecipitated protein). (b) Assay with H₂O₂ treated samples. Lane 1: untreated. Lane 2: 0.5 mM H₂O₂ for 90 min, Lane 3: 2.0 mM H₂O₂ for 30 min, Lane 4: IP blank. (c) Densitometric analysis representing the relative kinase activities of the phosphorylated MAPK from E. histolytica trophozoites exposed to heat shock at 43°C for 60 min with respect to that of control untreated trophozoites. (d) Densitometric analysis representing the relative kinase activities of the phosphorylated MAPK from E. histolytica trophozoites treated with either 0.5 mM H₂O₂ for 90 min or 2.0 mM H₂O₂ for 30 min with respect to that of control untreated trophozoites. Data represent mean ± S.D. of two different experiments.

(a) Control HS60 — IgG heavy chain
Phospho MAPK

(b) Control P0.5/90 P2/30
Phospho MAPK

Fig 9. Western immunoblotting of the phosphorylated EhMAPK that is immunoprecipitated with anti-phospho ERK1/2, with anti EhMAPK antibody. Phosphorylated EhMAPK was immunoprecipitated with antiphospho ERK1/2 antibody from the total cell lysate of E. histolytica trophozoites either untreated or treated and probed with anti EhMAPK antibody (a) Assay with heat shock samples. Lane 1: untreated, Lane 2: heat shock at 43°C for 60 min. (b) Assay with H₂O₂ treated samples. Lane 1: untreated, Lane 2: 0.5 mM H₂O₂ for 90 min, Lane 3: 2.0 mM H₂O₂ for 30 min.
Chapter V Functional characterization of E. histolytica MAPK

DISCUSSION

E. histolytica genome codes for a single typical MAPK that clusters with the ERK7/8 family of MAPKs. Although its sequence has already been characterized in a previous publication from our group [87] its functional implication is largely unexplored till date. Our present study however indicates a differential kinase activity of the protein in response to different stresses. In a number of different mammalian cell lines ERK/MAPK activation has often been associated not only with cellular growth and proliferation induced by a number of proliferation-inducing agents [92, 92] but also with different stresses like heat shock, arsenite and oxidative stress [93-95]. Our present study emphatically indicates a possible involvement of EhMAPK in heat shock and oxidative stress response in the protozoan. Moreover a moderate basal level of phosphorylation accompanied by a detectable amount of kinase activity indicates a probable critical role of EhMAPK in normal cellular homeostasis/maintenance as well which is in accordance with the general function of ERK subfamily of MAPKs. However a significant increase in the phospho-EhMAPK species in response to heat shock and 0.5 mM H$_2$O$_2$ treatment, the stresses which the protozoan can withstand and a corresponding decrease in response to 2.0 mM H$_2$O$_2$ treatment which is lethal for the parasite points towards a possible role of EhMAPK at the cross-road of cell survival and cell death in response to a specific stress. Our data also prompts us to assume the presence of some upstream elements that regulate the phosphorylation of EhMAPK and activities of which may be directly altered in response to the stresses. Since the parasite genome lacks MEK, the kinases that usually phosphorylate and activate MAPKs in mammals, there seems to be the existence of some other mechanisms of increasing phosphorylation of EhMAPK during stress survival.

The phylogenetic closeness between protozoan MAPKs and mammalian ERK 7/8 has already been noted by Ward et al. [96] in P. falciparum, by our group in E. histolytica [87] and in T. gondii by Lacey et al [81]. The mammalian ERK 7/8 are also phosphorylated on the TEY motif but not by the MEK. Autophosphorylation has been identified as a predominant mechanism of activation of ERK7/8 [97, 48, 98]. Also, enhanced phosphorylation of ERK8 is observed under DNA-damaging stress conditions.
Chapter V  Functional characterization of E. histolytica MAPK

including peroxide stress [98]. Therefore, a distinct possibility exists that an autophosphorylation mechanism may be responsible for the increase in phosphorylation of EhMAPK during heat and 0.5 mM hydrogen peroxide stresses. The Entamoeba genome codes for a number of protein phosphatases [99] and a possible involvement of these upstream phosphatases in the maintenance of the phosphorylation status of EhMAPK may be envisaged.

The kinase assays with the recombinant EhMAPK and subsequent immunoblot analysis using anti-phospho tyrosine, anti-phospho serine/threonine and anti-phospho ERK1/2 antibodies have indeed shown the ability of the protein to undergo autophosphorylation on both threonine as well as tyrosine residues within its TXY activation motif. Moreover one of the interesting features found to be associated with the kinase activity of the recombinant protein towards its substrate MBP was the inability of the singly phosphorylated forms of the protein to carry out substrate phosphorylation as revealed by the kinase assay experiments with the recombinant EhMAPK in the presence of either PTP1B, a tyrosine phosphatase or PP2A, a serine/threonine phosphatase and with the T178A and Y180A mutant EhMAPK proteins.

So we conclude that E. histolytica MAPK probably functions as a key signaling molecule for the parasite, responsible for regulating its survival and death strategies in response to different environmental stress. Moreover our data reveals that the autophosphorylating ability of EhMAPK may be an important regulatory mechanism. Besides, the Entamoeba genome contains several MAPK phosphatase and dual phosphatase sequences [99]. Whether these phosphatases play a role in determining the level of phosphorylation of EhMAPK remains an intriguing question. A study of these upstream elements in relation to EhMAPK activation during stress exposure of E. histolytica will shed significant light on the stress response mechanism of the parasite. Based on our data we have hypothesized a model to decipher the mechanisms of stress response mediated by EhMAPK. According to this model the activity of EhMAPK can be assumed to be regulated by a critical balance between its autophosphorylating ability and the activity of the inhibitory phosphatases under normal conditions of cellular homeostasis. Exposure to stress can increase the predominance of the autophosphorylation mechanism resulting in the activation of EhMAPK, thereby enabling
Chapter V  Functional characterization of *E. histolytica* MAPK

the parasite to withstand/overcome the stress while a lethal stress probably results in the predominance of the protein phosphatases (fig 10) leading to the inactivation of MAPK and its subsequent inability to rescue the trophozoites from the stress.

![Diagram of stress response mechanism of EhMAPK](image)

**Fig 10. Model depicting the stress response mechanism of EhMAPK.** Under normal unstressed conditions a basal level of phosphorylated EhMAPK is maintained through three distinct phosphorylation and dephosphorylation events which include the EhMAPK autophosphorylation, the phosphorylation by some unknown upstream kinases and the dephosphorylation by some unknown upstream phosphatases. In response to a stress however this critical balance between the phosphorylation and dephosphorylation events is disturbed. While a non-lethal stress may lead to the predominance of the phosphorylation event mediated through either upstream kinases or the autophosphorylation ability of EhMAPK which results in the increased phospho EhMAPK species in the trophozoites that promotes its survival, a lethal stress on the other hand results in the predominance of the dephosphorylation event thereby leading to a decreased level of phosphorylated EhMAPK and a subsequent loss in viability of the trophozoites.
REFERENCES


Chapter V Functional characterization of E. histolytica MAPK


Chapter V  Functional characterization of E. histolytica MAPK


Chapter V Functional characterization of E. histolytica MAPK


Chapter V Functional characterization of E. histolytica MAPK


