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Identification and functional analysis of a stress-responsive MAPK15 in *Entamoeba invadens*

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ABSTRACT

E. histolytica, a protozoan parasite is the causative agent of amoebiasis in human beings. It exists in two different forms – the motile trophozoite form which undergoes encystation under starvation conditions to form the non-motile, osmotically resistant cyst form. Cellular stresses stimulate several signaling cascades which assist the parasite in counter-attacking such conditions thereby, promoting cell survival. To study the stress-associated pathways activated during encystation, we have used *Entamoeba invadens*, a reptilian parasite as a model organism because of its ability to undergo encystation under *in vitro* conditions. In this study, we have identified a stress-responsive MAPK which gets upregulated under different stress conditions, including encystation. Sequence analysis and phylogenetic classification show that the MAPK belongs to the atypical MAPK15 family (henceforth, named EiMAPK15), which does not require an upstream MAPKK for its phosphorylation and activation. The *in vitro* kinase activity of recombinant EiMAPK15 exhibits its auto-phosphorylation ability. Immunolocalization studies reveal that the protein is mainly cytosolic under normal growing conditions but gets translocated into the nucleus under stress conditions. Knockdown of EiMAPK15 using double-stranded RNA was found to reduce the expression of other encystation-specific genes which in turn, resulted in the decline of the overall encystation efficiency of the cells. Overall, the present work has laid the platform for further characterization of this important MAPK gene in *Entamoeba invadens*.

1. Introduction

Entamoeba histolytica is a monogenetic protozoan endoparasite which causes amoebiasis in human beings. Amoebiasis is a diarrheal disease in humans caused by the protozoan parasite *Entamoeba histolytica*. It is estimated that about 40,000–100,000 people worldwide die annually due to amebiasis [1]. This disease is transmitted by ingestion of cyst-contaminated food or water [2]. *Entamoeba histolytica* has a biphasic life cycle alternating between the motile trophozoite form, which upon starvation undergoes encystation to form the dormant, four-nucleated cyst form [3]. Throughout its life cycle, the parasite is exposed to wide variety of harsh environmental conditions such as high concentration of reactive oxygen species (ROS) and reactive nitrogen species (RNS) during tissue invasion, high oxygen pressure inside host tissues, high temperature, pH fluctuations and osmolarity difference inside and outside the host body [4]. Such changes trigger specific signaling cascades which eventually prepare the parasite to cope with the stressful environment. Mitogen-activated protein kinase (MAPK) signaling pathway is one of those stress-responsive pathways. MAPKs are a family of serine/threonine kinases that play a pivotal role in the transduction of extracellular signals into a diverse range of intracellular responses like cell proliferation and differentiation, cell survival, cell death and immune responses [5,6].

Based on the ability of mode of phosphorylation and activation, MAPKs are classified into two types: conventional MAPKs and atypical MAPKs. A conventional MAPK signaling pathway is a three-tiered cascade of kinase enzymes wherein a MAPK kinase kinase (MAP3K) phosphorylates and activates a MAPK kinase (MAP2K) which further phosphorylates and activates the effector MAPKs. Conventional MAPKs consist of ERK1/ERK2, p38s, JNKs, and ERK5 [7,8]. The conventional MAPKs are also characterized by the presence of a highly conserved TxY (Threonine-x-Tyrosine) motif in its activation loop that gets phosphorylated at threonine and tyrosine residues to attain a catalytically active conformation [9]. On the contrary, atypical MAPKs do not require any upstream kinase enzyme for its phosphorylation and activation. They have an inherent ability to undergo auto-phosphorylation. Atypical MAPKs include ERK3/ERK4, NLK (Nemo-like Kinases) and ERK7 (also referred as, MAPK15) [10]. ERK3, ERK4, and Nemo-like kinase lack the TxY motif in its activation loop. Their tyrosine residue is replaced by glycine or glutamic acid residue [11]. Interestingly, ERK7/

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MAPK15 contains the TxY motif in its activation loop, but there is no evidence that it gets phosphorylated by any upstream MAPKK family. Recent studies have revealed that phosphorylation of this TxY motif is catalyzed by MAPK15 itself [12].

MAPK signaling pathway is an evolutionarily highly conserved pathway across all eukaryotic organisms including plants, fungi, and animals [13]. However, not much has been investigated in this regard in *Entamoeba*. Only single MAPK homologue, associated with cell survival has been described so far in *E. histolytica* [14], but its downstream targets or upstream activating kinases are yet to be explored.

In this study, a novel MAPK belonging to the MAPK15 family has been identified in *E. invadens* (EiMAPK15). MAPK15 is the most recent addition to the MAPK family, which is reported to be involved in cell transformation [15], protection of genomic integrity [16], DNA damage repair [17], and autophagy [18]. To understand the significance of MAPK signaling pathway during encystation, *Entamoeba invadens*, a reptilian parasite, has been chosen as a model organism because of its ability to undergo encystation under *in vitro* conditions as there is no available encystation medium for *E. histolytica* [19].

For functional analysis of EiMAPK15, RNAi technique was used [20]. Knockdown of EiMAPK15 resulted in the reduced expression of several encystation-specific genes involved in chitin wall formation, suggesting its significance in overall encystation process. Here, we also provide evidence that EiMAPK15 is predominantly localized in the cytoplasm of the trophozoite under the normal growing conditions but a fraction of this protein gets translocated inside nucleus under stress conditions. Further, the kinase activity of both, the recombinant and native EiMAPK15 suggests that the protein can auto-phosphorylate itself and does not require any upstream kinase for its activation.

To the author's knowledge, this is the first report for the involvement of an atypical MAPK15 gene in encystation of *Entamoeba*, and the information presented here will lay the foundation for further characterization and importance of this gene in the survival of the parasite within the host body.

2. Materials and methods

2.1. Sequence characterization

2.1.1. Multiple sequence alignment

MAPK15 protein sequences from different organisms were retrieved from publicly available UniProt database [21] (http://www.uniprot.org) and aligned with EiMAPK15 using Clustal Omega [22] (http://www.ebi.ac.uk).

2.1.2. Identification of conserved motifs

The conserved motifs present in EiMAPK15 were identified by using MEME software available online [23] (http://meme-suite.org/tools/meme). The putative Nuclear Localization Signal present in EiMAPK15 was predicted by using cNLS Mapper [24] (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi). The presence of putative SH3 binding motif was predicted using SH3PepInt [25] (http://modpepint.informatik.uni-freiburg.de) while the putative ubiquitination site was predicted using UbPred [26] (http://www.ubpred.org)

2.1.3. Conserved domain analysis

The domains present in EiMAPK15 were analyzed by CD-Search tool [27] (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

2.1.4. Phylogenetic tree construction

Protein sequences of 73 MAPKs belonging to different families of MAPK were retrieved from UniProt database for phylogenetic classification of EiMAPK. These sequences were aligned using CLUSTALW. The tree based on this amino acid sequence alignment was reconstructed by employing Maximum Likelihood (ML) method using the MEGA software package 6.06 version [28]. WAG model was used to estimate the evolutionary distance between the pair of amino acid sequences. The ML estimation of the rate heterogeneity among sites was explored under the assumption that the rate variation followed an invariant + gamma distribution. Gaps were handled using "partial deletion" option in which sites with missing data were not completely removed. The reliability of the tree topology was assessed by bootstrap (parsimony) analysis with 1000 resampling replicates.

2.2. Cloning, overexpression of recombinant EiMAPK15 and antibody production

2.2.1. Plasmid constructs for overexpression of recombinant EiMAPK15

The 1053 base pair gene encoding EiMAPK15 was amplified by PCR using specific primers (Supplement Table 1) and subcloned into a TA cloning vector, pGEM®-T Easy Vector (Promega) followed by cloning into the pQE-30 expression vector (Qiagen). The plasmid was then used to transform *E. coli,* SG13009 cells for recombinant Histidine-tagged protein expression. The sequence of the cloned gene was confirmed by sequencing.

2.2.2. Purification of recombinant his-tagged protein and antibody production

The transformed *E. coli* SG13009 cells were grown overnight at 37 °C in the shaker incubator. The following day, the overnight culture was diluted to 1/100 in fresh LB medium and grown at 37 °C until OD_{600} reached 0.6. At this point, the bacterial cells were induced to express the recombinant protein by adding 1 mM IPTG followed by 3 h post-induction incubation at 37 °C. Cells were then centrifuged at 5000 rpm for 15 min. To purify the recombinant protein from inclusion body, the cell pellet was suspended in lysis buffer (50 mM Tris, 300 mM NaCl, 5 mM β-mercaptoethanol, pH 8.0 with 1X protease inhibitor cocktail (Sigma) and sonicated till the lysate became clear. The lysate was then centrifuged at 10,000 rpm for 30 min to remove all the proteins present in the soluble fraction. The pellet was solubilized in solubilization buffer (50 mM Tris, 300 mM NaCl, 8 M Urea and 5 mM Imidazole, pH 8.0) for 2 h at room temperature. The solubilized fraction was then purified by Ni-NTA affinity chromatography. The purified and denatured protein was refolded by gradual dialysis steps in refolding buffer containing 6 M urea, 4 M urea, 2 M urea, and Tris-HCl buffer (pH 8.0) at 4 °C. The purified proteins were separated by SDS-PAGE, and the protein bands were cut and outsourced to Abgenex, India for antibody production. The Antibody was raised against the purified protein in New Zealand rabbits. The immune sera received, was further purified by Protein A-sepharose affinity chromatography and concentrated for further application.

2.2.3. Western blotting

For western blot analysis, proteins obtained from cell lysates were resolved by SDS-PAGE, transferred to PVDF membrane (Millipore), probed with appropriate antibodies and detected with DAB system using DAB (3,3´- diaminobenzidine) substrate (Genei) and hydrogen peroxide or enhanced chemiluminescence detection (Merck Millipore).

2.3. RNAi interference

2.3.1. Plasmid constructs for expression of double-stranded RNA

To downregulate the expression of EiMAPK15 at RNA level, a 254 bp region at C-terminal was identified as a target for RNA interference. To make the double-stranded RNA highly specific to EiMAPK15 gene, it was ensured that the target sequence did not contain complete 19-mer homology to any other gene of *Entamoeba invadens* [29]. For the construction of dsRNA expression vectors, the target region was PCR amplified and cloned into the MCS region of the pL4440 vector which is bidirectionally flanked by T7 promoters. The resulting recombinant plasmid was used to transform RNaseIII-deficient *E. coli* strain, HT115(DE3) (rnc14::DTn10) for dsRNA expression. As a negative