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Role of kinases in virulence and pathogenesis of protozoan parasite *E. Histolytica*

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1. ABSTRACT

Protein kinases are known to regulate several cellular processes like metabolism, motility and endocytosis through phosphorylation of specific target proteins which forms a communication system relaying extracellular signals to intracellular milieu for an adaptive response. One of the protozoan parasite *Entamoeba histolytica*, which causes amoebiasis and is one of the prominent reason for causing diarrhoea in infants of developing countries, where it remains the third leading cause of deaths in infants(1). The genome of this parasite codes for 331 putative protein kinases which accounts for 3.7% of the proteome. The kinome of the parasite is composed of several conserved and as well as kinase with unusual domain architecture. About one-third of kinome codes for transmembrane kinases (TMK) which is proposed to help the parasite to sense and adapt to the gut environment which is constantly changing. Many kinases are known to be involved in virulence but, the kinome of this important parasite is unexplored. In this review, we present an overview of *E. histolytica* kinases and their role in amoebic biology understood till now.

2. INTRODUCTION

Entamoeba histolytica is a human enteric protozoan parasite which causes amoebiasis. It is one of the leading parasitic burdens in developing countries, contributing to an estimated 100,000 deaths annually(2, 3). A large fraction of infected individual remains asymptomatic, and only 10% of infected individuals show symptoms of the disease. Primary clinical manifestation includes diarrhoea, dysentery, intestinal ulcers and extraintestinal invasive form of the disease show liver, lungs and brain abscesses in less than one percent infected individuals(4). However, factors responsible for turning asymptomatic condition into clinical manifestation are not clear but gut microbiota has been shown to play an important role (3). National Institute of Allergy and Infectious Disease (NIAID) classifies *E. histolytica* as a category B biodefence pathogen because of its environmental stability, low

infectious dose, chlorine resistance, and easy dissemination through contaminated food and water. Even in low-incidence settings, these properties of the parasite leading to outbreaks among military and general populations(5).

The life cycle of *E. histolytica* is simple, consisting of two stages, either an infectious cyst or invasive trophozoite form. The infection is transmitted after the ingestion of the food or water contaminated with *E. histolytica* cysts and according to new finding it may also occur during sexual contact, among homosexual man groups(6). Following ingestion, excystation to trophozoites occurs, and the released trophozoites migrate to the large intestine, multiplying by binary fission to produce more cysts or trophozoites may invade host tissue and may cause disease.

Tissue destruction mediated by *E. histolytica* occurs in three steps: attachment to host cells, lysis and phagocytosis of dead cells. The death of host cells may lead to inflammation or invasion of tissues by a parasite. Several molecules, such as Gal/GalNAc lectin, serine-rich *Entamoeba histolytica* proteins, and lipopeptidephosphoglycan, have been suggested as possible molecules involved in the attachment of *E. histolytica* to intestinal epithelial cells of the host(7). Upon adherence, *E. histolytica* induces multiple cytotoxic effects on host cells including increased intracellular Ca^{2+}, reactive oxygen species production, loss of membrane integrity, DNA fragmentation, phosphatidylserine (PS) exposure, and caspase-3 activation(8). Past experiments have established a strong link between amoebic cytotoxicity and amoebic phagocytosis and motility. A great deal of subsequent work established a model of sequential adherence, contact-dependent cytotoxicity, and amoebic phagocytosis(9). *In vitro*, *E. histolytica* preferentially ingests apoptotic cells via recognition of exposure of PS and collectins on the surface of apoptotic cells, though the molecular interactions were not defined until recently. Amoebic calreticulin (CAL) was found to be the surface receptor for host C1q. CAL was required for phagocytosis of apoptotic cells but did not mediate killing(10). Following attachment, parasite releases pore forming peptides called ‘amoebapore’, which are structurally similar to mammalian granulolysin and Natural killer cells lysin(11). Apart from amoebapore peptide homologue of haemolysin III has been found in the parasitic genome which may be involved in cell lysis. Hence, multiple factors might be involved in host cell lysis. Another means of killing host cells is the activation of the apoptotic pathway(12). Following the death of cells, amoeba phagocytoses the dead cell mass. The amoebic kinase EhC2PK was found to recruit amoebic Calcium binding protein 1 (EhCaBP1) and subsequently initiate the phagocytosis(13). The initiation of phagocytosis is accompanied with actin polymerization which is again an area of active research in parasite involving alpha kinases, p21 activated kinases, Rho GTPases etc(14). The process finally completes with membrane scission and formation of the endosome.

A recent discovery of trogocytosis by parasite changed the paradigm of sequential adherence, cytotoxicity, and ingestion of host cells by *E. histolytica* trophozoites. The parasite was found to ingest pieces of intact living cells via trogocytosis which contributed to the invasion and destruction of host tissues. Trogocytosis is an active process that is similar to phagocytosis at the molecular level, as the involvement of EhC2PK, Phosphoinositide kinase and actin(15). These similarities suggest that the relationship between amoebic adherence, cytotoxicity, and ingestion is much more dynamic than previously appreciated. Although trogocytosis and phagocytosis are mechanistically similar, but recently an AGC family kinase, EhAGCK1 was shown to be exclusively involved in amoebic trogocytosis(16). This is the first report on trogocytosis specific molecule being identified. This finding indicates that kinases play an important role in activating specific signalling cascade even in processes which are very similar like, phagocytosis and trogocytosis. But the mechanistic details of the operation and regulation are yet to be understood. Furthermore, now it is widely accepted that kinases play an important role in processes which regulate virulence of the parasite, so it becomes more important to understand the role of these kinases in amoebic biology. Moreover, *E. histolytica* strains which are tolerant to higher doses of metronidazole are already reported so it becomes a priority to find an alternative line of treatment timely(17). Many

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classes amoebic kinases are divergent from host kinases, hence it will worthwhile to develop them as drug targets. This review will summarise the bioinformatics and experimental information available for amoebic kinase for better assessment and analysis.

3. KINOME OF E. *HISTOLYTICA*

Despite being a single cell organism, the genome codes for about half the number of kinases as in human. Presence of a large number of protein kinase indicates that protein phosphorylation is a crucial mechanism of the regulation of cellular activities in this parasite(18). *E. histolytica* used an array of receptors present mainly on the cell surface that transduces signal intracellularly through various pathways. The diversity of these pathways helps organisms to adapt to the host environment and respond appropriately. It is likely that some of the signaling pathways may play an essential role in the host-parasite relationship.

E. histolytica genome codes for 331 putative protein kinases like sequences. Twenty-four sequences were found to lack conserved aspartate in the catalytic loop(19, 20). These sequences which lack conserved aspartate are unlikely to functional and are referred to as kinase homology domain. But their functional status can be validated through experimental methods only as these are assumptions made based on existing databases. The roles of such pseudokinases in *E. histolytica* are currently unclear and not worked upon. However, the presence of kinase homology domains (KHD) in other unicellular parasites like *Plasmodium falciparum* suggests an important role, possibly in the regulation of the phosphorylation network(21). The remaining 307 sequences of putative protein kinases with conserved catalytic aspartate can be expected to be functional. Out of 307 sequences selected, 296 sequences of putative kinase domains contain at least one glycine-rich loop motif GXGXXG in subdomain I of kinase catalytic domain. However, in 11 sequences glycine-rich loop was found to be absent but it is possible that these kinases might be functional as it has been seen in other organisms, a kinase with no glycyl residue in the “Gly-rich” region can bind ATP(22). In *E. histolytica* high divergence is observed in key residues involved in nucleotide binding as compared to other eukaryotic kinases of similar class so the possibility of these 11 putative kinases being functional cannot be ruled out.

Based on the amino acid sequence of the catalytic domains, 195 putative kinase sequences of *E. histolytica* could be classified into various subfamilies as described by Hanks and co-workers(23). Furthermore, 195 putative kinase sequences could be classified into 24 AGC family kinase, 36 Calmodulin-dependent protein kinase (CAMK), 42 cyclin-dependent protein kinase, mitogen activated protein kinase, glycogen synthase kinase, casein kinase-2 (CMGC), 55 protein tyrosine kinase (PTK) and 38 other protein kinase (OPK). However, 112 sequences could not be assigned any class, and this was largely due to unusual domain compositions and structural differences. Thirty-seven of these unclassified protein kinases are predicted to have single span transmembrane region. Although, classification of these sequences is not possible, but these possess conserved domains with known functions. Mostly, these kinase domains are present in combination with SH2 domain, Laminin EGF domain, Rho-GEF, Leucine rich repeats and Ankyrin repeats etc. Some of the unclassified kinases form close paralogues and hence may be performing important functions. The sequences of unclassified kinases show a maximum identity of 30% with plant or other protozoan kinases. There are several other domain compositions which have only been found in *E. histolytica* till date like, CaM like kinase with endonuclease, Src like kinase with a transmembrane domain. Although the function of these kinases is yet unknown but research leading to discovering their function in the biology of parasite will also reveal the evolutionary details of these kinases and their functions.

E. histolytica genome encodes 307 putative protein kinases, which is approximately three times the number of kinases coded by the genome of the malarial parasite *Plasmodium falciparum(2*4, 25) and doubles the number of kinases coded by *Leishmania major*. The number of kinases expressed by the host of the parasite i.e. human is only double(26, 27) . In Figure 1, we compare the total number of kinases encoded by different organisms. This indicates the

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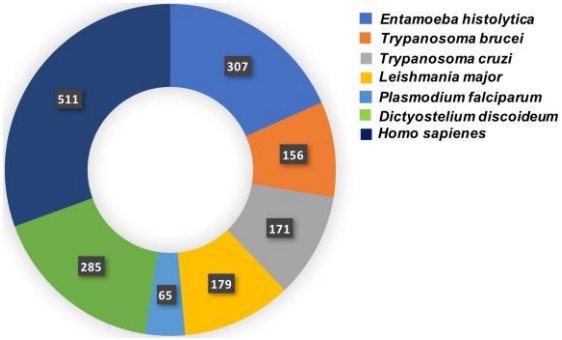

Figure 1. The pie-chart showing the number of kinases encoded by genomes of different organisms. The parasite *E. histolytica* codes for the highest number of kinases than many other parasites and 50% of the human. This is an indication that this parasite can sense the rapidly changing gut environment and generating appropriate response for survival and pathogenesis by the parasite through various classes of kinases(18, 24-27).

existence of a very extensive and intricate signalling system in the parasite which enables it to adapt and survive in the rapidly changing gut environment and turn into invasive form when provided the opportunity.

4. TRANSMEMBRANE KINASES INVOLVED AS A RECEPTOR

Genome analysis of *E. histolytica* reveals that parasite possesses 90 trans-Membrane kinases (TMKs) which are responsible for sensing the extracellular environment and host tissues and membranes(28). The invasion by *E. histolytica* involves adherence as the first step, here TMK can possibly play a great role in identifying the ligand present on host cells and subsequently activating the corresponding pathway for internalization. It is interesting to note that Human genome codes for about 80 TMKs, which is lower than this parasite(29). Figure 2 shows the comparison between TMKs encoded by other known model organisms.

All members of the TMK family contain an N-terminal signal sequence, an extracellular domain, and a single transmembrane helix followed by a cytosolic kinase domain. TMK is of interest for not only their potential role in antigenic variation but also for their role in cell signaling. It is surprising that *E. histolytica* also codes for tyrosine kinase-like domain, which is unique to this parasite. Beck *et al*, has classified these TMKs in 6 families and all of them express in trophozoite stage and analysis of motifs presents in the sequences of TMK revealed that in spite of the presence of conserved motifs these are quite divergent from the known kinases and likely to be dual specificity kinases(28). Table 1 summarizes the TMK classification(28). Out of 6 families, only group B family has been studied to an extent. This group is further sub-classified in B1, 2, 3 families. The B1 family includes 35 members and they exhibit ≥95% sequence identity at both nucleotide and amino acid sequence level. Mostly divergence is observed at 5'- end while 3' end is conserved(30). Thirteen members of this family do not have clear open reading frame due to presence of stop codon and hence may not code for functional kinases. TMKB1-3 kinase is constitutively expressed at low levels while expression of TMKB1-9 has been shown to be regulated by serum. And this is most abundant TMK expressed during proliferation(31). Further unsaturated lipid associated with BSA is shown to regulate the expression(32). Another member Phagosome-associated TMK96 (PATMK), which belongs to B3 family was identified to participate in erythrophagocytosis and is uniquely required for intestinal infection but not hepatic infections. Over-expression of truncated PATMK in trophozoites revealed that it reduces the ability to infect the intestine but its ability to cause liver abcess was unaffected when directly introduced in the organ(33). More detailed studies are required to understand if this TMK is acting as a receptor for apoptotic dead cells or as a regulator of ingestion via its kinase domain. TMK39 belonging to Group C is found to participate in phagocytosis. Trophozoites expressing kinase dead mutant of TMK39 displayed defect in phagocytosis of *E. coli* but not of LDL particles(34). This indicates that there exists specific receptor-ligand based endocytosis in this parasite is yet to be discovered in detail. Another group E member, TMK54 is expected to be involved in the regulation of expression of Gal/GalNAc lectin heavy subunit. TMK54 can possibly regulate the virulence of the parasite as Gal/GalNAc lectins are involved in adhering to host cells(28). Although a great deal of research is required to uncover the role of TMKs in amoebic biology but current data available indicates that TMKs are involved in sensing the extracellular environment, identifying the ligands to be internalised

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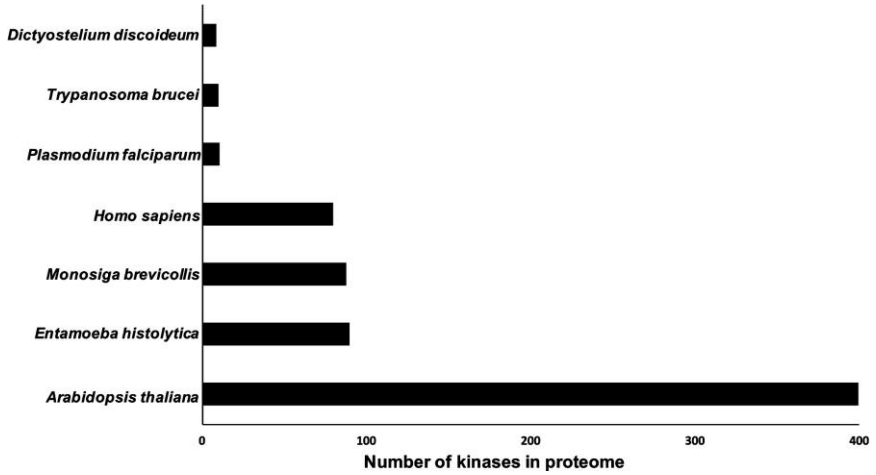

Figure 2. Bar diagram showing a number of predicted transmembrane kinases in kinome of various organisms. Comparatively *E. histolytica* genome codes for the highest number of TMKs amongst protozoans and the number is more than encoded by the human genome. This information indicates that this parasite efficiently senses and adapts to gut environment while undergoing excystation and encystation in small and large intestine respectively. The signalling mediated by TMKs is also important during the invasion of host tissues by the parasite under favourable circumstances(28, 29).

and respond to chemical or hormonal signals. More detailed understanding of protozoan TMKs will help define the mechanisms that these organisms use to respond to their environment.

5. PHOSPHOINOSITIDE KINASES

Although phosphoinositide kinases are not protein kinases but still, we have considered them in this review, as they play a very crucial role in endocytic processes and motility of the parasite. Phosphoinositide is ubiquitous and minor constituents of the cell membrane. After adherence to the host cells signals is transduced by activation kinases and phosphatases which modify phosphoinositides in the plasma membrane of a parasite. The formation and breakdown of various kinds of phosphoinositides occur in a controlled manner. It plays an important role in regulating diverse cellular process such as cytoskeleton reorganization, membrane trafficking endocytosis process(35). Like in other organisms, phosphoinositides are central to processes like phagocytosis and motility which determine the virulence of the parasite(36).

In higher eukaryotes, phosphoinositide kinases are classified into 3 types based on their substrate specificity, subcellular localization and function despite this they have significant amino acid sequence homology in the catalytic kinase domain(37, 38). Genome analysis of *E. histolytica* reveals that its genome codes for only type I and type III PIPK family while type II PIPK is missing. It was also found that *E. histolytica* has a single gene encoding EhPIPKI for a generation of Phosphoinositide (4,5)bisphosphate (PtdIns (4,5)P_2) from Phosphoinositide 4-phosphate (PtdIns (4)P)(39). Both homologs shared 30%-40% overall sequence identity to the corresponding human PIPK proteins. Based on activation loop amino acid sequences phosphoinositide is named as type I (EhPIPKI) and type III (EhPIPKIII) subfamily. In higher eukaryotes, the type I and II PIPK exhibit three isoforms (α, βand γ). However, *E. histolytica* PIPK families do not have any isoforms. Amoebic type I phosphoinositide kinase, EhPIPKI shows different substrate specificity as compared to mammalian counterparts(39).

Although, EhPIPKI can phosphorylate substrate other than preferred PtdIns (4)P, but the specificity is very high as compared to mammalian homologue. By sequence analysis, it is found that conserved residues ‘GXSGS’ which has G loop and Lys147 residue is present in EhPIPKI which is

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Table 1. Classification of *E. histolytica* TMKs

TMK Group	Signature motif in the kinase domain	Extracellular domain size	No. of amino acid between TM to the kinase domain	No. of motifs			
				CXC	CXXC	CXXXC	CXXCXXGYY
A	CC(I/V)KITDFGTSR	517-532	220	4	4/5	3/4	3
B_1	KLTDFGS(A/S)R	897-916	135	1	25	2	9
B_2	KLTDFGS(A/S)R	822-1762	133	0	90	0	25
B_3	KLTDFGS(A/S)R	830-2117	145	0	24	0	7
C	C(A/G)KLTDFGTC	547-624	160	0	33	0	11
D_1	PITAKVTDFGTS	520-619	234	3	5	3	7
D_2	V(T/V)(C/V)KV(T/S)DFGTS	399-614	233	4	5	3	9
E	AKLSDFGTSR	401-412	150	1	0	0	0
F	VKVSDFGLS and WXAPE	231-368	24	0	0	0	0

Note: The table shows the conserved features of kinase domains present in TMKs and the length of the extracellular domain in the peptide sequence. The large number of CXXC-motif containing TMKs might be involved in antigenic variation and cell signalling required for survival or invasion of host tissues(28).

involved in nucleotide (ATP) binding(40). Kinase domain of EhPIPKI is larger than that seen in mammalian orthologs and makes up almost the entire length of the protein (residues 20-646). Several pieces of evidence indicated that plasma membrane-bound full-length EhPIPKI undergoes cleavage and gives rise to two fragments, N-terminal cytosolic and C-terminal associated with the plasma membrane. This processing of EhPIPKI is unique to amoeba and is not known elsewhere. Experimentally it is shown that both wild type and C-terminal part of the protein is targeted to the plasma membrane by dilysine motif (KK) and glutamate residue in the activation loop(39). These membrane targeting residues are found to be conserved from yeast to mammals(41). In many ways, EhPIPKI is different from homologues found in other organisms as it displays high substrate specificity, post-translational processing, and the presence of unique insertion tandem repeat (RD) sequence.

EhPIPKIII has smaller PIPK domain and lacks both conserved FYVE and CCT domains. This enzyme phosphorylates PtdIns (3)P at D-5 and yields PtdIns (3,5)P_2. The potential role of PtdIns (3,5)P_2 is not clear in this parasite, although it has been suggested that PtdIns (3)P takes part in phagosome maturation(39). But it can be expected that this protein may work with one of the many FYVE domain containing protein expressed by amoeba.

6. CAM-LIKE KINASES INVOLVED IN PHAGOCYTOSIS

Following the Phosphoinositide generation after adherence to a target host cell, there is a high probability of calcium flux, which has not been demonstrated experimentally. Nevertheless, it has been shown that chelating cellular Ca^{2+} ions by BAPTA-AM cause a decrease in phagocytosis by the parasite(42). The role of calcium signalling is indirectly unveiled through a C2 domain containing kinase, EhC2PK(13). This protein shows maximums sequence similarity in the kinase domain, i.e. 45% with calcium/CaM dependent protein kinases of human(13). EhC2PK is one of the crucial kinases that helps in initiating phagocytosis in *Entamoeba histolytica.* The C2 domain of the protein binds phosphatidylserine in a Ca^{2+} dependent manner and recruits EhCaBP1 (Calcium Binding Protein 1) to the membrane to the site of phagocytosis. The calcium-binding defective mutant of EhCaBP1 binds EhC2PK and localizes to the site of phagocytosis, hence the recruitment of former protein is Ca^{2+} independent. EhCaBP1 further helps in the recruitment of other molecules like actin and Arp2/3 complex to newly initiated phagocytic cups(43). The recruitment of multiple proteins by EhCaBP1, which itself is a small EF-hand containing proteins can be explained by its trimeric structure which is formed by the interaction

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Table 2. Percentage of kinome represented by the Ca^{2+}/CaM-like kinases in various parasites

Organisms	Total kinases in the genome	CAMK (Ca^{2+}/Calmodulin dependent protein kinase)	Percentage of CAMK with respect to total kinase in the genome
Entamoeba histolytica	307	36	12%
Plasmodium falciparum	65	13	20%
Trypanosoma brucei	156	14	8%
Leishmania major	179	16	8%
Dictyostelium discoideum	285	21	7%
Note: It is important to note that *E. histolytica* is next to *P. falciparum* in order(25, 26). Presence of 36 Ca^{2+}/CaM-like kinases indicates the intricate Ca^{2+} signalling networks present in the parasite which are unknown.			

of each molecule in head to tails manner(44). The overexpression of the C2 domain and kinase dead domain in trophozoites showed dominant negative phenotype. The activity of the kinase domain was found to be essential for initiation of the phagocytic cup, but the enzyme was completely lost during the progression of the cup. The protein autophosphorylates itself at serine 428 positions. This kinase is also unique in terms of domain composition as mostly PKC like kinases are present with C1/C2 domains. The mass spectrometric analysis of phagosome proteome has also revealed the presence of some CaM-like kinases(45). Thirty-six CaM-like kinases are encoded by the genome of amoeba account for roughly 12% of kinome (Table 2) which indicates that this parasite has extensive Ca^{2+} dependent signalling pathways which are yet to be explored. Although many amoebic CaM-like kinases lack conserved CaM- binding motifs like "IQ" motif but, it is interesting to note that *E. histolytica* genome codes for 27 calcium binding proteins and some of which are shown to be involved in phagocytosis it is tempting to expect that CaM-like kinases may relay the signalling in concert with them. Moreover, presence of six out of 36 Ca^{2+}/CaM-like kinases (EHI_011180, EHI_143800, EHI_155280, EHI_069290, EHI_151730, EHI_186820) in phagosome proteome also indicates their involvement in endocytic processes and actin cytoskeleton remodelling(18).

7. ALPHA KINASES INVOLVED IN THE VIRULENCE OF *E. HISTOLYTICA*

Once the process of phagocytosis has initiated at the plasma membrane, the actin dynamics come in to play. There is rapid polymerisation of actin beneath the membrane which drives the process. *E. histolytica* genome codes for Arp (Actin related protein) subunits, formin, and coactosin which all contribute in actin polymerisation but however conserved WASP protein could not be identified in the genome although WASP related protein could be annotated. In *E. histolytica* actin phosphorylation has been found to be important and it is regulated by alpha kinases. There are five alpha kinases encoded by *E. histolytica* genome, in which two kinases reported as EhAK1 and EhAK2 possess unusual domain organization. Their structure consists of the alpha kinase at the N terminal and SH3 domain at the C terminus. The combination of the SH3 domain with catalytic alpha kinase domain has not been reported for known alpha kinases till now(46). In the remaining three kinases the C terminus consists of the kinase domain and no other domain appear in these proteins. The kinase domain of EhAK1 shows maximum sequence similarity of 30% with the alpha kinase domain from *D. discoideum.* The kinase core of EhAK1 comprises of 8 conserved subdomains and the C terminus of the protein and has zinc finger motifs, which is common among other known alpha kinases(46). In EhAK1 kinase domain, some conserved residues are present like lysine at eighty-fifth positions, which help in the binding of dATP and mutating this amino acid leads to a kinase dead mutant. EhAK1 is recruited by EhCaBP1 to the site of phagocytosis in a Ca^{2+} dependent manner. It is worth noting that EhAK1 binds with EhCaBP1 in calcium dependent manner which is different from EhC2PK-EhCaBP1 binding where no calcium is required. Hence phagocytic pathways have steps dependent and independent of Ca^{2+}. The major substrate for EhAK1 is G-actin; these alpha kinases phosphorylate the actin at threonine 107. The phosphorylation has

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been shown to enhance the rate of polymerisation. The kinase itself has been shown to trans-autophosphotylates at three positions. Threonine at 279 has been an important phosphorylation site as phosphorylation mutants are defective in autophosphorylation as well as substrate phosphorylation. Also, it has been found that overexpress phosphorylation site mutant (T279A) leads to a defect in the phagocytosis process and thus cell loses its invasive abilities (14). These alpha kinases have unusual substrate specificity and phosphorylate specific serine and threonine residues of the alpha helix present in the substrate(47). The kinase activity of EhAK1 is indispensable for the progression of a phagocytic cup and hence the completion of the process. Still, the role of other alpha kinases in amoebic biology is yet unexplored and further work will reveal their functional relevance.

8. AGC FAMILY KINASES

The interaction of an amoebic cell with target host cells leads to changes in phosphoinositides in the plasma membrane of a parasite. The generation of PtdIns (4,5)P_2 and PtdIns (3,4,5)P_3 due to activation of PIPK results in recruitment further downstream AGC family kinases. AGC kinases are the subgroup of serine/ threonine protein kinases and their subfamily consist of PKA, PKG and PKC. Total of 24 AGC family kinases are encoded by *E.histolytica* genome and so far only one has been found to be involved in the endocytic process recently(16). Their maximal activity and activation are achieved by phosphorylation at two highly conserved regulatory motifs at T/activation loop and hydrophobic motif. These T loops are present in the catalytic domain while the hydrophobic motif is presented at non-catalytic region following the kinase domain. In spite of these regulatory motifs, AGC possesses an important phosphorylation site which is responsible for their integrity and activation referred to as ‘turn motifs’(48). Even though it has, that PI3K-PKC activity is needed for host cell killing by *E. histolytica,* but the detailed mechanism and protein involved in this pathway is still needed to be identified(49). In previous studies, it has been found that *E. histolytica* trophozoites interact with fibronectin by 37 KDa fibronectin receptor located at their surface, this interaction helps in the activation of an enzyme referred as PKC(50). The activity of the enzyme is necessary for protein phosphorylation, actin cytoskeleton reorganization, and polymerization and in the degradation of the bound protein. This study reveals the pathogenic capacity and toxicity of *E. histolytica* by a stimulatory role of phorbol ester. It also explores the vast role of calcium ion and PKC during cell adhesion and cell killing by amoebas. Another AGC family protein is known to be involved in the reorganization of the actin cytoskeleton in *E. histolytica* is PKA (cyclic AMP-dependent protein kinase). Like PKC, in these kinases, the interactions of fibronectin with trophozoites initiate the signalling cascade inside the trophozoite cell. These kinases phosphorylate only that protein which helps in actin cytoskeleton reorganization, which leads to the activation of another regulatory enzyme i.e. adenylase cyclase. *E. histolytica* has been recently shown to ingest live host cells by trogocytosis, which literally means nibbling. The process is shown to be essential for host tissue invasion by the parasite(16). Although mechanistically so far, the process looked similar to phagocytosis with evidence of involvement of EhC2PK in both the processes(15). But in 2017, an AGC family kinase EhAGCK1 was found to be involved in the trogocytosis uniquely(16). This is the first report of a kinase to be specifically associated with trogocytosis. This kinase sequence contains pleckstrin homology (PH) domain at N-terminal of the protein which binds PtdIns (3,4,5)P3. This kinase beautifully decorates the plasma membrane at the site of trogocytosis and it leaves the site as soon as the fragment of the host cell is ingested by the trophozoites. Another AGC family kinase, EhAGCK2 which is 51% identical to EhAGCK1 is involved in all actin-dependent endocytic processes, like phagocytosis, trogocytosis and pinocytosis. This kinase also has PH domain at N-terminal of the protein and binds PtdIns (3,4,5)P3. But downstream substrate for both, EhAGCK1 and EhAGCK2 are unknown and further work is required to characterize the signalling pathway operating through these kinases. Both kinases are very transiently present at the site of endocytosis and leave the site by the

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time process complete, hence these could not be detected in any phagosome proteome analysis. But the report indicates that kinases belonging to the same family may have a different role in very similar processes like trogocytosis and phagocytosis and hence give distinction to the process.

9. P21 ACTIVATED KINASE FAMILY (EhPAK)

As endocytic processes progress, the actin dynamics are exponentially scaled so that process is fast and efficient. Following the role of alpha kinases in G-actin phosphorylation, p21 activated kinases (PAK) come in the picture. In eukaryotes, p21 activated kinase controls the cell motility and their morphology. Actin dynamics play a major role in controlling cell morphology, polarization, adherence, and the force required for translocation(51). In alignment with the previous findings, *E.histolytica* EhPAK is found to be a key molecule that regulates cell polarity, motility and phagocytosis(52). PAKs kinases are serine/threonine kinases and except Rho, they act as an effector molecule for small GTPase family-like Rac and Cdc42(53). *E. histolytica* Genome codes for 14 putative PAK kinases and two PAK-like proteins in which the kinase domain has diverged from the conserved protein. The N-terminal of the protein possesses conserved motifs known as p21 binding domain (PBD) and Cdc42/Rac interactive binding domain (CRIB)(54). The N-terminal of the protein also has a proline-rich sequence that has the ability to bind with SH3 domain and guanine nucleotide exchange factor PIX (Pak interacting exchange protein)(55, 56). The C-terminus of the protein has sequences that are conserved among the species. Six PAKs harbour CRIB (cdc42/Rac binding) domain but it is not certain whether these and other PAKs are regulated by small GTPase.

EhPAK1 (putative p21 activated kinase 1) is 53 kDa protein which shows maximum sequence similarity with human PAK2 (58% similarity), human PAK1 (45% similarity) and with *D.discoideum* MICH (50% similarity). EhPAK1 sequence lacks consensus Cdc42/Rac interactive binding domain (CRIB) that helps in the association of Rac and Cdc42. But from the previous studies, it has been found that N-terminus of the protein can bind with activated Rac1 but not with the activated Cdc42. Thus, the N-terminal of EhPAK acts as a regulatory domain. Elisabeth *et al* have shown the constitutive kinase activity of C- terminus of EhPAK *in vitro* and overexpression of this kinase domain in amoeba cause a significant decrease in the cell migration, which is proven by dynamic image analysis. This study elaborates the role of EhPAK in cell motility and hence in phagocytosis in *E. histolytica(*52*)*.

Another protein from the PAK family which plays an important role in collagen invasion and capping in *E. histolytica* is named as EhPAK2. This kinase is the first member of the PAK family that contains a conserved PH domain and a highly conserved CRIB domain. EhPAK2 CRIB domain show maximum sequence similarity with Cla4p from *S.cerivisiae (*53%) and with DdPAKC from *D.discoideum* (29%). The conserved CRIB domain possesses conserved residues like, His123, Phe134, and Trp141 which help in binding with the effector loop & strand beta2 of the GTPase. The residues which are important for the interaction of EhPAK2 and EhRac are Met121 and Phe145(57). Moreover, the functional studies of EhPAK2 show that C terminal of the domain had activity toward the myelin basic protein(57). Another member EhPAK3 is a serine/threonine kinase which phosphorylates histone *in vitro* in absence of any small GTPase(58). So far only biochemical properties of these kinases are known but they have not been linked to cellular processes, more research on this kinase is required to establish the role of these kinases in amoebic biology.

10. ROLE OF OTHER KINASES IN THE VIRULENCE OF *E. HISTOLYTICA*

As we have previously discussed that motility and endocytic processes are crucial for virulence of the parasite but interfering with the growth and metabolism of the parasite also exerts a negative effect on parasite virulence. Kinases like cyclin-dependent kinase (CDKs), mitogen-activated protein kinase (MAPKs) and glycogen synthase kinase (GSKs) belong to a group of the family known as CMGC kinase (cyclin dependent protein kinase,

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mitogen activated protein kinase, glycogen synthase kinase, casein kinase-2). MAPKs are serine/threonine kinases, which are responsible for cell proliferation and differentiation in *E. histolytica*(59, 60). In *E. histolytica* EhMAPKs is associated with their survivability and death during stress condition like heat shock and oxidative stress(61). It is well known that CDKs are typically involved in controlling cell cycle and their activities are regulated by cyclin which binds at the N-terminal conserved sequence of the kinase(62). The amoebic CDKs possess conserved cyclin binding sites known as PSTAIRE. In *E. histolytica* genome seven cyclin coding genes have been identified that possess 17% to 19% of sequence similarity among themselves and around 31% with human cyclin kinase domain. The regulatory subunit of CDKs is still not identified in *E. histolytica*. As it is known that parasite lacks essential checkpoints of the cell cycle(62). It is worth investigating the existence of an alternative mechanism of cell cycle control. Another metabolic enzyme of interest is Glycogen Synthase kinase (GSK) which are involved in controlling glycogen metabolism and it serves as an important component for the Wnt pathway in most of the metazoans(63). But their functional role in *E. histolytica* is still not clear. So far 4 putative GSKs are identified in amoeba genome, which shows maximum sequence similarity of 46% with human GSK3(63). Like CDKs, casein kinase 2 (CK2) is also involved in regulating the cell cycle and in DNA repair. CK2 are serine/threonine kinase and they exist in both forms either in the monomeric or heteromeric state. Their structure consists of two alpha subunits and two beta subunits(64). In amoeba 3 putative alpha subunits which serve as catalytic kinase domain and 5 homologs of the beta subunit that acts as the regulatory domain has been identified in the proteome. The mechanism of action and signalling pathway that is operated through these kinases is still unclear and needs further investigations.

11. DISCUSSION

The data and findings presented in the review indicate that *E. histolytica* is unique in terms of its kinome composition from other protozoan parasites. This parasite has extensive signalling network to sense its surroundings as the highest number of TMKs are expressed by the parasite in comparison to other protozoan parasites. The signals perceived by these TMKs are translated via downstream effectors and might help parasite adapt to, rapidly changing host gut environment. The amoebic TMKs are also shown to be receptors for the extracellular signals that may result in simple endocytic processes like phagocytosis of bacteria and fluid or in an invasion of host tissues. In both cases, a complex signalling cascade is initiated in which kinases play an important role. The invasive and endocytic processes are dependent on Ca^{2+} but the molecular mechanism responsible for this is not known till now(13). In silico analysis has revealed that *E. histolytica* genome codes for 27 Ca^{2+} binding proteins and 36 CaM- like kinases, so it is worth assuming that these proteins may be acting in coordination to carry out the Ca^{2+} dependent signalling. But these signalling networks are yet to be revealed and offer to be attractive therapeutic intervention target as amoebic CaM like kinases of the parasite is different from the host kinases. However, the role of C2 domain containing kinase belonging to CaM like kinases family, in recruiting CaBP1 in erythrophagocytosis and trogocytosis is now established. This recruitment of CaBP1 is essential for the initiation of the process, as it is responsible for bringing other proteins at the site of phagocytosis. This signalling pathway involving CaBP1 is now extended and alpha kinase, EhAK1 is known to be recruited at later steps in Ca^{2+} dependent manner, which phosphorylates amoebic actin as well recruits Arp2/3 complex as shown in schematic presentation (Figure 3). The different Ca^{2+} binding affinities of different EF-hands in EhCaBPs are considered to govern the Ca^{2+} dependent or independent recruitment of downstream effectors in the erythrophagocytic pathway(65). Further, Recruitment of Arp2/3 complex is essential to kick-start the actin polymerisation at the site of phagocytosis. Phosphorylation of amoebic G-Actin by EhAK1 seems to be essential for actin dynamics *in vivo* as well. The sequential recruitment of kinases in an orchestrated manner is essential for the progression of the process, and this can be regulated by lipid modifications, sequential phosphorylation etc. As recently shown that *in vivo* amoeba behaves differently to the dead and live host cells in terms of

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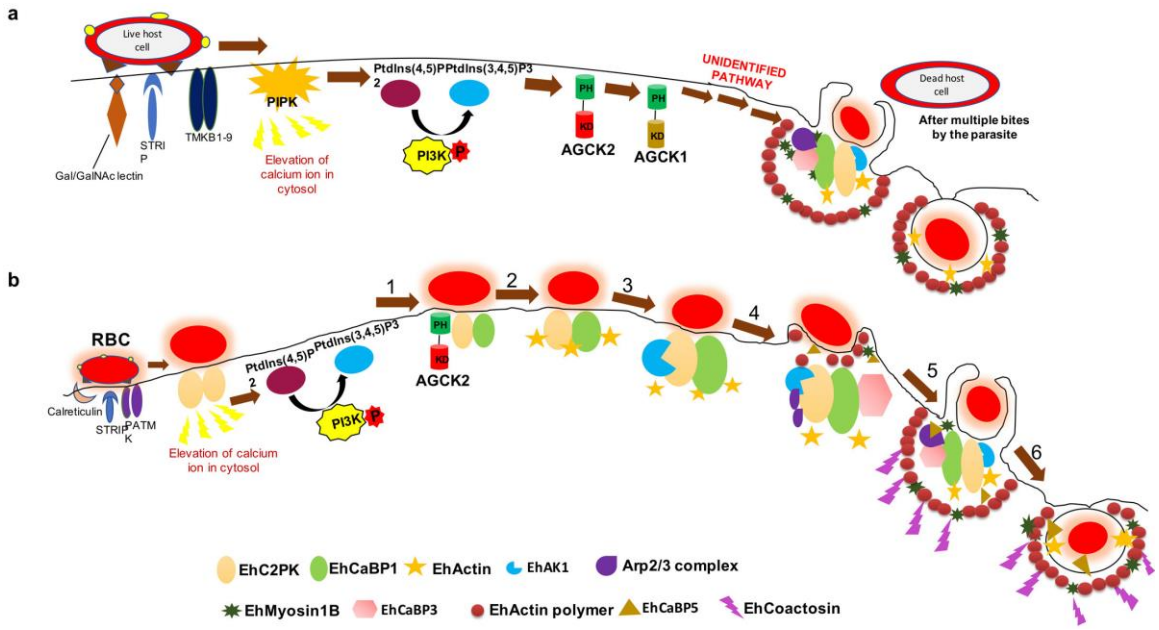

Figure 3. Schematic diagram depicts the signaling pathway activated during trogocytosis and phagocytosis in *E. histolytica* trophozoites. (a) Trogocytosis is initiated with the adhesion of the amoebic cell to the live host cell (RBC, intestinal epithelial cells) with the help of Gal/GalNac lectin, TMKs, and several other proteins increase the intracellular Ca^{2+} level inside the parasite. Due to adhesion and several other signalling PIPK is activated that help in the generation of PtdIns (4, 5)P_2 and PtdIns (3,4,5)P_3 by EhPIPK1 that aid in the recruitment of AGC kinase family, EhAGCK1 and 2. The EhAGCK1 involve in the nibbling of a live host cell but do not participate in phagocytosis of dead cells. The AGC family are involved in the reorganization of the actin cytoskeleton in *E.histolytica* during this process. (b) The schematic diagram shows the details of erythrophagocytosis mechanism in trophozoites, the process initiates as erythrocyte interacts with receptors and there is local Ca^{2+} elevation on the cytosolic side along with a localised generation of PtdIns (4,5)P2 PtdIns (3,4,5)P3 in the plasma membrane inner leaflet by EhPIPK1. This step is followed by the recruitment of EhC2PK and EhCaBP1 in calcium dependent manner, while PtdIns (3,4,5)P3 leads to recruitment of EhAGCK2. Following, EhCaBP1 then recruits an alpha kinase, EhAK1, both of which bind to G and F actin and increase the local actin concentration. EhAK1 phosphorylate G actin and enhances polymerisation. EhAK1 also recruits Arp2/3 which further enhances polymerisation efficiency. Arp2/3 recruits another Ca^{2+} binding protein, EhCaBP3 which helps in the bundling of actin and progression of phagocytic cups. Further, another protein EhCaBP5 interacts with the IQ motif of myosin 1B while EhCoactosin, which is an actin-binding protein binds to G & F actin and stabilize the actin polymer(68, 69). The process is completed with the scission of the plasma membrane forming an endosome.

ingestion. Adherence to host cells leads to the activation of several kinases and phosphatases that help in the modification phosphoinositides which are located in the plasma membrane of the amoeba. Phosphoinositides regulate various cellular processes such as cytoskeleton reorganization, membrane trafficking and endocytosis process. Recently, type I PIPK has been shown to be involved in erythrophagocytosis and is responsible for the generation of PtdIns (4,5)P_2 in the plasma membrane. PtdIns (3,4,5)P_3 is mostly produced by the phosphorylation of PtdIns (4,5)P_2 upon interaction with ligands or host cells (dead or live). This production of PtdIns (3,4,5)P_3 is crucial for downstream signalling of the endocytic process. PtdIns (3,4,5)P_3 is known to recruit a trogocytosis specific AGC family kinase, EhAGCK1, to ingest the live host cells. Otherwise, another kinase of same family EhAGCK2 is recruited for phagocytosis of dead host cells. The mechanism of recruitment of different kinases in response to live or dead host cells and how these molecules dictate different signalling cascade is still under investigation. But, as it is known for the mammalian system that Akt phosphorylates β-actin in PtdIns (3,4,5)P_3 dependent manner and PKC also regulated actin dynamics via phosphorylation of myristoylated alanine-rich C-kinase substrate(50). We assume that EhAGCK1 may relay signalling to form tunnel like structures during trogocytosis while AGCK2 may be involved at early stages of actin polymerisation. Furthermore, PAK is another important class of kinases involved in regulating actin

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Table 3. List of the kinases that play a key role in cellular processes, regulating the virulence in *E. histolytica*

S.no	Protein Accession number	Protein name	Function	Localization	Type of protein	References
1	EHI_073660	TMKB1-9	Serum responsiveness, Proliferation	N-terminal extracellular domain and cytoplasmic kinase domain	Transmembrane	(31)
2	EHI_037140	TMK39	Receptor for Phagocytosis	N-terminal extracellular domain and cytoplasmic kinase domain	Transmembrane	(34)
3	EHI_188110	TMK54	Growth and Expression of the Gal/GalNAc lectin heavy subunit	N-terminal extracellular domain and cytoplasmic kinase domain	Transmembrane	(28)
4	EHI_167650	TMK96/PATMK	Ingestion of apoptotic cell	N-terminal extracellular domain and cytoplasmic kinase domain	Transmembrane	(33)
5	EHI_153770	PIPKI	Pseudopods formation, Phagocytosis	Plasma membrane	Membrane	(39)
6.	EHI_053060	EhC2PK	Initiate phagocytosis	C2 domain on the membrane and kinase domain in the cytoplasm	Membrane	(13)
7.	EHI_188930	EhAGCK1	Participate in trogocytosis	Localised near the plasma membrane & associated with pseudopods	Membrane	(16)
8.	EHI_053040	EhAGCK2	actin dependent endocytic process	Cytoplasm	Cytosolic	(16)
9.	XP_656642	EHAK1	Regulate phagocytosis by actin phosphorylation	Phagocytic cup	Membrane	(14)
10.	X98048	EhPAK	Maintain cell polarity, motility and regulate phagocytosis	Dispersed throughout the cytoplasm and in nascent pseudopods.	Cytosolic	(52)
11	EHI_148900	EhPAK2	Required for collagen invasion and capping	PH domain membrane interacting and cytosolic	Cytosolic	(57)
12	EHI_073650	EhMAPK	Cell survivability in stress condition		Cytosolic	(61)

Note: Kinases in this table are found to be important for the survival of trophozoites and many are down regulated in phagocytosis defective cell lines. Hence, these kinases are attractive drug targets as apart for being important for a parasite, many are sequentially divergent from host kinases.

dynamics(66). The N- terminal of EhPAK acts as a regulatory domain while the C terminus of the protein shows the kinase activity. *In vitro* studies and over expression of these kinase domains in amoeba cause a significant decrease in cell migration. Hence, EhPAK is a key molecule that is involved in regulating actin dynamics during cell polarity, motility, and phagocytosis. Although detailed studies regarding PAKs are not available, but evidence shows their importance in actin dynamics. It is known that amoebic genome codes for Rab GTPases and Rho GTPases, these small regulators along with kinases may play a role in altering actin dynamics as per the requirement of endocytic processes like phagocytosis or trogocytosis. So far, we can see that actin dynamics is an important process in cellular processes and many signalling cascades converge at this point. Most of the kinases involved in the endocytic processes like EhC2PK, EhAK1 and EhAGCK1 are highly dynamic and leave the site of

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endocytosis before the completion of process or formation of the new endosome, so they are undetected in phagosome proteome. So, these dynamic molecules need multiple approaches to be identified and reveal their role in amoebic biology. List of kinases in Table 3 summarizes the role of kinases known in influencing the virulence of the parasite. The kinases in amoeba are also known to be divergent in terms of the domain composition and conserved motifs. But they are excellent models to study the evolution of signalling mechanism mediated by them. From data so far generated by researchers shows that kinases form important activation points of specific signalling pathways, which might be overlapping for example as in the case of phagocytosis and trogocytosis. Moreover, amoebic kinases show sequential and structural differences from host kinases which implies that they can be safe targets for drug development. As a problem of drug resistance against the choice of drug metronidazole has been reported in Giardia and increased tolerance towards metronidazole, showing higher MIC values, has also been reported in clinical isolates from patients compared with the reference strain of *E. histolytica* HM1: IMSS(17, 67). There is an urgent need to address the challenge of drug resistance by exploring novel drug targets. The unconventional amoebic kinases form an unexplored and potential mine of targets for drug development against amoebiasis. Overall*, E. histolytica* presents a model system for studying and understanding the evolution of many intricate and complex signalling network present in higher organisms. And a detailed study of these networks will help in the development of effective drugs against amoebiasis.

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Azhar Ahmad and Shalini Mishra, have contributed equally to the review. Somlata and S. Gourinath have critically analysed the manuscript contributed to the drafting.

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Abbreviations: TMK(Trans-Membrane kinase), GalNAc (N-Acetylgalactosamine), PTK (Protein Tyrosine Kinase), PI (phosphoinositide), PtdIns(4,5)P2 (Phosphatidylinositol 4,5-bisphosphate), PtdIns(3,4,5)P3 (Phosphatidylinositol (3,4,5)-trisphosphate), CAMK (Calmodulin dependent kinase), AK (Alpha Kinase), PAK (p21 Activated Kinase)

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