

## Expression and Function of a Family of Transmembrane Kinases from the Protozoan Parasite *Entamoeba histolytica*†

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**The signaling proteome of *Entamoeba histolytica* is made of transmembrane kinases (TMKs) that are rarely found in unicellular eukaryotes. There are 90 TMK genes reported for *E. histolytica*, and these have been grouped into nine distinct families based on motifs present on both extracellular and kinase domains. Of these, the B1 family was chosen for further analysis. Genomic sequencing revealed the presence of 28 members belonging to this family. Genes corresponding to the majority of these were truncated and not considered for further analysis. Only five members were full length and contained both extracellular and cytosolic kinase domains. BLAST analysis revealed the presence of homologs of these B1 TMKs in the nonpathogenic *Entamoeba dispar*. However, the ligand binding domains of the orthologous B1 TMKs of the two species showed considerable divergence, indicating the possibility of a correlation with the pathogenic potential of the organism. Only two of the five full-length copies (B1.I.1 and B1.I.2) were expressed in *E. histolytica* under the culture conditions used. Antisera generated against the extracellular domain of B1.I.1 stained the cell surface, particularly the areas of contact between the trophozoites. Staining was also seen in the frontal and posterior regions of the motile amoeba. An amoebic cell line expressing a truncated version of the B1.I.1 that lacked the kinase domain was generated. Inducible expression of the truncated TMK resulted in a decrease in cellular proliferation and an increase in sensitivity to serum starvation. Our data indicate that the B1.I class of TMKs is involved in parasite proliferation.**

The intestinal protozoan parasite *Entamoeba histolytica* is the causative organism of invasive amoebiasis, which is responsible for about 40,000 deaths every year (41). It is endemic in many developing countries. A large fraction of infected individuals do not display any symptoms of invasive disease and remain asymptomatic. It is not yet clear what factor(s) or signal(s) is particularly involved in turning a commensal *E. histolytica* isolate into a virulent cell capable of tissue invasion.

All living organisms have developed extensive mechanisms to respond to environmental signals. These signals are perceived through an array of receptors present mainly on the cell surface. The diversity of these receptors helps organisms to interact with different extracellular signals and respond appropriately. It is likely that some of the signaling pathways play important roles in the host-parasite relationship.

A number of signaling pathways have been reported in *E. histolytica*. The binding of extracellular matrix components to amoebae through focal adhesions is thought to be involved in generating signals needed for motility and cell killing (7, 12, 16, 19, 22, 23). For example, the binding of trophozoites to the extracellular matrix component collagen resulted in tyrosine phosphorylation of pp125<sup>FAK</sup> and the recruitment of pp60<sup>src</sup> and paxillin to the pseudopods (26). The involvement of p<sup>21</sup>-activated kinase in phagocytosis has also been demonstrated

(17). Signal transduction pathways involving calcium have also been identified (27, 28). These pathways are mediated through a number of calcium binding proteins, including calmodulin (24), a novel calcium binding transcription regulator (11), and a novel protein involved in actin cytoskeletal dynamics (30).

The presence of an extensive signaling network in *E. histolytica* was revealed by its genome analysis. More than 200 putative kinases, including an estimated 90 novel transmembrane kinases (TMKs), have been tentatively identified (20). The *E. histolytica* TMK is predicted to contain an N-terminal signal sequence, an extracellular domain, and a single transmembrane helix followed by a cytosolic kinase domain. These kinases fall into three groups based on the predicted extracellular domains (20). The first group of 50 transmembrane kinases has CXXC-rich repeats that are also found in the intermediate subunit (Igl) of Gal/GalNAc lectin and the *Giardia lamblia* variant-specific surface protein. The other two groups either carry cysteine-rich domains containing CXC repeats or lack the cysteine-rich extracellular domains (20). Each group can be further subdivided based on sequence features in subdomain VII of the kinase domain. The B family has the signature KLTFDGS in subdomain VII. This family is further classified into three families (B1, B2, and B3) based on the size of the protein and the distribution of CXXCXXGY motifs in the extracellular domain. The B1 family was predicted to have 11 members (4). It is likely that the nine families represent functionally different receptors in terms of sensing a variety of extracellular signals and relaying them to a number of downstream signaling pathways. Recently, the expressions of some of these TMKs have been studied, but no clear conclusion can be drawn about the function of these molecules (4).

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† Supplemental material for this article may be found at <http://iai.asm.org/>.

TABLE 1. Homologs of B1 TMK in different species of *Entamoeba*<sup>a</sup>

| Species                      | Database <sup>b</sup> | Unique portion of the extracellular domain |   |            | Kinase portion of the intracellular domain |   |            |
|------------------------------|-----------------------|--|---|------------|--|---|------------|
|                              |                       | Accession no. of the topmost hit           | Region of identity to gene with ID no. 56467968 | % Identity | Accession no. of the topmost hit           | Region of identity to gene with ID no. 56467968 | % Identity |
| <i>Entamoeba dispar</i>      | Sanger                | dispar40b03.p1k                            | 283–987   | 57         | dispar81a01.p1k                            | 3046–3915                                       | 86         |
|                              | TIGR                  | EDGH482TR                                  | 67–966  | 53         | 97861                                      | 39–1067   | 84         |
| <i>Entamoeba invadens</i>    | Sanger                | inv122h05.q1k                              | 481–1083  | 42         | inv060b06.p1k                              | 3040–3852                                       | 63         |
|                              | TIGR                  | 205413                                     | 1–963   | 34         | 205446                                     | 3–1055  | 57         |
| <i>Entamoeba terrapinae</i>  | Sanger                | terra181a06.q1k                            | 295–954   | 36         | terra214b01.p1k                            | 24–887  | 56         |
| <i>Entamoeba moshkovskii</i> | Sanger                | mosh129g09.q1k                             | 85–939  | 38         | mosh129g09.q1k                             | 228–1034  | 71         |

<sup>a</sup> One-kilobase stretches from the 5' and the 3' ends of the gene (B1.I.1) were used for searching the databases. Numbers in the regions of identity refer to nucleotide positions. ID, identification.

<sup>b</sup> All searches were carried out using tBLASTx.

TMKs play a critical role as signal perceivers and transducers in higher eukaryotes. Among the organisms studied so far, plants carry the largest number of TMKs (3, 33). Most of these have not been functionally characterized (34). In humans, about 70 TMKs have been reported (29, 36). TMKs regulate a great diversity of cellular processes in animals, including cell survival, proliferation, differentiation, metabolism, and migration (31). Plant TMKs are also known to participate in development, hormone perception, and pathogen response (5, 15). While most TMKs in animal systems are tyrosine kinases, plants have mainly serine/threonine kinases, with a few exhibiting dual specificities (21, 32).

Among unicellular eukaryotes, there are only a few known examples of TMKs, such as the epidermal growth factor receptor-like cell surface molecules of *Trypanosoma brucei* (14). A few have been tentatively identified in the genome of the free-living amoeba *Dictyostelium discoideum* (8). Therefore, the presence of such a large number of TMKs in *E. histolytica* was surprising. At present, it is not clear whether these have any roles in amoebic biology or are the remnants of past evolution with no current significance. Here we present results from genome sequence analysis of the B1 family of TMK and show that at least one of the B1.I genes is involved in cellular proliferation.

#### MATERIALS AND METHODS

**Sequence analysis.** Nucleotide and protein sequences of all target genes were retrieved from the *E. histolytica* genome database (<http://www.ncbi.nlm.nih.gov>). Indicated sequences were taken and used for a BLAST sequence similarity search against the *E. histolytica* genome database (12.5 times coverage, that is, the final assembled genome sequence) at the Institute of Genome Research (<http://www.tigr.org/tdb/e2k1/cha1>), the Wellcome Trust's Sanger Institute ([http://www.sanger.ac.uk/Projects/E\\_histolytica](http://www.sanger.ac.uk/Projects/E_histolytica)), and the NCBI. For primary screening, the nucleotide sequence corresponding to B1.I.4 (Table 1) was used. All of the hits that showed  $\geq 95\%$  sequence identity at the nucleotide level against B1.I.4 over a minimum stretch of 500 nucleotides were extracted. Conserved domains and motifs were identified by CD-search (<http://www.ncbi.nlm.nih.gov/BLAST>), Pfam (<http://pfam.wustl.edu/hmmsearch.shtml>), MPSrch (<http://www.ebi.ac.uk/MPSrch>), and ProDom (<http://www.sanger.ac.uk/software/Pfam>). The determination of amino acid composition and multiple alignments (CLUSTALW) were performed using the BioEdit sequence alignment editor (version 7.0; Tom Hall). Secondary structure, topology, and membrane organization were predicted by using the set of tools available at [www.expasy.ch](http://www.expasy.ch).

**Cells and transformation.** All experiments were carried out with *E. histolytica* strain HM-1:IMSS clone 6, which was obtained from William A. Petri (Univer-

sity of Virginia). The cells were maintained and grown in TYI-33 medium supplemented with 15% adult bovine serum, 2% Diamond's vitamin mix, and antibiotics (0.3 units/ml penicillin and 0.25 mg/ml streptomycin). Hygromycin (Sigma) was added at 10  $\mu$ g/ml for maintaining the transformed cell lines. Cell viability was determined by microscopy using a trypan blue dye exclusion test.

Transfection was performed by electroporation as described previously (30, 40). Two consecutive pulses were applied, each with an exponential discharge of 3,000 V/cm at a capacitance of 25  $\mu$ F (Gene Pulser II; Bio-Rad). For each transfection,  $4 \times 10^6$  trophozoites in Cytomix buffer (10 mM  $K_2HPO_4/KH_2PO_4$  [pH 7.6], 120 mM KCl, 0.15 mM  $CaCl_2$ , 25 mM HEPES, 2 mM EGTA, 5 mM  $MgCl_2$ ) and 300  $\mu$ g DNA were used. Drug selection with 5  $\mu$ g/ml hygromycin (Sigma) was started 2 days after transfection. The concentration was increased to 10  $\mu$ g/ml after a few days.

**Antibody generation.** Antibody was raised against a peptide from the N-terminal (unique) region of the receptor corresponding to amino acids 152 to 171 of B1.I.1 TMK. The peptide was conjugated to bovine serum albumin (BSA) and injected into rabbits subcutaneously. After the initial injection, the rabbits were given three booster injections at an interval of 21 days. The peptide was designed based on the output of a set of programs that help predict antigenic regions. All immunoassays were carried out in the presence of BSA. Under these conditions, the anti-N-terminal antiserum was found to have no reaction to BSA or bovine serum (data not shown).

**Southern and Northern hybridizations.** Southern blots were hybridized in a solution containing 1% sodium dodecyl sulfate (SDS), 1 M NaCl, and  $3 \times 10^5$  cpm  $ml^{-1}$  of DNA probe at 65°C for 16 h. Blots were washed according to the manufacturer's instructions and exposed for autoradiography. Radioactive DNA probes were prepared using a NEB blot random priming kit.

Total RNA was purified using TriPure reagent (Boehringer Mannheim) according to the manufacturers' instructions. RNA samples (30  $\mu$ g) were denatured by incubation with 6 M deionized glyoxal, 50% (vol/vol) dimethyl sulfoxide, and 1 $\times$  gel running buffer (12 mM Tris-HCl, 6 mM sodium acetate, and 0.3 M EDTA [pH 7.0]) at 50°C and electrophoresed in a 1.2% (wt/vol) Tris acetate EDTA agarose gel. After electrophoresis, the gel was blotted onto a GeneScreen Plus nylon membrane (NEN) and hybridization was carried out at 42°C in 50% formamide-containing buffer according to the manufacturer's instructions.

**PCR and cloning of the PCR products.** Primers to amplify the indicated genes were designed based on the *Entamoeba* genome sequence database. Appropriate restriction enzyme sites were incorporated in the primers used for PCR amplification for molecular cloning of the products. The primers used were NTF1, 5' CG GGTACC TGT TAT TCT TTA TAT TCA TTG ATG 3', and DN-RP-c-myc, 5' CG GGA TCC TTA TAA ATC TTC TTC TGA AAT TAA TTT TTG TTC TGA TTC TTC TTG TGC TTT ATT 3'. These were obtained from Microsynth, Switzerland. PCR was performed with 100 ng of *E. histolytica* genomic DNA. The conditions were 92°C for 5 min, 40°C for 1 min, and 70°C for 2 min (5 cycles), followed by 92°C for 1 min, 52°C for 1 min, and 70°C for 2 min (25 cycles). For expression in amoebic cells, the shuttle vector pEH YHG-tetR-O-CAT (ptoc) (30, 40) was used. The CAT gene was excised using KpnI and BamHI restriction endonucleases, and the truncated B1 TMK gene was inserted in the sense orientation. A plasmid containing the truncated TMK gene was constructed and named p $\Delta$ kinase. The cell lines generated with ptoc and p $\Delta$ kinase were referred to as Eh toc and Eh  $\Delta$ kinase, respectively.

**Reverse transcription-PCR (RT-PCR).** Five micrograms of total RNA was reverse transcribed following standard protocols using 100 pmol of specific primer, 2  $\mu$ l of 5 $\times$  RT buffer (50 mM Tris-Cl [pH 8.3], 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 0.8 mM denucleotide triphosphates) in a total reaction volume of 17  $\mu$ l. The reaction mixture also contained 1  $\mu$ l of RNasin (40 U/ $\mu$ l) and 1  $\mu$ l of Moloney murine leukemia virus reverse transcriptase (200 U/ $\mu$ l; MBI Fermentas). The primers used in this study were NTF, 5' CC GGT ACC CAT GCT AAA GCT TAT GAT TG 3'; NTR, 5' CC GGA TCC AAT TAG TCG TTC AAA TGA GA 3'; NT 1, 5' TGG TAA ATA ATG ATT ATA TG 3'; and NT 2, 5' ATT ACA AGT GAG ATT ACC 3'. The conditions used for PCR were 92°C for 1 min, 42°C for 1 min, and 70°C for 1 min (5 cycles), followed by 92°C for 1 min, 52°C for 1 min, and 70°C for 1 min (25 cycles).

**Immunostaining.** *E. histolytica* isolates (2  $\times$  10<sup>5</sup> cells) resuspended in incomplete TYI-33 medium at 36°C were transferred onto coverslips, placed in a petri dish, and allowed to adhere for 10 min at 36°C, and cells were fixed with 3.7% prewarmed paraformaldehyde for 30 min at 36°C. After fixation, some of the cells were permeabilized with 0.1% Triton X-100-phosphate-buffered saline (PBS) for 2 min and then quenched with 50 mM NH<sub>4</sub>Cl-PBS for 30 min. The coverslips were blocked with 3% BSA-PBS for 30 min, followed by incubation with primary antibody (1:50) for 1 h at 36°C. These were first washed with PBS and then with 1% BSA-PBS before incubation with secondary antibodies at a 1:200 dilution for 30 min at 36°C. The preparations were further washed with PBS, mounted on a glass slide using DABCO [10 mg/ml 1,4-diazabicyclo(2,2,2)octane (Sigma)] in 80% glycerol, and sealed. Alexa 488-labeled cells were visualized using a laser scanning confocal microscope (Zeiss LSM Meta model) after excitation at 488 nm.

**Cellular proliferation.** *E. histolytica* cell transfectants Eh  $\Delta$ kinase and Eh *toc* were grown in the presence of 20  $\mu$ g/ml hygromycin B, and growth was measured in the absence and the presence of 10  $\mu$ g/ml tetracycline after the indicated time periods. The cells were counted by hemocytometer. Cell viability was determined by microscopy using trypan blue dye exclusion test.

**Immunoprecipitation of the B1.I TMKs from *E. histolytica* trophozoites.** *E. histolytica* cells were harvested and washed three times with phosphate-buffered saline (0.37% K<sub>2</sub>HPO<sub>4</sub>, 0.11% KH<sub>2</sub>PO<sub>4</sub>, 0.95% NaCl). The cells were then lysed in lysis buffer (10 mM Tris-Cl [pH 7.5], 150 mM NaCl containing protease inhibitors like 2 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 1% Triton X-100, 1% BSA). The lysate was centrifuged to remove cellular debris, and immunoprecipitation was carried out using protein G-Sepharose beads, partially purified indicated antisera, and preadsorbed lysate essentially as described before (30). The pellet was resuspended in 50  $\mu$ l of SDS-polyacrylamide gel electrophoresis (PAGE) buffer and boiled for 5 min, and the bound proteins were separated from beads by centrifugation, followed by 7.5% SDS-PAGE, followed by Western blotting. The anti-N-terminal antiserum was used at a dilution of 1:2,000.

**Immunoprecipitation of the truncated TMK from Eh  $\Delta$ kinase cells.** Trophozoites induced for 48 h (20  $\mu$ g/ml hygromycin and 10  $\mu$ g/ml tetracycline) were harvested by washing once with cold PBS (pH 7.4) and resuspending in 1 $\times$  lysis buffer (150 mM NaCl, 50 mM Tris [pH 8.3], 1% NP-40) with general-use protease inhibitor cocktail (Sigma). Cells were lysed by vortexing. Magnetic protein G-coated beads (Dyna) were coupled to monoclonal anti-*c-myc* antibody (clone 9E10; Santa Cruz Biotechnology) by incubating 50  $\mu$ l of beads with 50  $\mu$ g of antibody in a total volume of 50  $\mu$ l for 1 h. Beads were washed twice in cold PBS (pH 5.5), and 500  $\mu$ l of amoebic lysate was added. The immunoprecipitation reaction was incubated overnight at 4°C with rocking. The beads were then washed twice with 500  $\mu$ l of cold lysis buffer containing only 0.5% NP-40 and once with cold PBS. Beads were resuspended in 1 $\times$  SDS-PAGE sample buffer, boiled for 5 min, and analyzed on an 8% SDS-polyacrylamide gel. The gel was electrophoretically transferred onto polyvinylidene difluoride membranes for Western blot analysis. The blot was incubated with a 1:2,500 dilution of anti-*c-myc* monoclonal antibody clone 4A6 directly conjugated to horseradish peroxidase (Upstate Biotech) in 5% nonfat dry milk for 1.5 h and washed three times in Tris-buffered saline-0.1% Tween 20. The blot was developed with an ECL reaction kit (Amersham).

**Miscellaneous methods.** The concentration of protein in a sample was estimated by bicinchoninic acid assay using BSA as a standard (37). SDS-PAGE analysis was carried out in 10% acrylamide gels under reducing conditions according to the method of Laemmli (18).

## RESULTS

**In silico analysis of B1 family of TMKs. (i) Genomic organization of B1 family.** In order to understand the structural and functional diversity of the B1 family, computational analysis

was carried out using standard bioinformatic tools. For this study, the B1.I.4 (protein identification no. EAL44808; gene identification no. 56466774) (Fig. 1) gene was used as the reference for comparison with other members, as this was the first member to be identified. We repeated all of the analysis with B1.I.1 as the reference, and the results obtained were nearly identical. The similarity shown here is with respect to the reference sequence and not with each other. In order to identify the members of the family, a BLAST search was carried out and 27 sequences showing  $\geq$ 95% identity at the nucleotide level were selected for further analysis. At this stringent level, there was only one hit with members of another family (B2). Figure 1 describes the sequence features of the 28 members of the B1 family. The various functional domains identifiable by sequence homology with known domains are marked. It is apparent that the majority of members were truncated. The members were classified based on the functional domains retained by them. Class I includes members that encode both extracellular and intracellular domains. The members of class II lack the N-terminal portion but contain the cysteine-rich region (Fig. 1). Class IV contained the highest number of members. This group encodes only the cytoplasmic domain. On the other hand, members of class V lacked this domain. One member of this class contained the complete extracellular domain (B1.V.5). When sequences upstream of the start codon (in B1.I class) were analyzed, the sequence identity did not extend beyond a few hundred base pairs. Thus, these are unlikely to be allelic copies. Since the other classes are truncated, only members of the B1.I family are likely to encode functional TMKs. However, this inference is drawn from the genome sequence data available now, which may not be highly accurate due to problems in sequencing and assembly, especially of repetitive sequences.

**(ii) Domain organization of a typical B1 TMK.** B1.I.4 was chosen as a representative member of the B1 TMK family of homologous genes. Conceptual translation suggested that B1.I.4 encodes 1,355 amino acids with a theoretical pI of 5.98 and computed molecular mass of 155.6 kDa. The protein is predicted to have two hydrophobic regions, as determined by Kyte-Doolittle hydropathy plotting (data not shown). The first stretch from amino acid residues 1 to 20 may be the signal sequence. Amino acid residues 900 to 940 were predicted to be the transmembrane domain. The rest of the protein was found to be hydrophilic. Overall predictions suggested that this protein is likely to be anchored to the membrane by a single transmembrane domain with an intracellular C-terminal segment (residues 932 to 1350) and an extracellular N-terminal region (residues 1 to 900) (Fig. 2). The cytoplasmic region appeared to be a serine/threonine kinase as revealed by motif and domain search algorithms. A conserved domain search using the NCBI server revealed statistically significant similarity ( $E = 3 \times 10^{-12}$ ) with the variant-specific surface protein-like domain of *Giardia lamblia*. The similarity mapped to amino acid residues 400 to 900. Further, this region was comprised of cysteine-rich repeats similar to the epidermal growth factor, laminin, and furin repeats found in many growth factor receptors (Fig. 2). This protein also contained modules rich in other amino acids, such as asparagine, cysteine, and isoleucine. Based on the analysis given above, the schematic diagram of B1.I.4 is shown in Fig. 2.

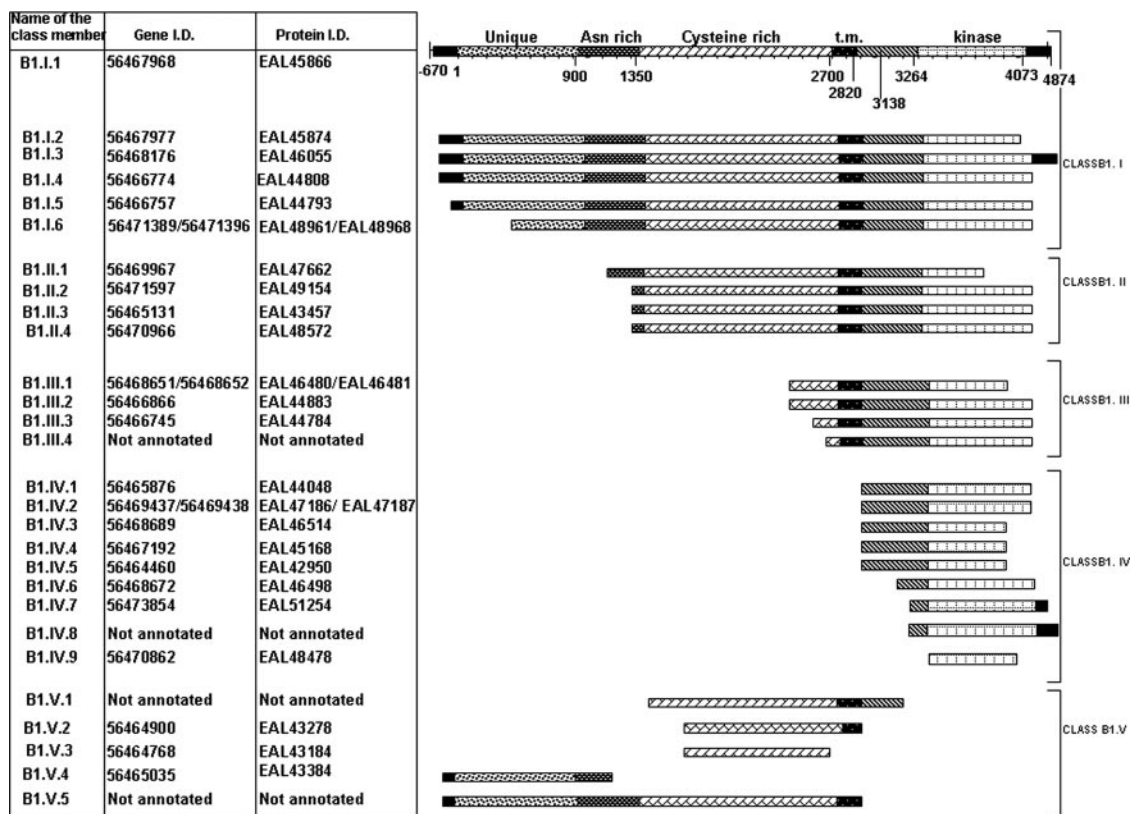


FIG. 1. Mapping and classification of different homologs of B1 TMK identified in *E. histolytica* genome. Multiple copies of the B1 TMK were identified by sequence search algorithms as described in the text. Only those sequences that showed  $\geq 95\%$  identity in a minimum stretch of 500 nucleotides at the nucleotide level with the gene B1.I.1 were selected for analysis. Numbers below the line indicate nucleotide positions of the regions encoding different domains. t.m., the region encoding transmembrane segment. I.D., identification.

**(iii) Presence of B1.I TMKs in other species of *Entamoeba*.**

Normally, genes needed for important biological processes are conserved across closely related species and this property has been extensively used in many bioinformatic predictions. In

order to see whether members of the B1.I class of TMKs are conserved, the presence and sequence similarities of TMKs were assessed in different species of *Entamoeba* for which the sequences are available. Sequence searches were carried out

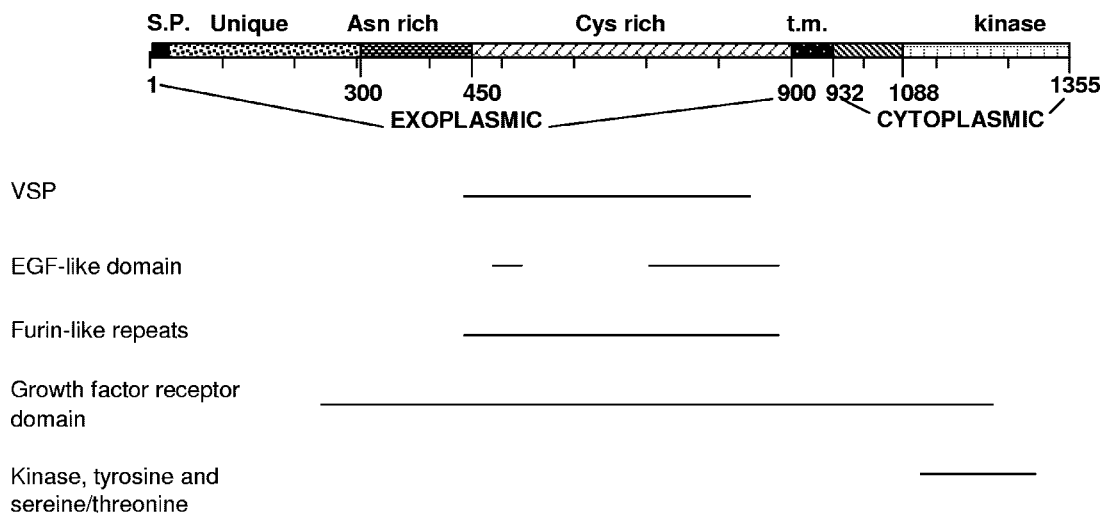


FIG. 2. Domain organization of a full-length B1 TMK. Schematic representation of the predicted orientation and different regions of a typical B1.I TMK based on amino acid composition. Numbers below the line indicate amino acid residues. S.P., signal peptide; t.m., transmembrane segment. Regions showing similarities with known motifs and domains from other systems are marked by thin lines (see text for details). EGF, epidermal growth factor; VSP, variant-specific surface protein.

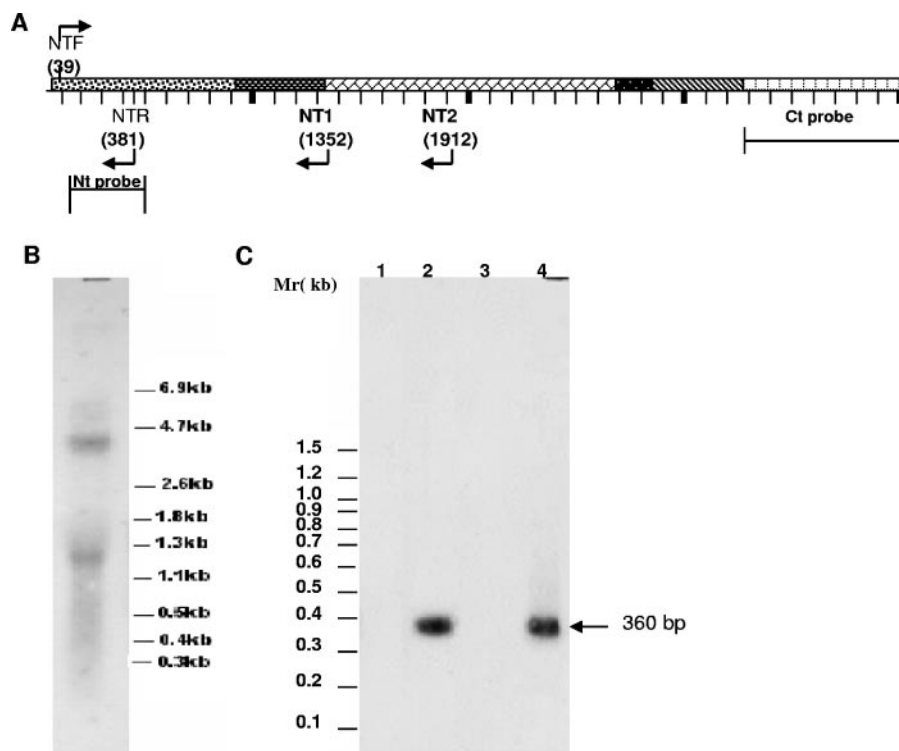


FIG. 3. Expression of B1 TMK transcript in *E. histolytica* trophozoites. (A) Primers and probes used in this study are shown schematically; the numbers below the line indicate nucleotide bases. The expression of the TMK was shown by Northern hybridization (B) and RT-PCR (C). For Northern hybridization, 30  $\mu$ g of total RNA from mid-log-phase trophozoites of *E. histolytica* cells was used and the filters were hybridized with a labeled Ct probe and autoradiographed. Five micrograms of total cellular RNA was used for RT-PCR using a set of indicated primers following standard methods described in the text. Southern hybridization of the PCR is shown in panel C. The probe used was the Nt fragment. Lanes 1 and 3, PCR in presence of NT 1 and NT 2 primers, respectively, but without reverse transcriptase; lane 2, PCR of the RT reaction performed with NT 1 as the primer; lane 4, PCR of the RT reaction performed with NT 2 as the primer.

using 1-kb regions from both ends of the B1.I.1, representing the unique parts of the extracellular and kinase domains. Sequences were compared at both the nucleotide and amino acid levels. The homolog of the kinase domain was found in the nonpathogenic sibling species *E. dispar*. The identity at the amino acid level was observed to be 84%, suggesting that these two may have similar substrate recognition capabilities (Table 1). However, no homolog of the N-terminal ligand binding domain was found in *E. dispar*. Similar results were also observed with other species of *Entamoeba*, suggesting high levels of sequence diversity in the ligand binding regions but conservation of the kinase domains. Since the complete genomic sequences are not yet available for many species, our inability to identify homologs of ligand binding domains does not necessarily mean they are absent.

**(iv) Expression of B1 TMK in *E. histolytica* trophozoites.** Transcription profiling of a number of *E. histolytica* TMKs has been carried out using microarrays (4). However, there is no information on the B1 family. In order to study the function of this family of TMKs, it was necessary to check whether the family was expressed under normal growth conditions in the trophozoites of *E. histolytica*. This was carried out using Northern blot analysis and RT-PCR. Northern analysis showed two RNA bands of sizes 4.1 and 1.2 kb using the Ct probe derived from the entire kinase domain (Fig. 3B). The 4.1-kb band corresponds with the expected size of the transcript based on

the size of the open reading frame. The 1.2-kb RNA species may arise from a transcriptionally active truncated copy of the TMK, such as the one carried by the B1.IV class of TMKs due to high level of sequence identity. Reverse transcriptase PCR was also carried out to check for expression using the primers shown in Fig. 3A. The RT reaction was performed with primers NT1 and NT2, followed by PCR with primers NTF and NTR. The products of RT-PCR were separated and hybridized with the Nt probe derived from the 5' end of the gene. Amplicons of expected size (360 bp) were obtained (Fig. 3C). The overall results showed that the members of the B1.I class of TMKs were expressed in *E. histolytica* cells. In order to check which genomic copy(ies) of B1.I may be expressed, the RT-PCR products were cloned and the nucleotide sequences were determined for five randomly isolated colonies. The sequences of the clones were aligned with the full-length B1.I members. If the transcript is derived from a particular member of the gene family, the sequence identity after alignment should be nearly 100% with that member. The results showed that the sequences were 99% identical to two members, B1.I.1 and B1.I.2 (see Fig. S1 in the supplemental material). The sequence identity with other B1.I and two B1.V members (Fig. 1) was found to be  $\leq 96\%$ .

In order to look for typical promoter motifs present in the upstream regions of B1.I members, a 1-kb sequence upstream of the start site in each of these copies was extracted and

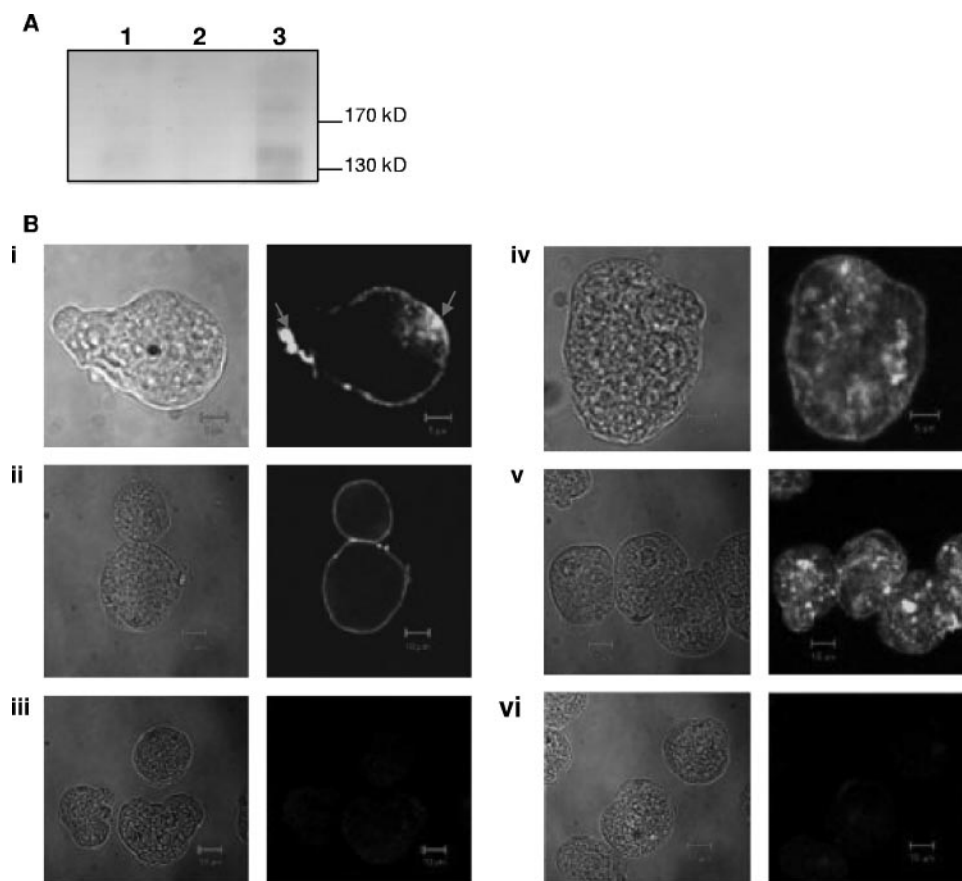


FIG. 4. Immunostaining of the B1.I TMK. (A) Immunoprecipitation of *E. histolytica* total lysate using anti-N-terminal antisera. One microgram of precleared lysate was incubated with the antibody-bound protein G beads. The immune complexes were washed, eluted by boiling in SDS-PAGE sample buffer, and resolved on a 7.5% SDS-PAGE gel. They were blotted onto nitrocellulose membranes and probed with the anti-N-terminal antisera followed by anti-rabbit horseradish peroxidase using an ECL chemiluminescence kit. Lane 1, beads used for preclearing the lysate; lane 2, preimmune serum; lane 3, anti-N-terminal serum. (B) Immunofluorescence. *E. histolytica* HM-1:IMSS cells were visualized by confocal microscopy (Zeiss LSM Meta model) (magnification,  $\times 100$ ) after staining with anti-N-terminal antiserum followed by anti-rabbit (Alexa 488) antibodies. The cells were fixed with paraformaldehyde and are shown in panels i, ii, and iii, and the ones that were permeabilized after fixation are shown in panels iv, v, and vi. (Panels i and ii) Staining with anti-N-terminal antisera. (Panel iii) Staining with the control serum. (Panels iv and v) Staining with anti-N-terminal antiserum. (Panel vi) Staining with control serum. Bar, 10  $\mu\text{m}$ .

checked for sequence similarity and motif identification (see Fig. S2 in the supplemental material). The canonical transcription start site ATTCA was found close to the translation start site ATG in expressed genes B1.I.1 and B1.I.2 (discussed in a later section), suggesting that the 5' untranslated region was very small, as expected of *E. histolytica* genes. Four out of five full-length B1.I TMKs showed conservation of the upstream sequences up to 670 nucleotides.

The expression of the B1.I TMKs was also checked by staining *E. histolytica* cells using antibody raised against the N terminus of the molecule. Given the level of sequence similarity in the N-terminal region, it was not possible to generate specific antibody against a particular B1.I TMK. The antibody is expected to recognize all members of B1.I family and the two B1.V members (B1.V.4 and B1.V.5) containing complete extracellular domains. BLAST analysis using the peptide sequence suggested that the epitope is not present in any other group of protein molecules expected to be present in *E. histolytica*. Immunoprecipitation suggested that the antibody recognized a molecule of 150 kDa, which is the expected size of B1.I

TMK (Fig. 4A). The higher-molecular-weight species seen are likely to be aggregates. The cells were fixed with paraformaldehyde, followed by indirect staining in the presence of BSA and visualization by fluorescence microscopy. The fixed cells showed significant cell surface staining with the antibody (Fig. 4B, panels i and ii). The fluorescence was visible in the frontal part of the cell, resembling a pseudopod, and the posterior region, which is likely to be the uroid (Fig. 4B, panel i) (42). The cells were also stained after permeabilization (Fig. 4, panels iv and v). The results suggested that these molecules were present in multiple foci intracellularly, similar to what was observed by Beck et al. (4).

**Functional analysis. (i) Generation of a dominant-negative cell line.** As mentioned before, there was no significant similarity of the extracellular domain of B1.I TMKs with any known transmembrane kinase from any other species. This precluded the prediction of a probable function or potential ligand based on sequence similarity. Therefore a functional genetic approach was used to decipher the biological role of the gene. Since the ploidy of *E. histolytica* is high and no

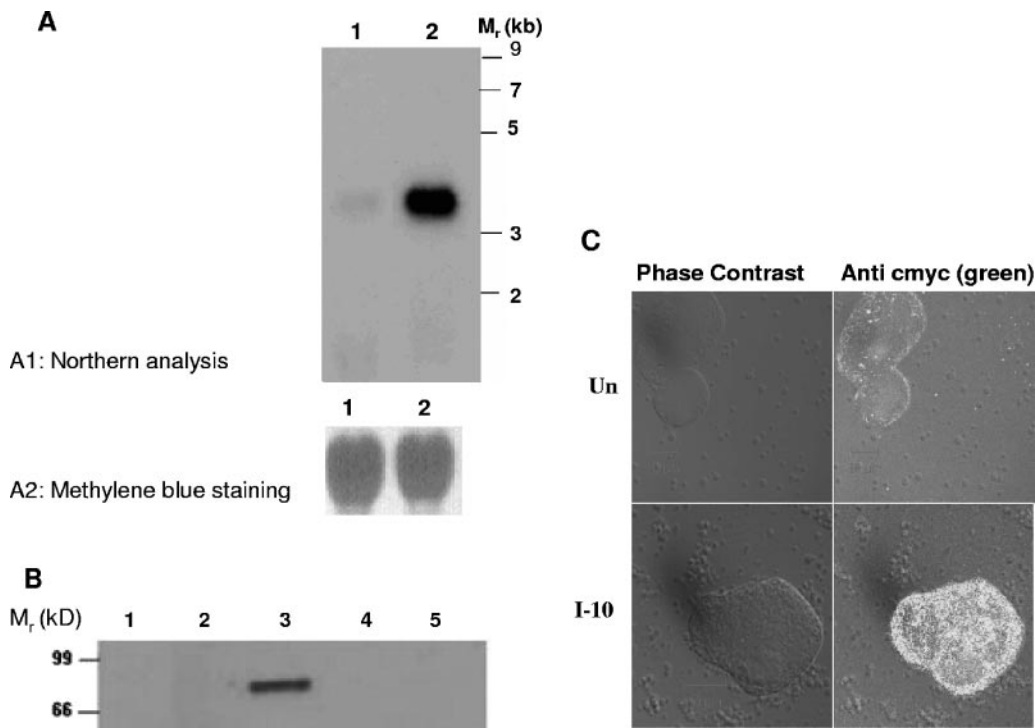


FIG. 5. The expression of the truncated kinase. (A) Expression in *E. histolytica* transfectants by Northern analysis. All experiments were carried out with the Eh  $\Delta$ kinase cells grown in the presence of 20  $\mu$ g/ml of hygromycin B. In addition, some cells were also grown in the presence of the inducer tetracycline at 10  $\mu$ g/ml for 60 h unless otherwise mentioned. The expression was analyzed by Northern hybridization (A1). Thirty micrograms of the total RNA from indicated cells was resolved on 1.2% formaldehyde denaturing gel at 3 V/cm. Hybridization was carried out with  $^{32}$ P-labeled Nts probe (A1). The bands were visualized by autoradiography. Lane 1, uninduced; lane 2, induced at 10  $\mu$ g/ml of tetracycline. (A2) Methylene blue staining of the same blot showing the amount of rRNA loaded. (B) Expression in *E. histolytica* transfectants by Western analysis. The total protein was extracted from Eh  $\Delta$ kinase cells grown with (I-10) or without 10  $\mu$ g/ml tetracycline for 48 h using lysis buffer as described in Materials and Methods.  $\Delta$ kinase was immunoprecipitated using anti-*c-myc* antibody and resolved on an 8% SDS-PAGE gel. The gel was electroblotted onto a polyvinylidene difluoride membrane and probed with anti-*c-myc* horseradish peroxidase. Lane 1, untransfected; lane 2, uninduced at 48 h; lane 3, induced at 48 h; lane 4, magnetic beads alone coated with induced 48-h lysate; lane 5, Magnetic beads alone coated with untransfected. (C) Expression of the truncated B1 TMK protein by immunostaining. Truncated B1 TMK contained a *c-myc* tag fused to the C-terminal end. Eh  $\Delta$ kinase cells were grown with (I-10) or without (un; untransfected) 10  $\mu$ g/ml tetracycline for 72 h, and the cells were immunostained with anti-*c-myc* antibody using indirect immunofluorescence. The cells were also stained with propidium iodide to locate the nucleus. Magnification,  $\times 60$ . Bar, 10  $\mu$ m.

method is yet available for integrating DNA fragments to chromosomes, a dominant-negative mutant approach was taken, whereby a truncated form of the TMK was overexpressed. We expect the expressed truncated putative receptor to be able to bind ligand through the exoplasmically oriented ligand binding domain but not transduce a signal downstream through the kinase domain. Such approaches have been used successfully in other organisms to generate cells displaying dominant-negative phenotypes, for example, for the FGFR and ERECTA leucine-rich repeat receptor-like kinases (35, 39).

The tetracycline-inducible *E. histolytica* expression vector pEh tet-O-CAT (ptoc) (13) was used for cloning where the restriction sites KpnI and BamHI were used to replace the CAT gene with a part of the B1.I TMK without the kinase domain, that is, containing only the exoplasmic and the transmembrane domains (from 1 to 3,138 nucleotides) (Fig. 1), and called p  $\Delta$ kinase. The p  $\Delta$ kinase construct carried an in-frame *c-myc* epitope tag followed by a stop codon. This was expected to attach a *c-myc* peptide to the truncated B1.I.1 TMK for use as a tag in immunoprecipitation. *E. histolytica* cells were transfected with the p  $\Delta$ kinase by electroporation. The  $\Delta$ kinase

transfectant (Eh  $\Delta$ kinase) and a cell line carrying the parental vector (Eh toc) were used in all of the subsequent experiments.

The inducible expression of the truncated TMK was demonstrated by Northern and Western analysis. Thirty-microgram portions of total RNA from uninduced and tetracycline-induced transformed cells under the indicated conditions were hybridized to the Nt probe (marked in Fig. 3) derived from the extreme 5' end of the gene (Fig. 5A). This probe is expected to hybridize to the members of B1.I and B1.V.5 based on sequence similarity. A band of 3.23 kb was observed in lanes where RNA was extracted from cells treated with tetracycline. A low level of expression was also noticed in cells grown in the absence of the inducer, suggesting a low level of leaky expression of the truncated transcript, as previously observed for this vector (30). Densitometric analysis of the amount of RNA induced in the presence of 10  $\mu$ g/ml of tetracycline revealed about a 10-fold stimulation compared to that for the cells with no tetracycline. The induction of the truncated protein in the transformed cells was further confirmed by Western blot analysis of the expressed protein. The *c-myc* tag was used to specifically demonstrate the induction of the cloned protein. The

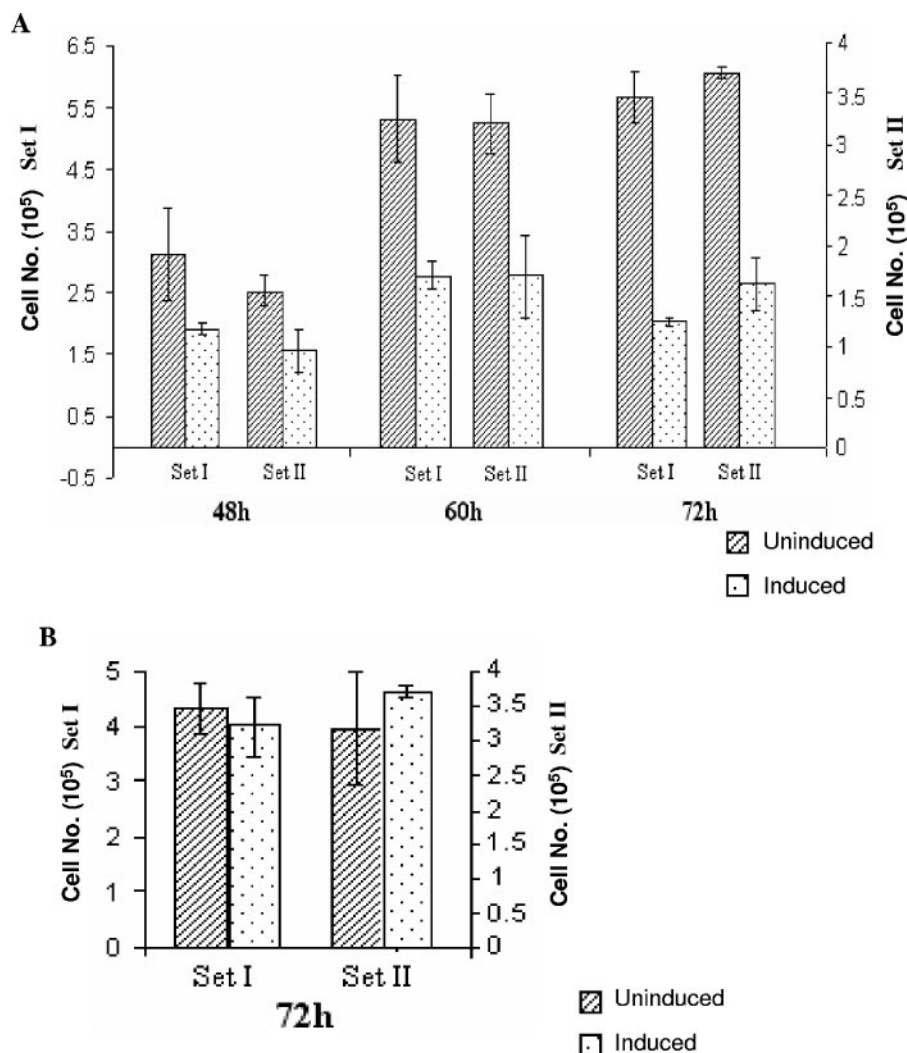


FIG. 6. Growth of cells transfected with  $\Delta$ kinase and toc (a vector with CAT gene). (A) Equal numbers of Eh  $\Delta$ kinase were grown in the presence of 20  $\mu$ g/ml of hygromycin B alone (uninduced) or along with 10  $\mu$ g/ml of tetracycline (induced) for indicated times. The cells were harvested and counted after trypan blue staining. The results of two independent experiments (sets I and II) are shown. (B) Control transfectants (Eh toc) were grown in the presence of 20  $\mu$ g/ml of hygromycin B alone (uninduced) or along with 10  $\mu$ g/ml of tetracycline (induced) for 72 h. The cells were harvested and counted after trypan blue staining. The results of two independent experiments (sets I and II) are shown. Error bars indicate the range of variation.

immunostained blot showed an 82-kDa band in cells that were induced with 10  $\mu$ g/ml of tetracycline. There was no visible band in the lanes where there was no inducer, suggesting that the protein is produced only on induction. These experiments suggested that the truncated TMK gene is expressed in the transformed cells. The size of the band was found to be smaller than the expected size of 120 kDa. This could be attributed to the nature of the protein, particularly since it is rich in cysteine and asparagine. The expressed truncated protein was expected to be on the cell surface as it contained a membrane-spanning domain. In order to verify this, the transformed cells were stained with anti-*c-myc* tag antibody and visualized by confocal microscopy (Fig. 5C). Low levels of cell surface staining could be seen in the Eh  $\Delta$ kinase cells in the absence of tetracycline, suggesting leaky repression. The staining was restricted to the cell surface. Upon induction for 48 h, the staining was still seen

at the cell surface. However after 72 h, high levels of  $\Delta$ kinase were observed in both the plasma membrane and intracellular compartments.

(ii) **Phenotypic characterization of Eh  $\Delta$ kinase cells.** A number of different parameters, such as growth, erythrophagocytosis, fluid-phase pinocytosis, and cytopathic ability were used to assess phenotypic changes in Eh  $\Delta$ kinase cells. These properties are quite often used as markers for assessing the virulent potential of *E. histolytica* cells (1, 2, 25).

TMK has been known to play a key role in cell proliferation and survival in different organisms (31). It is likely to be involved in a similar function in *E. histolytica*. A marked reduction (53% of uninduced at 60 h) in cell number was observed when Eh  $\Delta$ kinase cells were grown in the presence of 10  $\mu$ g/ml of tetracycline to induce the truncated kinase (Fig. 6A). The cells transfected with the parental vector



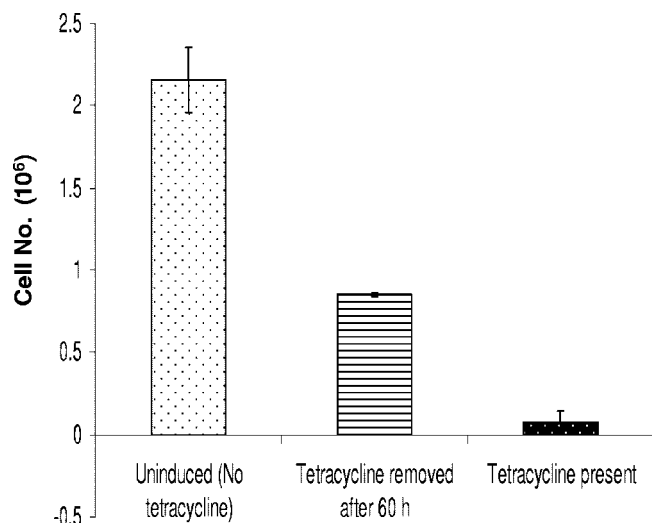


FIG. 7. Effect of serum on growth of the Eh  $\Delta$ kinase cells. Cells were inoculated in 125-by-16-mm glass tubes in three sets in complete medium containing 20  $\mu$ g/ml of hygromycin. To one set, no tetracycline was added (uninduced), while two sets were induced with 10  $\mu$ g/ml of tetracycline for 60 h. After 60 h, medium was replaced and tetracycline was added back to only one of the induced sets. Thereafter, the cells were starved in a medium containing 0.1% serum for 36 h, followed by replenishment in complete medium containing 15% serum for 55 h. The cells were then harvested, and the number was determined by with a hemocytometer. Error bars indicate the range of variation.

alone (Eh toc) (13) did not show any significant change in growth over 72 h in the presence of 10  $\mu$ g/ml of tetracycline (Fig. 6B). Since Eh toc cells express the CAT gene, the results suggest that overexpression of the truncated kinase affects either viability or the growth potential of *E. histolytica* cells (Fig. 6A and B). Viability was not found to change significantly, as determined by trypan blue uptake (data not shown). The proliferation defect was reversible as there was an increase in cell number of the transformed cells after the removal of tetracycline. To demonstrate this, Eh  $\Delta$ kinase cells were grown in the presence of tetracycline for 60 h and divided into two sets for subsequent treatment. One set was allowed to grow in fresh medium without tetracycline, while the other was grown in fresh medium with tetracycline for 48 h. The cell number increased by 76% for the set where tetracycline had been removed relative to an 18% increase in the set where it was continuously present (data not shown here). This indicated that the tetracycline induction of Eh  $\Delta$ kinase cells did not result in cell death but essentially reduced the rate of cellular proliferation. The defect could be reversed by removal of the inducer.

Serum is known to contain growth factors for a variety of cells. Many of these are known to function through receptor kinases. In order to check whether there was any role of serum in the dominant-negative phenotype of Eh  $\Delta$ kinase, these cells were subjected to serum starvation followed by replenishment. It has been shown before that *E. histolytica* cells stop proliferating in the absence of serum and resume cell division after a few hours upon serum repletion (10). The cells were grown in complete medium with or without tetracycline, followed by serum starvation and replenishment as indicated in Fig. 7. Eh

$\Delta$ kinase cells showed very poor recovery after serum replenishment following starvation when tetracycline was present.

The assays for measuring erythrophagocytosis, fluid-phase pinocytosis, and target cell killing were carried out as described in Materials and Methods. There was no significant difference in any of these in Eh  $\Delta$ kinase relative to Eh toc cells in the presence or absence of tetracycline (data not shown).

## DISCUSSION

The prototypical *E. histolytica* TMK contains an N-terminal signal peptide, a predicted extracellular domain, and a single transmembrane helix followed by a cytosolic protein kinase-like domain, an organization similar to those of TMKs of other organisms. Based on this organization, the B1.I family is thought to be one of receptor kinases and can be defined as TMKs, though these properties have not been shown functionally. Other homologs are truncated copies lacking either the 5' or the 3' end of the genes and were not considered for this study. Based on extracellular and kinase domains, *E. histolytica* B1 family TMKs were suggested to have 11 members (4). Our analysis showed that this classification gave only a broad overview and that the actual number of different family members may be much higher. The high level ( $\geq 95\%$ ) of sequence identity also supports the idea that all of the members of B1.I class may have a common ligand and share the same downstream pathways; that is, these may be functionally identical. Moreover, due to this high level of sequence identity, it was difficult for us to distinguish between different members of the B1.I family, except when we sequenced the RT-PCR products. We found a few members of the B1 family (B1.IV) that encoded nearly identical copies of the kinase region but lacked the extracellular and transmembrane domains. This suggests that the kinase may also function independently without the ligand binding domain.

We have analyzed a few cloned RT-PCR products, and the results showed that only B1.I.1 and B1.I.2 appear to be expressed in growing axenic cultures. It is likely that other members are expressed at low levels, and the sequencing of a large number of RT-PCR products may indicate their expression. A previous study also showed that a subset of the TMKs of different families was expressed under in vitro axenic culture conditions (4). These studies indicate the possibility that many TMK homologs may not be functional or are not expressed during trophozoite growth in axenic conditions. It is also not possible for us to conclude that the rest of the B1.I TMKs are pseudogenes as these genes may be switched on under different conditions. Our analysis suggests a possibility of distinguishing the expressed genes from the silent genes based on the 5' upstream sequences of B1.I genes. The upstream sequences of B1.I.1 and B1.I.2 were different from others. The differences were noticed beyond 670 nucleotides upstream of the translation initiation codon ATG. Sequence conservation of the upstream 670 nucleotides was surprising as most of the amoebic promoters have been found very close to the gene (11). It is likely that either the specific promoters or the controlling elements are beyond 670 nucleotides or that an epigenetic mechanism may be involved in determining the expression status of these genes (6).

Members of the B1 TMK did not show any significant clustering in any segment of the genome. Also, no large-scale duplication of any segment of the *E. histolytica* genome was observed (data not shown). Therefore, the origin of multiple copies of the B1 TMKs is of great interest. An intriguing possibility is through retrotransposition, for which there is currently no evidence.

For a TMK protein to be functional, it should be able to bind the ligand through the extracellular domain and transduce the signal to the downstream effector pathway through the activated kinase domain. A large number of studies with different organisms have shown that overexpression of a TMK with either a truncated kinase or mutant kinase domain confers a dominant-negative phenotype (9, 35, 38, 39). The overexpression of truncated *E. histolytica* B1.I.1 TMK also gave similar results, making it likely that this kinase is mechanistically similar to other TMKs. A dominant-negative phenotype may be due to the ability of the cell to bind to the ligand but not transduce a signal. This can happen if the truncated putative receptor either removes the ligands so that the ligand is not available for binding or forms nonfunctional heterodimers with the wild-type molecule. Currently, we are unable to suggest the likely mechanism leading to the dominant-negative phenotype we observed. Experiments are being planned to address this issue.

The nature of the ligand at present is not known. The inability of Eh  $\Delta$ kinase cells to recover from serum starvation when grown in the presence of the inducer indicates that the truncated B1.I TMK is likely to mediate its effect on growth by removing a potential ligand from the serum. If this is true, the potential ligand may be present in the serum. However, it cannot be ruled out that the growth defect due to the truncated TMK and the defect due to serum starvation may be through different pathways having an additive effect on the overall growth defect.

Can we speculate on the role of a B1.I TMK in amoebic biology and pathogenesis? The data presented here gave an indication of a B1.I TMK in cell proliferation and sensitivity to growth factors present in serum. The inhibition of the functioning of these molecules and downstream pathways should, consequently, block the proliferation in *E. histolytica*. Therefore, it may be a potential target for new drug discovery. Although the dominant-negative cell line did not show any reduced pathogenesis by in vitro assays, there are indications that this molecule may have a role in pathogenesis. In preliminary experiments, the expression of B1 TMKs was found to be highest among all B family TMKs in amoebic cells that were isolated directly from a patient, as seen by microarray analysis (W. Petri, J. Frederick, C. Gilchrist, and R. Haque, unpublished data).

In conclusion, this study describes computational identification and domain organization of different members of the B1 family of TMKs in *Entamoeba*. It also reports the expression and localization of the B1.I.1 TMK in *E. histolytica* trophozoites. The experiments described here suggest a likely role of the B1.I.1 TMK in *E. histolytica* cell proliferation. This gives a useful molecular insight into environmental sensing by the parasite.

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