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Functional characterization of EhADH112: An *Entamoeba histolytica* Bro1 domain-containing protein

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Abstract

EhADH112 is part of the EhCPADH complex, a protein involved in key events of the *Entamoeba histolytica* host invasion. EhADH112 participates in trophozoite adherence to target cells and in phagocytosis. We report here the finding of two EhADH112 homologues in the *E. histolytica* genome (EhADH112-like proteins). EhADH112 and its relatives have a Bro1 domain at their amino-terminus and a consensus context for phosphorylation by Src-tyrosine kinases, both involved in signal transduction processes in other organisms. Our findings associate EhADH112 to supplementary functions related to those reported for the Alix/AIP1 family. To elucidate the precise function of EhADH112, we studied the phenotypes displayed by trophozoites transfected with the *Ehadh112* full gene. Transfected trophozoites overexpressed a 78 kDa protein, which was mainly targeted to the EhCPADH complex. Moreover, these trophozoites exhibited enhanced phagocytic rates, providing further evidence of EhADH112 contribution to adhesion and phagocytosis activities.

Index Descriptors and Abbreviations: Neo^r, *neomycin resistance* gene; PBS, phosphate-buffered saline solution; bp, base pairs; kb, kilobase pairs; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction

Keywords: EhADH112; EhCPADH; Alix/AIP1; Bro1 domain; Adhesin; Phagocytosis

1. Introduction

Entamoeba histolytica destroys almost all tissues of human using macromolecules participating in adhesion, contact-dependent cytolysis and proteolytic, and phagocytic activities. The EhCPADH complex (124 kDa), formed by a cysteine protease (EhCP112) and an adhesin (EhADH112), has been involved in all these processes (García-Rivera et al., 1999). EhCPADH is modified in adherence- and virulence-deficient mutant trophozoites and is recognized by sera of patients with intestinal and amoebic liver abscesses (Arroyo and Orozco, 1987). Also, this complex has been found to be secreted in active form by trophozoites (Ocádiz et al., 2005). EhCPADH is encoded by the *Ehcp112* and *Ehadh112* adjacent genes, located within a *locus* flanked by *RabB* and *Ras-related racA* genes at the 5′ end, and by a hypothetical protein-coding gene at the 3′ end (Flores et al., accompanying paper).

The EhADH112 polypeptide contains three putative transmembrane segments and four possible glycosylation sites. It also has an epitope (from 444 to 601 amino acids) recognized by anti-EhCPADH monoclonal antibodies (MAb112) at its carboxy-terminus. These antibodies inhibit adherence to target cells and phagocytosis (García-Rivera et al., 1999). An EhADH112 recombinant protein which contains the last 243 residues (EhADH243), and antibodies against it, inhibit

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adherence and destruction to target cells by live trophozoites. Moreover, immunization of hamsters with EhADH243 led to the development of antibodies against EhCPADH and to a significant reduction of liver abscesses formation after challenge with virulent trophozoites (Martínez-López et al., 2004). Additional experiments, using plasmids containing the *Ehcp112* gene or an *Ehadh112* gene fragment which lacks 306 bp at the 5′ end, successfully expressed these proteins in fibroblasts, where they were sorted to the plasma membrane, in the same way in which native proteins do in live trophozoites (Madriz et al., 2004).

The particular function of EhCP112 and EhADH112 polypeptides needs to be separately studied to elucidate the relationships existing between them and among other trophozoite proteins. Molecular cross-talk allows signaling triggers in cells to perform different functions, including those related to virulence. In other organisms, the Alix/AIP1 protein family has been associated to distinct biological processes as signal or adaptor proteins. However, no Alix/AIP1 proteins had been identified in *E. histolytica*. We report here the finding of two EhADH112-like proteins in the *E. histolytica* genome. EhADH112 and its relatives show similarity with Alix/AIP1 family members. Furthermore, to better understand the function of EhADH112, we transfected trophozoites with the *Ehadh112* gene and analyzed its influence on phagocytosis.

2. Materials and methods

2.1. In silico analysis

We searched for *Ehadh112*-related genes at the *E. histolytica* genome project databases (Sanger Institute, www.sanger.ac.uk; and The Institute for Genomic Research, TIGR, www.tigr.org). Similarity between predicted EhADH112-like proteins and EhADH112 was determined using the Expert Protein Analysis System (ExPASy) Proteomics Server by the NCBI BLAST2 service program. Sequence alignments were generated using the Clustal W program (version 1.81), the BLOSUM matrix and colored by the BoxShade server. Amino acid sequences from other organisms showing significant similarity to EhADH112 and EhADH112-like proteins were obtained from the DDBJ/GenBank/EBI databases using a BLASTP search.

2.2. Entamoeba histolytica cultures

Entamoeba histolytica clone A (strain HM1:IMSS) and transfected trophozoites (ANeo and ANeoADH populations) were axenically cultured in TYI-S-33 medium (Diamond et al., 1978). Medium for transfected trophozoites was supplemented with 10 or 40 μg/ml G418.

2.3. Construction of plasmids

A DNA fragment of 2061 bp encoding EhADH112 was PCR amplified using *E. histolytica* genomic DNA as template and the *Ehadh112* (5′-GGGGTACCATGAA TAGACAATTCATTCATTCCTGAA-3′) sense, and (5′-CGGGATCCTTACTTATCGTCGTCATCCTTGT AATCAAGAGATGGAAACATAGGATTG-3′) antisense oligonucleotides, which contain *Kpn*I and *Bam*H1 recognition sites, respectively. The *Ehadh112* gene was cloned into pExEhNeo plasmid (Hamann et al., 1995), generating the pExEhNeo-*Ehadh112* construction. This plasmid was automatically sequenced to corroborate the *Ehadh112* gene identity. pExEhNeo and pExEhNeo-*Ehadh112* plasmids were used to transform the bacterial *Escherichia coli* DH5α strain and purified with the Qiagen Maxi kit (Chatsworth, CA).

2.4. Transfection of E. histolytica

Transfection of pExEhNeo and pExEhNeo-*Ehadh112* plasmids into trophozoites was performed by electroporation to generate the ANeo and ANeoADH populations, respectively. Amoebae (10^7) were washed twice in cold PBS and once in cold cytomix buffer (Hamann et al., 1995). Electroporation was performed with the Bio-Rad Gene Pulser using 1200 V/cm and 25 μF, with a time constant of 0.4 ms. Electroporated amoebae were transferred into culture medium for 48 h before selecting them with 10 μg/ml G418 (Life Technologies, Gaithersburg, MD) first, and then with 40 μg/ml G418.

2.5. Reverse transcriptase (RT)-PCR experiments

Total RNA (1 μg) from A and ANeoADH trophozoites were extracted to synthesize cDNAs using a reverse transcriptase sequencing kit (Invitrogen) (Sambrook et al., 1989). Then, PCRs for *Neo*^{r}, *Ehadh112* and *actin* (as internal control) cDNAs were performed with the following primers: *Neo*^{r} 5′-ATGATTGAACAAGATG G-3′ sense, and 5′-TTAGAAGAACTCGTC-3′ antisense oligonucleotides; *Ehadh112* 5′-CATACCAATG AGAAAGTCAGATCC-3′ sense, and 5′-CCTTAGC GTTAGGATGTGCTC-3′ antisense internal primers; and *actin* 5′-AGCTGTTCTTTCATTATATGC-3′ sense, and 5′-TTCTCTTTCAGCAGTAGTGGT-3′ antisense oligonucleotides. Amplified products were visualized in 1.5% agarose gels, and documented and analyzed by densitometry.

2.6. Western blot assays

Proteins (30 μg) from transfected trophozoites were resolved by SDS-10% PAGE and electrotransferred onto nitrocellulose membranes. Membranes were incubated for 2 h at room temperature with mouse

anti-EhCPADH (MAb112) (Arroyo and Orozco, 1987) or mouse anti-actin monoclonal antibodies. Then, we used peroxidase-conjugated anti-mouse IgM (Zymed) as secondary antibodies. Immunoblots were revealed with 4-Cl-naphtol. Relative intensities were documented and analyzed by densitometry.

2.7. Erythrophagocytosis assays

Type O human erythrocytes (Rh^+) were freshly obtained, washed, and diluted to 10^8 cells/ml. *E. histolytica* trophozoites (10^6 cells/ml of TYI-S-33 without bovine serum) were incubated with erythrocytes (1:100) at 37 °C for 5, 10, and 15 min (García-Rivera et al., 1982). Percentage of ingested erythrocytes was determined by counting 200 amoebae at random from at least three independent duplicate experiments. Before these experiments, cell viability was determined by trypan blue exclusion and in all cases it was greater than 95%.

3. Results and discussion

3.1. EhADH112 has relatives in the E. histolytica genome

We searched for *Ehadh112*-related genes at the TIGR/Sanger genome databases. Two independent contigs (of 2693 and 2757 kb) that we named EhADH112-like 1 (898 amino acids) and EhADH112-like 2 (919 amino acids), respectively, were found to contain sequences 40% homologous to the *Ehadh112* gene product. EhADH112 showed amino acid identities between 21 and 23% with these proteins (Fig. 1A). The *Ehadh112-like 1* gene was flanked towards its 5′ end by a large non-coding region but towards its 3′ end, by a putative polyadenylate-binding protein gene. Meanwhile, the *Ehadh112-like 2* gene was found to be flanked by large non-coding regions at both ends. Although *Ehadh112*, *Ehadh112-like 1*, and *Ehadh112-like 2* genes share structural relationships, they were located at different *loci*. Since EhADH112 is functionally associated with EhCP112 within the EhCPADH complex, EhADH112-like 1 and EhADH112-like 2 proteins could display or not virulence-related properties. Further work shall be directed to study their independent functions.

3.2. EhADH112 and its relatives exhibit similarity with Alix/AIP1 proteins

EhADH112 has an adherence domain at its carboxy-terminus. However, its identity with other proteins in the databases had remained unknown (Martínez-López et al., 2004). To elucidate its specific function in trophozoites, we looked for EhADH112 homologues, using its primary sequence (GenBank Accession No. AF127375). In silico analysis showed that EhADH112 possesses 36–42% homology with Alix/AIP1 family members (Fig. 1B). The highest homology was found with *Dictyostelium discoideum* and mammalian Alix/AIP1. Identities ranged between 17 and 21% along the evolutionary scale. Like other Alix/AIP1 family members, EhADH112 and its relatives have the conserved Bro1 domain at their amino-terminus (Figs. 1A and B). This conserved domain constitutes the putative interaction site for Snf7, a protein involved in yeast multivesicular body formation (Xu et al., 2004). Also, EhADH112 and its relatives contain a potential recognition sequence for Src-tyrosine kinases, present in all Alix/AIP1 proteins and located in a conserved position within the consensus KDNDFIYD context (Figs. 1A and B). Besides, they display predicted coiled-coil regions distributed along distinct sections of their central region (Fig. 1B). Interestingly, these proteins lack the typical Alix/AIP1 proline-rich (PXXP) tract at their carboxy-terminus, which represents a potential SH3-binding site (Fig. 1A). However, in EhADH112 the adherence domain is located at the carboxy-terminus. Additionally, other proteins such as Rim20 and Rim20p, the *Saccharomyces cerevisiae* and *Candida albicans* Alix/AIP1 homologues, have no PXXP motifs (Fig. 1B) (Davis et al., 2000).

Alix/AIP1 family members have been involved as signal or adaptor proteins in several biological processes such as apoptosis (Vito et al., 1999), intracellular protein sorting and endosomal transport (Katoh et al., 2003; Odorizzi et al., 2003), virus budding (Marsh and Thai, 2003), pH regulation (Peñalva and Arst, 2004), development of multicellular organisms (Che et al., 1999), cell adhesion (Schmidt et al., 2003), and phagocytic pathways (Garin et al., 2001). Our results suggest that EhADH112 and its relatives may have functions related with Alix/AIP1 proteins.

3.3. EhADH112 is mainly targeted to the EhCPADH complex in transfected trophozoites

To study the EhADH112 function we transfected trophozoites with the *Ehadh112* full gene. The presence of the pExEhNeo-*Ehadh112* plasmid in transfected trophozoites was confirmed by RT-PCR, using the Neo^r gene probe (Fig. 2A). ANeoADH trophozoites overexpressed the *Ehadh112* mRNA when compared with non-transfected trophozoites (Fig. 2B). Densitometric analysis of *actin* and *Ehadh112* bands showed that indeed, ANeoADH trophozoites overexpressed up to sevenfold the *Ehadh112* gene (Fig. 2C).

By Western blot experiments, MAb112 detected a 124 kDa band in total extracts from ANeo and ANeoADH trophozoites (Fig. 3A, lanes 2 and 3). However, the ANeoADH band was stronger (Fig. 3A, lane 3), providing evidence of the presence of a higher amount of EhCPADH complex in ANeoADH trophozoites. Monoclonal anti-actin antibodies revealed that approximately

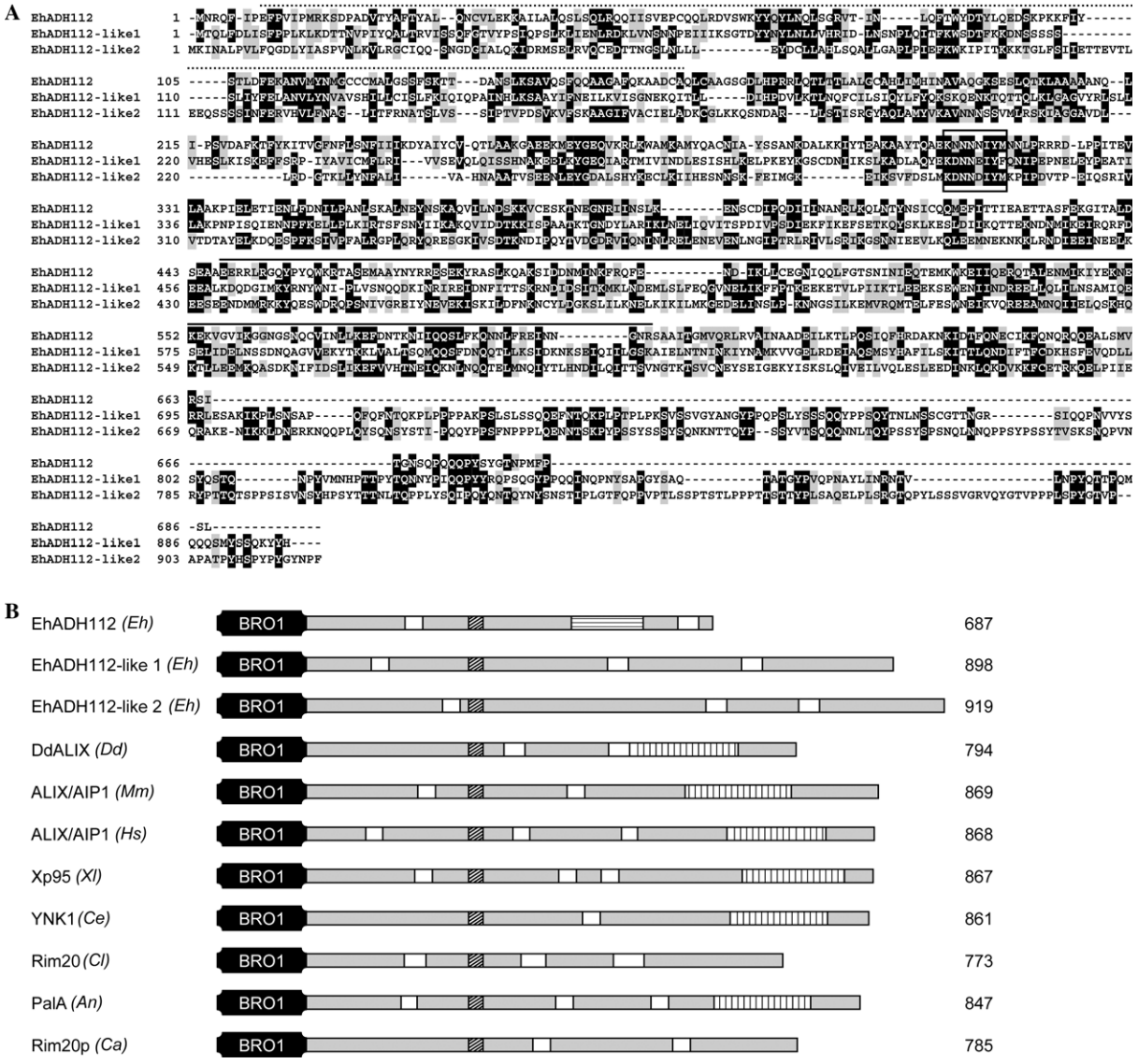

Fig. 1. EhADH112 and its relatives belong to the Alix/AIP1 family. (A) Multiple sequence alignment of EhADH112 and its relatives. Identical and conserved amino acids are in black and gray boxes, respectively. The Bro1 domain is shown above the alignment with an upper discontinuous line. The phosphorylation context is represented by a continuous square. The EhADH112 adhesion epitope is indicated by a continuous upper line. (B) Conserved architecture between Alix/AIP1 family. Structural characteristics of Alix/AIP1 and their homologues are indicated: Bro1 domain (black square), coiled-coil segments (white squares), the consensus KDNDFIYD phosphorylation context (square containing diagonal lines), proline-rich region (square containing vertical lines), and adhesion epitope (square containing horizontal lines). Each protein is denoted by its particular name followed by the species abbreviation (*Eh*, *Entamoeba histolytica*; *Dd*, *Dictyostelium discoideum*; *Mm*, *Mus musculus*; *Hs*, *Homo sapiens*; *Xl*, *Xenopus laevis*; *Ce*, *Caenohrabditis elegans*, *Cl*, *Candida lipolytica*, *An*, *Aspergillus nidulans*; *Ca*, *Candida albicans*). Residues number of each protein is indicated at the right end.

the same quantity of total proteins was loaded in both lanes (Fig. 3A, lanes 2 and 3). Additionally, in extracts from ANeoADH trophozoites we observed a 78 kDa slight band (Fig. 3A, lane 3), the expected mass of the EhADH112 subunit, which was absent in extracts from ANeo trophozoites. A 73 kDa band also appeared in the lane corresponding to ANeoADH trophozoites (Fig. 3A, lane 3). It is possible that EhADH112 as a single polypeptide results more unstable than protein forming part of the EhCPADH complex. Therefore, lower molecular weight bands could correspond to degradation products. Densitometric analysis of EhCPADH and actin bands demonstrated that ANeoADH trophozoites express almost sevenfold more EhCPADH than ANeo amoebae (Fig. 3B). Although *Ehadh112* gene transfection led to an EhCPADH overexpression, at this moment we do not know how complex formation is regulated.

3.4. ANeoADH trophozoites displayed increased phagocytic rates

We explored the influence of EhADH112 overexpression on phagocytosis. A and ANeo trophozoites ingested similar amounts of erythrocytes at all times tested,

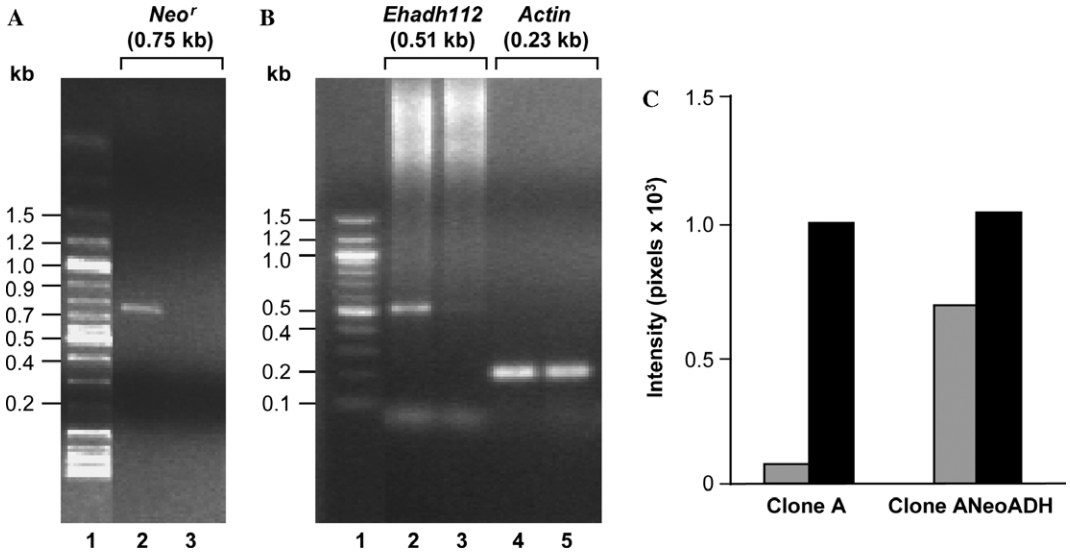

Fig. 2. ANeoADH trophozoites overexpress *Ehadh112* mRNA. (A) RT-PCR experiments using specific probes for *Neo*^{r} and (B) *Ehadh112* and *actin* genes. Lanes 1, molecular size markers. Amplified products using transfected trophozoites grown at 10 μg/ml G418 (lanes 2 and 4) or non-transfected clone A trophozoites (lanes 3 and 5). (C) Densitometric analysis for *Ehadh112* (gray bars) and *actin* (black bars) corresponding bands.

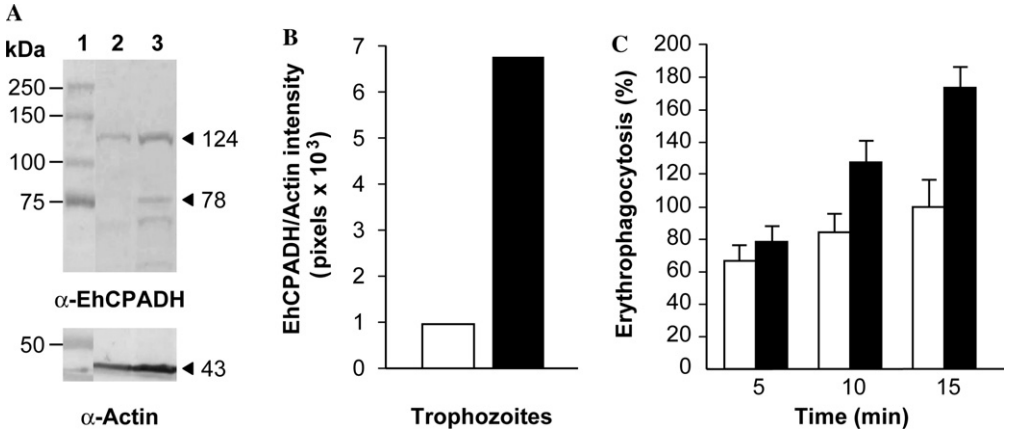

Fig. 3. ANeoADH trophozoites overexpress the EhCPADH complex and display enhanced phagocytic rates. (A) Western blot using anti-EhCPADH (MAb112) and anti-actin monoclonal antibodies. Lane 1, molecular weight markers. Lanes 2 and 3, extracts from ANeo and ANeoADH trophozoites (grown at 40 μg/ml G418), respectively. (B) Densitometric analysis for EhCPADH/actin bands intensity from ANeo and ANeoADH trophozoite extracts. (C) Erythrophagocytosis kinetics. White and black bars correspond to ANeo and ANeoADH trophozoites, respectively.

evidencing that the transfection procedure and G418 did not produce interference in assays. After 15 min, ANeo ingested in mean 13 ± 5 erythrocytes per trophozoite. This number was taken as 100% of phagocytosis (Fig. 3C). At this time, ANeoADH trophozoites ingested 23 ± 5 erythrocytes (75% more than ANeo trophozoites). At 10 min of phagocytosis, ANeoADH ingested 45% more erythrocytes than ANeo, whereas at 5 min, ANeoADH displayed only 15% more ingested erythrocytes. Therefore, EhADH112 overexpression positively influenced the phagocytic rates of trophozoites.

In conclusion, in this work we present evidence that *E. histolytica* EhADH112 is structurally related to the evolutionarily conserved and ubiquitously distributed Alix/AIP1 protein family. Considering that several members of this family have been involved in signal transduction processes, the presence of the Bro1 domain and the consensus KDNDFIYD phosphorylation motif in EhADH112, as well as the increase of erythrophagocytosis in transfected trophozoites suggest that EhADH112 could play a central role in signal transduction during this event, hence influencing the virulence of *E. histolytica.*

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