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EhNCABP166: A nucleocytoplasmic actin-binding protein from *Entamoeba histolytica*

A.D. Campos-Parra, N.A. Hernández-Cuevas, R. Hernandez-Rivas, M. Vargas*

Departamento de Biomedicina Molecular, Centro de Investigación y de Estudios, Avanzados del I.P.N. 2508, Col. San. Pedro Zacatenco, 07360 México, D.F., Mexico

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ABSTRACT

The actin cytoskeleton consists of multiple actin binding proteins (ABPs) that participate cooperatively in different cellular functions such as the maintenance of polarity and cell motility as well as the invasion of target cells and regulation of gene expression, among others. Due to the important role of ABPs in the pathogenesis of *Entamoeba histolytica*, the role of a new nucleocytoplasmic ABP from *E. histolytica* named EhNCABP166 was investigated. The EhNCABP166 gene encodes a protein with an estimated molecular weight of 166 kDa. Structurally, this peptide is composed of two CH domains arranged in tandem at the N-terminus of the protein, followed by an alpha-helical region containing a number of different domains with a low level of homology. Two (Bin1/Amphiphysin/Rvs167) (BAR) domains, one GTPase-binding/formin 3 homology (GBD/FH3) domain, three Bcl2-associated athanogene (BAG) domains, one basic-leucine zipper (bZIP) domain and one poly(A)-binding protein C-terminal (PABC) domain were also present. Molecular and biochemical studies showed that the EhNCABP166 protein is transcribed and translated in trophozoites of *E. histolytica*. It was also shown that the CH domains are functional and bind to F-actin, whereas the BAR and GBD/FH3 domains interact in vitro and in vivo with different families of GTPases such as Rho and Ras, and with different phosphoinositides. These findings suggest that these domains have the conserved functional properties described in other eukaryotic systems. These domains also interacted with additional GTPase and lipid targets that have not been previously described. Finally, cellular studies showed that EhNCABP166 is localized to the cytoplasm and nucleus of *E. histolytica* and that it has an important role in phagocytosis, proliferation, and motility of *E. histolytica*.

1. Introduction

The actin cytoskeleton is a dynamic network of filaments that consist mainly of actin and more than 100 structural proteins; it is involved in different cellular processes such as the cell cycle, cytokinesis, intracellular transport, endocytic and exocytic processes, movement, maintenance of cell shape and polarity, and the formation of highly specialized structures such as pseudopodia, filopodia, and lamellipodia, among others [1,2]. The complex and dynamic properties of the actin cytoskeleton are regulated at multiple levels by a variety of actin binding proteins or ABPs, some of which e.g., formin and profilin, respectively promote nucleation and polymerization of F-actin. ADF/cofilin and gelsolin have the ability to block the reactions involved in the assembly of actin by inhibiting nucleation or elongation of actin filaments [3]. Other ABPs, e.g., fimbrin and α-actinin, allow the formation of contractile parallel bundles of actin that comprise stress fibers, whereas filamin and spectrin crosslink the actin filaments to form three-dimensional actin networks that provide structural support for the cell surface [4]. It has also been shown that proteins like ezrin/radixin/moesin (ERM) connect actin filaments with the plasma membrane through the N-ERMAD domain [5]. In this regard, it has been reported that ABP functions are regulated by association with different phosphoinositides [6] or small GTPases [7]. Apart from being a fundamental component of the cytoskeleton, actin carries out different functions in the cell nucleus including the organization of chromatin remodeling complexes during RNA processing [8]. In addition, actin plays a direct role in transcription of RNA polymerases I, II and III [9–12]. In this regard, ABPs such as nuclear myosin I, which participates coordinately with nuclear actin to regulate the transcriptional activity of RNA polymerase I, may also localize to the nucleus [13]. Additionally, N-WASP can form complexes with PSF-NonO to regulate the transcriptional activity of RNA polymerase II [14]. Has been shown that α-actinin 4 in the nuclei of human cells is a powerful activator of the transcriptional activity of MEF2 [15]; α-spectrin II has also been associated with repair of nuclear DNA [16].

Abbreviations: PIs, phosphoinositides; BAR, Bin/Amphiphysin/Rvsp domain; CH, calponin homology domain; GBD/FH3, GTPase-binding/formin 3 homology domain; BAG, Bcl2-associated athanogene domain; bZIP, basic-leucine zipper domain; PABC, poly(A)-binding protein C-terminal domain.

* Corresponding author. Tel.: +52 55 57473326; fax: +52 55 5747 3326.
E-mail address: mavargas@cinvestav.mx (M. Vargas).

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It has also been observed that a fragment of the C-terminal region of human filamin ABP280 can influence the transcription activity of the FOXC1 factor [17]. Recently, the activated form of mDia was shown to shuttle between the nucleus and the cytoplasm using specific importin-alpha/beta and CRM1 nuclear transport machinery [18].

Study of the actin cytoskeleton of the human parasite *E. histolytica* is interesting because it has been proposed that reorganization of the amoeba cytoskeleton is essential for several cellular functions involved in pathogenicity including the interaction between the amoebic cell and its target cell [19], phagocytosis of intestinal epithelial cells and human erythrocytes [20], cytolysis of target cells [21], cell migration [22], and avoidance of the host immune response through the capping and release of antibodies [23]. However, the cellular and molecular processes involved in the movement of *E. histolytica* are still poorly understood. To date, different cytoskeletal ABPs such as profilin [24], myosin IB [20], myosin II [25], vinculin, tropomyosin [26], EhCaBP1 [27], spectrin [28], EhABP120 [29], EhABPH [30] and the α-actinin and α-actinin 2 proteins [31–33] have been identified and characterized. Because ABPs play an important role in the cytoskeletal reorganization of *E. histolytica*, this work reports the study of a multifunctional nucleocytoplasmic ABP from *E. histolytica* called EhNCABP166 that displays a distinct structural organization.

2. Materials and methods

2.1. Strains and culture conditions

Trophozoites of the pathogenic strain of *E. histolytica* HM1:IMSS were axenically cultured in TYI-S-33 [34]. Bacterial strain *Escherichia coli* BL21 (DE3) was used for recombinant DNA techniques. This bacterial strain was cultured in Luria-Bertani medium at 37 °C in the presence of ampicillin (50 μg/ml).

2.2. Bioinformatic analysis

Homology searches were carried out at the National Center for Biotechnology Information by the BlastP network service. Protein domains were identified by using the ExPASy proteomics server'. The sequence of nucleotides from EhNCABP166 was obtained from the *E. histolytica* Genome Database (TIGR).

2.3. Reverse transcription PCR assays

Total *E. histolytica* RNA was obtained using TRIZOL (Invitrogen) according to the manufacturer's recommendations. cDNA was synthesized using the primer 5′-CAACCAAGAATACCAATCTTTTATAGG-3′, which is specific to EhNCABP166, using the First-strand Synthesis System kit (Stratagene). Polymerase chain reaction (PCR) was then performed using primers corresponding to the EhNCABP166 gene (sense: 5′-AAAAGTATTGAAGAAAAAAAGAAT-3′; antisense (5′-TGGCATTACCATTTTAATTCCTAAAGC-3′) and the actin gene (sense: 5′-AGCTGTTCTTTCATTATATGC-3′; antisense: 5′-TTCTCTTTCAGCAGTAGTGGT-3′).

2.4. Generation of antibodies and western blot assay

To generate anti-EhNCABP166 antisera, recombinant GST/EhAB166 (E241-N681) was purified according to standard procedures and as recommended by the manufacturer; eight Balb/c mice were intraperitoneally inoculated with 10 mg of fusion protein 4 times at 15 d intervals. For the first dose, protein was emulsified with complete Freud adjuvant (Sigma). After the inoculation series, the animals were sacrificed and the serum was collected. For western blot assays, total *E. histolytica* proteins were separated by SDS-PAGE (7.5%), transferred to a nitrocellulose membrane (BioRad), blocked and probed with antibodies against the EhNCABP166 (1:1000) and the preimmune serum. The membranes were then incubated with horseradish peroxidase-conjugated sheep anti-mouse Ig (1:5000; Zymed), and the reaction was developed with 4-chloro-1-naphthol solution and 0.04% H_2O_2.

2.5. Preparation of cytoplasmic and nuclear extract

The cytoplasmic and nuclear fractions were purified as previously described [35]. For western blot assays, nuclear (NE) and cytoplasmic (CE) extracts were separated by SDS-PAGE (10%) and transferred to nitrocellulose membranes. Membranes were incubated with sera against EhNCABP166 (1:1000) in 5% nonfat dry milk and 0.05% Tween-20 in phosphate-buffered saline (PBS) pH 7.4 overnight at 4 °C. Proteins were detected using a horseradish peroxidase-conjugated secondary antibody (1:1500) (Zymed) and the ECL-Plus system (GE Healthcare). As a purification control, antibodies that specifically bind the *E. histolytica* EhPC4 protein, which is localized to nuclear fraction, and EhGEF2, which is localized to the cytoplasmic fraction, were used [36].

2.6. Overexpression of EhNCABP166 protein

Amoebas transfected with the expression vector pExEhNeo/HSV-Tagged-EhNCABP166 were grown in the presence of 10 and 30 μg/ml of G418, and proteins from trophozoites were isolated and analyzed by immunoblotting using an antibody against EhNCABP166 (1:1000), as described above. The membranes were scanned and relative intensities were calculated with the AlphaEase FC program.

2.7. Generation, expression and purification of GST-fusion proteins

The EhNCGBD/FH3-166 (E438-Q852), EhNCBAR1-166 (R248-E512), EhNCBAR2-166 (K1057-E1314), and EhNCABP166ΔC (M1-K1022) sequences were amplified by PCR using genomic DNA from *E. histolytica* as a template and cloned in-frame into the prokaryotic pGEX-2TK vector (Amersham Bioscience), which allowed the production of different GST-fusion proteins that contained an optimal phosphorylation motif for the catalytic subunit of cAMP-dependent Protein Kinase (PKA). The sequence of these constructs was verified by automated DNA sequencing. Briefly, the four domains from the EhNCABP166 protein were amplified using the following specific primers: EhNCGBD/FH3-166: 5′-CGCGGATCCGCGGAAGAAAATAAAGAAGATTTTGAAGCAAAA-3′ and 5′-CCCCCGGGGGTTGTTCTTTCTTTCTTTCTTCTTCAATTTG-3′; EhNCBAR1-166: 5′-CGCGGATCCGCGAGAGAACGAACTAAAGAAGT-AAGAAGAGCT-3′ and 5′-CCCCCGGGGGTTCATTCTTTTTCTTATTATC-AATTATTTC-3′; EhNCBAR2-166: 5′-CGCGGATCCGCGAAAATCAAA-CTAATTGAAATGATTAAAAAT-3′ and 5′-CCCCCGGGGGCATTATTTT-CTGACTTAATGTTTCTAACAA-3′; EhNCABP166ΔC: 5′-CGCGGAT-CCGCGATGAGTTCTTTACTTAAACTCTCAAGTACA-3′ and 5′-CCCCCGGGGGCTTTTTATTTTCTTTTTTTATTATTTCATT-3′. Each sense and antisense primer was flanked with BamHI and SmaI restriction sites, respectively. PCR products were digested, purified and cloned into the BamHI and SmaI sites of the pGEX2TK vector to obtain the following recombinant proteins: GST/EhNCGBD/FH3-166, GST/EhNCBAR1-166, GST/EhNCBAR2-166, and the GST/EhNCABP166ΔC. All of the fusion proteins were purified with Glutathione Sepharose 4B™ (Amersham Bioscience) according to the manufacturer's protocol and were analyzed by SDS-PAGE. Briefly, supernatants containing the recombinant fusion proteins were incubated with Glutathione Sepharose 4B™