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LIFE SCIENCE AND BIOMEDICINE
NOVEL-RESULT

Entamoeba histolytica protein CaBP3 uses a calcium dependent nuclear localisation pathway in mammalian cells.

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Abstract

Entamoeba histolytica is a major cause of dysentery that leads to a high level of morbidity and mortality, especially in developing countries. Calmodulin-like calcium binding protein EhCaBP3 of *E. histolytica* is directly involved in disease mechanisms with roles in cytoskeleton dynamics and scission during erythrophagocytosis in a calcium dependent fashion. Interestingly, EhCaBP3 is also present in the nucleus of *E. histolytica*. We have used a transfected cell system to show that EhCaBP3 is capable of calcium dependent nucleocytoplasmic trafficking. Our data confirms and extends recent findings suggesting presence of a calcium dependent nuclear transport pathway in *E. histolytica*.

Keywords: *Entamoeba histolytica*; EhCaBP3; calcium mediated nuclear transport; thapsigargin.

1. Introduction

Entamoeba histolytica is a single cell protozoan parasite that causes amoebiasis in humans. It is one of the leading causes of parasitic disease burden in tropical regions and developing countries where hygiene and sanitation is limited (Aguilar-Diaz et al., 2011; Morf & Singh, 2012). Calcium binding proteins are involved in erythrophagocytosis that characterises invasive amoebasis, and in the associated modulation of the actin cytoskeleton dynamics (Christy & Petri, 2011; Somlata et al., 2012). Somlata & Bhattacharya (2011) and Somlata et al. (2012) showed that the initiation of erythrophagocytosis in *E. histolytica* depends on C2-domain containing protein kinase (EhC2PK), actin and calcium binding proteins (EhCaBP1 and EhCaBP3). EhCaBP3 shares homology with calmodulin (Aslam et al., 2012; Rout et al., 2011), and is present both in the nucleus as well as the cytoplasm of the parasite (Rout et al., 2011). Chemical depletion of cytoplasmic calcium in *E. histolytica* caused the localisation of a nuclear protein, EhCaBP6, to the cytoplasm (Verma et al., 2017) suggesting the presence of calcium dependent nuclear transport in the protozoan.

2. Objective

We examined the possibility that EhCaBP3 may be a nuclear shuttling protein capable of Ca^{2+} dependent nuclear import similar to calmodulin (CaM) in higher eukaryotes. CaM-dependent nuclear import was initially reported by Sweitzer and Hanover (Sweitzer et al., 2000), where CaM was shown to be able to

facilitate nuclear import under conditions where high intracellular Ca^{2+} inhibited conventional Ran-dependent nuclear transport. We used over-expression of EhCaBP3 in transfected mammalian cells to determine its localisation, followed by investigation of change in localisation on increased cytosolic calcium.

3. Methods

3.1. Cell culture, transfection

COS-7 cells (CRL-1651, American Type Culture Collection) were grown as previously (Ghildyal et al., 2009) and used for transfection of plasmids using Lipofectamine. Where indicated, cells were treated 18 h post-transfection with 1 μM Thapsigargin (Calbiochem, La Jolla, CA, USA) for 6 h before imaging (Kaur & Jans, 2011).

3.2. Plasmid Constructs

The sequence for EhCaBP3 (Amoeba Database) was synthesised and cloned into the pMK-RQ shuttle vector (Life Technologies, GeneArt) and subcloned into the Gateway™ compatible pEPI-DESTC vector (Ghildyal et al., 2009) for expression in mammalian cells as GFP-EhCaBP3. Plasmids encoding GFP alone (Ghildyal et al., 2005), GFP-SRY-wt, GFP-SRY-R133W (GFP-SRY with arginine at position 133 mutated to tryptophan) and GFP-SRY-R76P (GFP-SRY with arginine at position 76 mutated to proline) have been described previously (Kaur & Jans, 2011).

3.3. Quantitative Confocal laser scanning microscopy

Transfected cells were imaged live in serum free FluoroBrite DMEM, using Nikon Ti Eclipse confocal laser-scanning microscope with Nikon 60x/1.40 oil immersion lens (Plan Apo VC OFN25 DIC N2; optical section of 0.5 μm) and the NIS Elements AR software. Data from four individual scans was averaged to obtain the final images (Walker et al., 2013). Image analysis of digitized CLSM images was performed as previously (Shahriari et al., 2018). Data sets represent mean +/− SEM from at least 30 cells from three independent experiments.

3.5. Statistical Analysis

Mean and standard error of the mean values were calculated for each dataset, and significant differences in mean values determined by ANOVA; $p < 0.05$ was accepted as significant. All statistical analysis was performed with GraphPad Prism software.

4. Results

4.1. GFP-EhCaBP3 accumulates in the nucleus of eukaryotic cells

GFP-EhCaBP3 was present in both the nucleus and cytoplasm of COS-7 cells transfected to express the protein, with obvious accumulation in the nucleus relative to the cytoplasm (Fig. 1A, images labelled 'no treatment'). Some GFP-EhCaBP3 was also localised to the plasma membrane, to structures in the cytoplasm resembling microfilaments and formed punctate structures in some cells. In the same experiment, GFP alone was present diffused through the whole cell as expected. GFP diffuses freely across the nuclear envelope and is commonly found distributed almost equally throughout the whole cell (Ghildyal et al., 2009; Shahriari et al., 2018; Yu et al., 2016). Image analysis of the digital images (Fig. 1B, columns labelled 'no add') confirmed that GFP-EhCaBP3 was accumulated in nuclei relative to cytoplasm of cells in which it was expressed (Fn/c = 2.96 ± 0.62), while GFP was present diffused in the whole cell (Fn/c = 1.57 ± 0.07).

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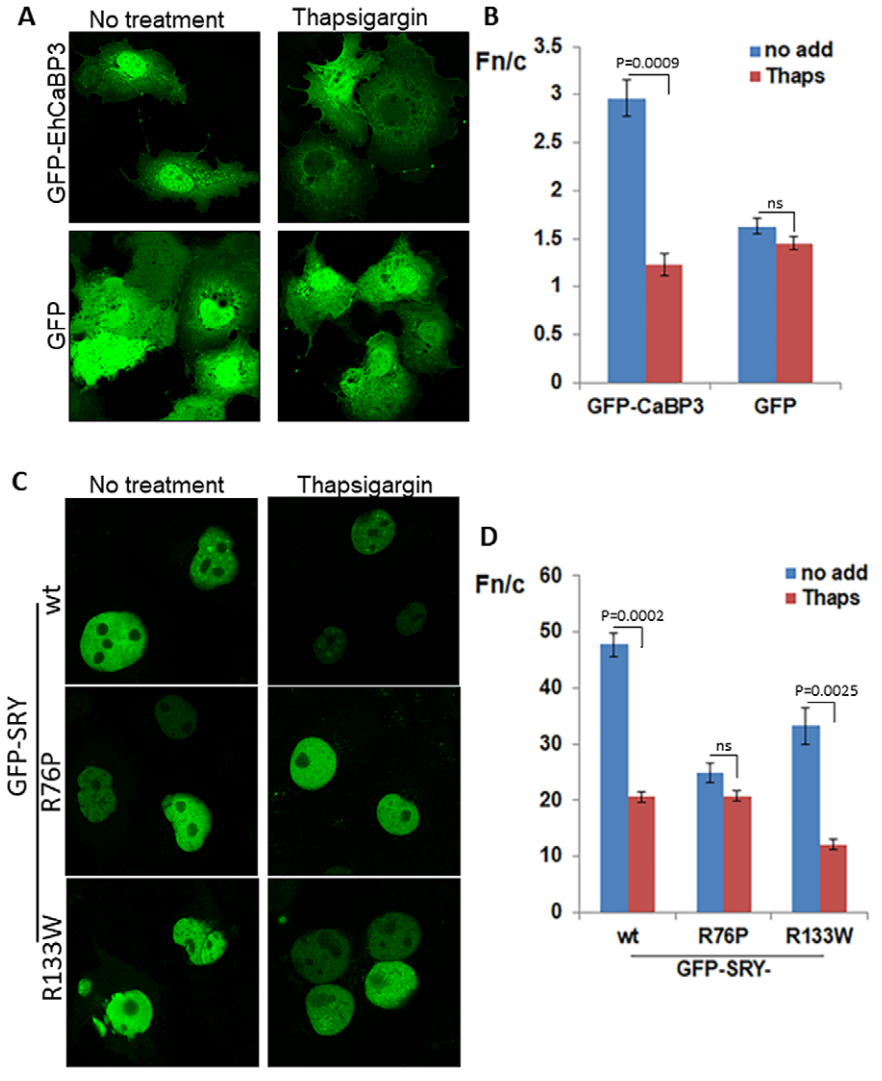

Figure 1. EhCaBP3 is localised in the nucleus and cytoplasm and has a calcium dependent nuclear transport
Overnight, subconfluent monolayers of COS-7 were transfected with plasmids to express GFP alone, GFP-EhCaBP3, GFP-SRY, GFP-SRY-R76P or GFP-SRY-R133W using Lipofectamine 2000, as per manufacturer's recommendations. Cells were either left untreated or treated with Thapsigargin (5 μM) for 6 h prior to live imaging on CLSM 24 h after transfection. Selected digital images are shown in A and C. Digital images were analysed with ImageJ to obtain relative fluorescence in the nucleus compared to that in the cytoplasm (Fn/c), shown in the histograms B, D. The yellow lines demarcate the cell outline. Two-way ANOVA followed by Sidak's test was used to determine statistical differences and values are shown on the histograms; ns = non-significant, significance was accepted at $p < 0.05$.

4.2. GFP-EhCaBP3 has calcium sensitive nuclear import

Thapsigargin treatment results in increased intracellular calcium due to release from endoplasmic reticulum (ER) and nuclear envelope (NE) stores (Thastrup et al., 1990). GFP-EhCaBP3 became significantly less nuclear on treatment with Thapsigargin (Fig. 1A, images labelled 'Thapsigargin') compared to in its absence. Quantitative image analysis for Fn/c confirmed this finding (Fig. 1B, columns labelled 'Thaps') with a statistically significant reduction in Fn/c on treatment compared to no treatment ($p = 0.0009$). Thapsigargin treatment had no effect on the subcellular localisation of GFP, as expected. Interestingly, GFP-EhCaBP3 appeared to be associated with cytoskeletal components under conditions

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of increased cytosolar calcium (Fig. 1A, image labelled GFP-EhCaBP3, Thapsigargin; note the cytosolar network localisation).

Cells transfected with GFP-SRY or its mutants (GFP-R76P, GFP-R133W) were used to control for Thapsigargin activity; SRY has two nuclear localisation signals (NLSs), one modulated by calcium and the other dependent on Importin-β (Sudbeck & Scherer, 1997). GFP-SRY-wt has both NLSs, GFP-SRY-R76P has the Importin-β dependent NLS only, while GFP-SRY-R133W has the calcium dependent NLS only. GFP-SRY-wt was highly accumulated in the nucleus while -R76P and -R133W less so as expected, due to presence of only one NLS (Fig. 1C, compare images labelled 'no treatment'). As expected (Kaur & Jans, 2011), treatment with Thapsigargin resulted in a statistically significant change in nuclear-cytoplasmic distribution of GFP-SRY-wt ($p = 0.0002$) and GFP-SRY-R133W ($p = 0.0025$) but had no effect on the subcellular localisation of GFP-SRY-R76P (Fig. 1C, compare images labelled 'Thapsigargin'); quantitative image analysis for Fn/c confirmed this finding (Fig. 1D). Data presented are representative of three independent experiments.

5. Conclusions

Our data show that nuclear transport of EhCaBP3 is calcium dependent. Eukaryotic calcium dependent nuclear transport is mediated by CaM (Sweitzer & Hanover, 1996) and is conserved from yeast to humans (Hanover et al., 2009). A classical CaM has not been identified in *E. histolytica* with the closest homolog being EhCaBP3 (Aslam et al., 2012). Taken together with a recent study (Verma et al., 2017) that showed that a calcium binding protein in *E. histolytica*, EhCaBP6, is a nuclear–cytoplasmic shuttling protein with calcium-dependent nuclear transport, our study strongly suggests the existence of a calcium modulated nuclear transport pathway in *E. histolytica*. Whole genome analysis of *E. histolytica* has shown the presence of putative protein components of the nuclear trafficking machinery. Indeed, our bioinformatics investigations predict the presence of all the core components of Ran dependent nuclear transport (Gwairgi & Ghildyal, 2018).

Taken together with the data presented in the current study, accumulating evidence strongly suggests the presence of complex nuclear transport pathways in *E. histolytica,* similar to higher eukaryotes.

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Conflicts of Interest. KW and RG declare no conflicts.

Author Contributions. KW performed the experiments and generated the figure. RG supervised the work and the experimental design and wrote the manuscript.

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Data Availability Statement. All data associated with this study are included in the manuscript. Reagents developed during this study are available from the corresponding author on request.

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Peer Reviews

Reviewing editor: Dr. André Luis Souza dos Santos
Universidade Federal do Rio de Janeiro, General Microbiology, Rio de Janeiro, Brazil, 21941-901

This article has been accepted because it is deemed to be scientifically sound, has the correct controls, has appropriate methodology and is statistically valid, and has been sent for additional statistical evaluation and met required revisions.

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Review 1: Entamoeba histolytica protein CaBP3 uses a calcium dependent nuclear localisation pathway

Reviewer: Dr. Jesús Valdés

Centro de Investigacion y de Estudios Avanzados del Instituto Politecnico Nacional, Bioquímica, Av. IPN 2508 - BQL11, Col San Pedro Zacatnco, Alc. GAM, CDMX, Mexico, 07360

Date of review: 02 October 2020

Conflict of interest statement. Reviewer declares none.

Comments to the Author: The authors expressed the calmodulin-like calcium binding protein EhCaBP3 was expressed in human background, and with carefully chosen controls demonstrated that localizes in the nucleus utilizing a calcium-dependent pathway. However, three points must be addressed:
1. The title of the paper should reflect that EhCaBP3 was tested in human background.
2. The introduction should give proper credit to EhCaBP precedent (Verma et al., 2017), not only discussing such previous findings.
3. Since the image is very well contrasted, marking at least one cell membrane could help untrained eyes to interpret Figure 1C.

Score Card

Presentation

4.4 /5

Is the article written in clear and proper English? (30%)	5/5
Is the data presented in the most useful manner? (40%)	5/5
Does the paper cite relevant and related articles appropriately? (30%)	3/5

Context

4.3 /5

Does the title suitably represent the article? (25%)	4/5
Does the abstract correctly embody the content of the article? (25%)	4/5

Does the introduction give appropriate context? (25%) 4/5

Is the objective of the experiment clearly defined? (25%) 5/5

Analysis

Does the discussion adequately interpret the results presented? (40%) 4/5

Is the conclusion consistent with the results and discussion? (40%) 5/5

Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%) 5/5

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Review 2: Entamoeba histolytica protein CaBP3 uses a calcium dependent nuclear localisation pathway

Reviewer: Dr. Marta Branquinha

UFRJ, Microbiologia Geral, Rio de Janeiro, RJ, Brazil

Date of review: 05 October 2020

Conflict of interest statement. Reviewer declares none.

Comments to the Author: In this paper, the authors set out to investigate whether calmodulin-like EhCaBP3 iscapable of calcium-dependent nucleocytoplasmic trafficking in Entamoeba histolytica through overexpression of EhCaBP3 in transfected mammalian cells and determination of its localisation, followed by investigation of changes on increased cytosolic calcium. The results of this well-conducted study suggested the presence of complex nuclear transport pathways in E. histolytica similar to higher eukaryotes. I strongly recommend the publication of this paper.

Score Card

Presentation

5.0 /5

Is the article written in clear and proper English? (30%)	5/5
Is the data presented in the most useful manner? (40%)	5/5
Does the paper cite relevant and related articles appropriately? (30%)	5/5

Context

5.0 /5

Does the title suitably represent the article? (25%)	5/5
Does the abstract correctly embody the content of the article? (25%)	5/5
Does the introduction give appropriate context? (25%)	5/5
Is the objective of the experiment clearly defined? (25%)	5/5

Analysis

5.0 /5

Does the discussion adequately interpret the results presented? (40%)	5/5
Is the conclusion consistent with the results and discussion? (40%)	5/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	5/5