Experimental Parasitology 126 (2010) 332–336

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Cellular analysis of host cell infection by different developmental stages of *Trypanosoma cruzi*

Luis Florencio-Martínez [a,b], Claudia Márquez-Dueñas [a], Gilberto Ballesteros-Rodea [a,c], Santiago Martínez-Calvillo [b], Rebeca Manning-Cela [a,*]

[a] Departamento de Biomedicina Molecular, Centro de Investigación y de Estudios Avanzados del IPN, México D.F., Mexico
[b] FES Iztacala, UBIMED, UNAM, Edo. de México, Mexico
[c] Facultad de Medicina Veterinaria y Zootecnia, UNAM, Mexico

ARTICLE INFO

Article history:
Received 11 November 2009
Received in revised form 17 March 2010
Accepted 6 April 2010
Available online 28 April 2010

Keywords:
Parasites
Transfection
Green fluorescent protein
Infectivity
Epimastigotes
Trypomastigotes
Amastigotes

ABSTRACT

Trypanosoma cruzi is an obligate intracellular parasite that infects phagocytic and non-phagocytic mammalian cells by a complex process that appears to involve several discrete steps. Even though the infection process was described many years ago, the molecular mechanisms involved remain poorly understood. As fluorescent proteins have proven to be excellent tools for live-cell imaging, we used *EGFP*- and *DsRed1-1*-transfected trypomastigotes, amastigotes and epimastigotes to study the infection process in living cells. Contrary to what has been reported, our results showed that epimastigotes are as infective as trypomastigotes and amastigotes. Besides, differences in replication, differentiation and parasite release times were observed among the stages. Our results suggest that the different developmental stages use distinct attachment and invasion mechanisms. We propose that fluorescent-based plasmid expression systems are good models for studying the infection process of intracellular microorganisms and could offers insights about the molecular mechanisms involved.

1. Introduction

Trypanosoma cruzi is the flagellated protozoan causative agent of Chagas' disease, an incurable fatal illness that affects 20 million people on the American Continent (Barrett et al., 2003). This parasite has a complex life cycle involving three distinct developmental stages that alternate between the invertebrate and the vertebrate hosts. Although the basic features of the infection process have been known for a century, diverse aspects of the molecular mechanisms involved have only recently been elucidated, and others aspects remain either controversial or unstudied (Espinoza and Manning-Cela, 2007). In humans as an obligate intracellular parasite, *T. cruzi* infects many different phagocytic and non-phagocytic cells through a complex cycle characterized by the presence of several discrete steps (attachment, invasion, multiplication, differentiation and parasite release from the host cell), the participation of multiple parasites and host cell molecules and the activation of bidirectional signaling cascades (Espinoza and Manning-Cela, 2007). In non-phagocytic cells, where disease pathogenesis takes place, some progress has been made in understanding the process of trypomastigote attachment and internalization into the host cell (Andrade and Andrews, 2005). Conversely, the available information about amastigotes is comparatively scarcer than that for trypomastigotes, and nothing is known about epimastigotes. Moreover, diverse aspects and key questions of the parasite escape from the parasitophorous vacuole, the parasite differentiation before and after the amastigote multiplication in the cytosol and the trypomastigote exit from the host cell remain poorly understood or unstudied (Espinoza and Manning-Cela, 2007). In the present work, we established the fluorescence-based monitoring of *in vitro* infections initiated with trypomastigotes, amastigotes and epimastigotes that were stably transfected with EGFP- and DsRed1-1-based plasmid expression systems, to study the infection kinetics of the different developmental forms of *T. cruzi*. Furthermore, the analysis of replication, differentiation and parasite release kinetics is also presented.

2. Experimental procedures

2.1. Cells and parasites

NIH 3T3 fibroblasts were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 5 mg/ml of penicillin–streptomycin at

* Corresponding author. Address: Av. IPN 2508, Col. San Pedro Zacatenco, C.P. 0736, México D.F., Mexico. Fax: +52 5557473938.
E-mail address: rmanning@cinvestav.mx (R. Manning-Cela).

0014-4894/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2010.04.015

37 °C in a humidified atmosphere containing 5% CO_2. Epimastigotes from the *T. cruzi* CL-Brenner strain and transfected lines were maintained in liver infusion tryptose (LIT) medium containing 10% FBS and 0.1 mg/ml hemine at 28 °C. The trypomastigotes and amastigotes were obtained from the supernatant of the NIH 3T3 monolayers infected as described below. The amastigotes were separated from trypomastigotes using the amastigote-specific antibody 2C2B6 against the Ssp-4 surface antigen of amastigotes, as previously described (Manning-Cela et al., 2001).

2.2. Construction of pTREXn–EGFP and pTREXn–DsRed1-1

The *EGFP* and *DsRed1-1* sequences were obtained by the digestion of pEGFP-N1 and pDsRed1-1 (Clontech) with *Hind*III/*Not*I and *Eco*RI/*Not*I, respectively, and after gel purification were sub-cloned in pTREXn (Vazquez and Levin, 1999) in the corresponding restriction enzymes sites. The resulted plasmids, pTREXn–EGFP and pTREXn–DsRed1-1, were used for transfection experiments after verifying the correct cloning by sequence analysis.

2.3. Generation of fluorescent parasites

Mid-log phase epimastigotes (3×10^8) resuspended in cold LYT medium were transfected by electroporation (BTX ECM 830) with 100 μg of cesium chloride-purified plasmid DNA at 300 V for 12 ms in 2 mm BTX electroporation cuvettes. After electroporation the transfected parasites were maintained for 5 min at 4 °C and then transferred to a fresh complemented LIT medium and incubated at 28 °C. After 48 h, the parasites were exposed to antibiotic selection with 500 μg/ml of G418 (No. Cat. 10131-035/GIBCO). Once antibiotic-resistant growth cultures were established, fluorescence clonal derivatives were isolated from each EGFP and DsRed1-1 parasite population by flow cytometry (FACSVantage, Becton, Dickinson).

2.4. Infectivity assay

For primary infections, monolayers of NIH 3T3 cells grown to 50% confluency in DMEM supplemented with 2% fetal calf serum were infected with 2×10^6 mid-log-phase fluorescent epimastigotes per ml, cultured in LIT media plus 10% FBS at 28 °C. Forty-eight hours later the cells were washed, and they were subsequently washed every 2 days with DMEM to remove non-adherent parasites, after which fresh DMEM plus 2% fetal calf serum was added. For the secondary infection experiments, 1×10^5/ml or 2×10^6/ml fluorescent epimastigotes cultured in LIT medium, or 1×10^5/ml or 2×10^6/ml fluorescent trypomastigotes or amastigotes obtained from the first infection and purified as described above, were used to infect NIH 3T3 fibroblasts (grown to 50% confluency) over 2 h. The cultures were washed with DMEM until non-adherent parasites were removed and fresh DMEM plus 2% fetal calf serum was added. Infections were monitored daily, and the number of amastigotes and trypomastigotes in the supernatant was determined. Using an inverted Olympus fluorescence microscope, the percentage of infected cells was calculated by comparing the number of cells containing parasites to the total number of cells, following the replication, differentiation and parasite-realized kinetics.

2.5. Epifluorescence and confocal microscopy

Cells grown over cover-slides were infected as described above and processed for fluorescence microscopy. The samples were fixed with 4% formaldehyde/PBS for 20 min, stained with 0.01 U/μl of rhodamine phalloidin (No. Cat. R415/Molecular Probes, Invitrogene) at room temperature for 20 min, nucleus was contra-stained with 5 μg/ml of DAPI (Molecular Probes), washed with PBS and mounted with vectashield® Mounting Medium (Vector Laboratories Cat. H-1000). The samples were analyzed in an Olympus fluorescence microscope BX41 equipped with a 60×/1.25 Oil Iris Ph3 UPlanFL N objective, and the images were captured with an Evolution VF Fast Cooled Color Media Cybernetics camera and analyzed with the Image-Pro Plus V 6.0 Media Cybernetics program. Besides, confocal laser microscope (Leica SP5, DM 16000, Mo) equipped with an HCXPLAPO lambda blue 63 × 1.4 NA oil objective was used and the images captured and analyzed using the software LAS AF (*Leica Application Suite Advanced Fluorescence Lite*/1.7.0 build 1240 Leica Microsystems).

2.6. Growth curves

The cultures were initiated with 1×10^6 epimastigotes per ml of complemented LIT medium incubated at 28 °C. The number of parasites was determined by direct counting with a Neubauer chamber every 24 h to determine the growing parasites. The viability of the cells was determined using the trypan blue exclusion test.

3. Results and discussion

3.1. Cloning and EGFP- and DsRed1-1-transfected parasite generation

To obtain green and red fluorescently tagged parasites, *EGFP* and *DsRed1-1* sequences were sub-cloned in the *T. cruzi*-derived cloning vector pTREXn and transfected into epimastigotes of the CL-Brenner strain, as described in Section 2. As shown in Fig. 1, epimastigotes expressing the EGFP or DsRed1-1 protein were uniformly fluorescent throughout the parasite body and flagellum as previously reported (Guevara et al., 2005; Pires et al., 2008). These results indicate that *EGFP*- and *DsRed1*-transfected parasites expressed the fluorescent proteins and demonstrate the successful generation of the florescent knock-in lines.

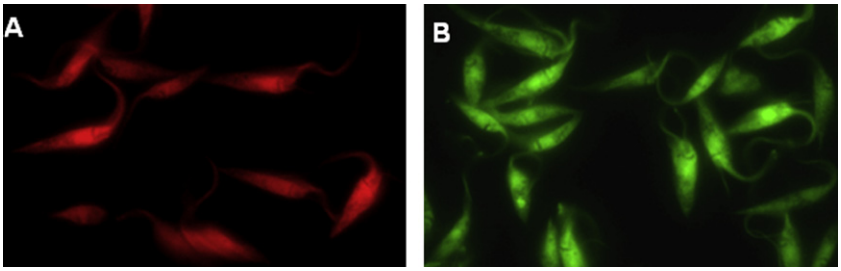

Fig. 1. Epifluorescence analysis of green and red fluorescently tagged epimastigotes. Direct observation of EGFP (A) and DsRed1-1 (B) knock-in parasites. Magnification is 60×.