INSTITUT PASTEUR

Microbes and Infection 10 (2008) 892–900

www.elsevier.com/locate/micinf

Original article

Calcineurin B of the human protozoan parasite *Trypanosoma cruzi* is involved in cell invasion

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Received 11 February 2008; accepted 15 May 2008
Available online 22 May 2008

Abstract

During *Trypanosoma cruzi* cell invasion, signal transduction pathways are triggered in parasite and host cells, leading to a rise in intracellular Ca^{2+} concentration. We posed the question whether calcineurin (CaN), in particular the functional regulatory subunit CaNB, a Ca^{2+}-binding EF-hand protein, was expressed in *T. cruzi* and whether it played a role in cell invasion. Here we report the cloning and characterization of CL strain CaNB gene, as well as the participation of CaNB in cell invasion. Treatment of metacyclic trypomastigotes (MT) or tissue-culture trypomastigotes (TCT) with the CaN inhibitors cyclosporin or cypermethrin strongly inhibited (62–64%) their entry into HeLa cells. In assays using anti-phospho-serine/threonine antibodies, a few proteins of MT were found to be dephosphorylated in a manner inhibitable by cyclosporin upon exposure to HeLa cell extract. The phosphatase activity of CaN was detected by a biochemical approach in both MT and TCT. Treatment of parasites with antisense phosphorothioate oligonucleotides directed to TcCaNB-CL, which reduced the expression of TcCaNB and affected TcCaN activity, resulted in ~50% inhibition of HeLa cell entry by MT or TCT. Given that TcCaNB-CL may play a key role in cell invasion and differs considerably in its primary structure from the human CaNB, it might be considered as a potential chemotherapeutic target.

Keywords: *Trypanosoma cruzi*; Cell invasion; Ca^{2+} mobilization; Calcineurin B; Inhibitors; Antisense oligonucleotides; Phosphatase activity

1. Introduction

An essential step in the life cycle of *Trypanosoma cruzi*, the causative agent of Chagas' disease, is the invasion of vertebrate cells, which is a multi-step process involving interactions between parasite and host cell molecules and the consequent activation of signal transduction pathways in both cells, resulting in intracellular Ca^{2+} mobilization [1–3]. The possible sources of Ca^{2+} in infective trypomastigote forms are intracellular compartments such as acidocalcisomes and endoplasmic reticulum [4].

In mammalian cells the protein phosphatase 2B, also known as calcineurin (CaN), is a serine/threonine phosphatase regulated by Ca^{2+} and calmodulin (CaM). The enzyme participates in several cellular processes including Ca^{2+}-dependent signal transduction pathways [5]. Purified CaN is a heterodimeric protein composed of a catalytic subunit called calcineurin A (CaNA), which contains four functional domains: the catalytic domain, the calcineurin B binding domain, the CaM binding domain and the autoinhibitory domain [5,6]. The catalytic activity of CaN is strictly dependent on its regulatory subunit calcineurin B (CaNB) [5], which is a Ca^{2+}-binding protein with

Abbreviations: MT, metacyclic trypomastigotes; TCT, tissue culture trypomastigotes; CaN, calcineurin or protein phosphatase 2B; PP2B, protein phosphatase 2B; CaNA, catalytic subunit of calcineurin; CaNB, regulatory subunit of calcineurin; CaM, calmodulin; CsA, cyclosporin A; Cype, cypermethrin; CyP, cyclophilin; TBS, Tris-buffered saline; DMEM, Dulbecco's modified Eagle's medium; PI, protease inhibitors; ORF, open reading frame.

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doi:10.1016/j.micinf.2008.05.003

high affinity for Ca^{2+}, being able to bind four Ca^{2+} per mol of protein [6]. When the four Ca^{2+}-binding motifs, also called ''EF-hand motifs'', were compared, the complete sequence of CaNB displayed 35% and 29% identity with CaM and troponin C, respectively [7]. Forty-five distinct subfamilies of EF-hand proteins have been identified to date. They contain two to eight EF-hands, whose amino acid sequences are similar to other EF-hand domains [8]. Based on the sequence alignment and identity among the inter spacer domains, CaNB can be considered a subfamily of the EF-hand proteins [8]. Increasing numbers of Ca^{2+}-binding proteins containing EF-hand motifs homologous to CaNB have been reported [9,10].

Recently, a novel protein homologous to CaN from *T. cruzi* clone CL Brener was identified and characterized. This enzyme possesses the catalytic and CaNB binding domains, but the CaM binding and autoinhibitory domains are absent [11]. Therefore, it is possible that CaN-like proteins lacking the CaM binding domain are activated and regulated by Ca^{2+} through their regulatory subunit CaNB. As *T. cruzi* cell entry is a Ca^{2+}-dependent process, we investigated whether calcineurin, in particular the regulatory subunit CaNB, was expressed in *T. cruzi* and played a role in host cell entry. Using *T. cruzi* CL strain, we demonstrate here that CaNB is present in this parasite and is implicated in target cell invasion.

2. Materials and methods

2.1. T. cruzi, *HeLa cell cultures and invasion assays*

The parasites (CL strain) were maintained cyclically in Balb/c mice and liver infusion tryptose (LIT) medium (5.0 g liver infusion; 5.0 g tryptose; 4.0 g NaCl; 0.4 g KCl; 8.0 g Na_2HPO_4; 2.0 g glucose; 10.0 mg hemin; 10% fetal calf serum). Grace's medium was used to obtain cultures enriched in MT, which were purified through DEAE-cellulose column. TCT were obtained from HeLa cells, grown in DMEM medium supplemented with 10% fetal bovine serum in 5% CO_2 atmosphere at 37 °C, by collecting the parasites released 5 days post infection. For invasion assays, 3×10^4 HeLa cells grown in 4-well Microchamber Slides (Nunc) were incubated with parasites for 3 h, washed with PBS, fixed in methanol and stained with Giemsa.

2.2. *Preparation of HeLa cell and parasite extracts*

HeLa cells, washed in PBS, were detached by scraping, suspended in PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 25 μg/ml leupeptin, 25 μg/ml antipain), and sonicated on ice. After 5 min centrifugation at 12,000 × *g*, the supernatant was immediately used or stored at −80 °C until use [12]. To prepare parasite extract, trypomastigotes (2.8×10^8) were washed with TBS pH 7.2 containing protease inhibitors (PI) (mini complete PI Cocktail Tablets, Roche). The pellet was resuspended in 4 ml autoclaved MilliQ water with PI, the cells disrupted in a French Press were diluted in 5× TBS buffer containing PI. After 1 h centrifugation at 200,000 × *g* at 4 °C, the supernatant was concentrated in Amicon tube.

2.3. *Detection of serine/threonine-phosphorylated* T. cruzi *proteins*

Trypomastigotes (5×10^7) were solubilized at 4 °C in a buffer containing protease and kinase inhibitors (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 mM iodocetamide, 1 mM $NaVO_4$, 1 mM NaF). The lysates, dissolved in loading buffer, were subjected to SDS–PAGE and electrotransferred to PVDF membrane. The proteins were analyzed by immunoblotting using anti-phospho-serine/threonine antibodies (Sigma–Aldrich) and the immunocomplexes revealed by chemiluminescence with ECL plus Western blotting detection reagent and ECL-Hyperfilm (GE Healthcare).

2.4. *Phosphatase activity assays*

CaN activity was assayed using 80 mM *p*-Nitrophenyl phosphate (*p*NPP) as substrate (Sigma–Aldrich), as described [13]. Briefly, tubes containing either 50 nM purified bovine brain calcineurin (Sigma–Aldrich) as an internal control, or 5–10 μl of parasite extract, in a final volume of 50 μl reaction buffer (0.4 mM $CaCl_2$, 100 mM Tris–HCl pH 7.2, 100 mM NaCl, 1 mM $MnCl_2$), buffer only or buffer plus CaN/CaM, were incubated for 5 min at 30 °C. Upon addition of 80 mM *p*NPP at 20 s intervals, and 30 min incubation, the reactions were terminated by adding 950 μl of 1 M NaOH at 20 s intervals. Ca^{2+}-independent CaN activity was determined under identical conditions by adding 5 mM EGTA. The release of *p*-nitrophenol (PNP) was measured at 405 nm in DU 64 spectrophotometer (Beckman). To calculate CaN activity, the average absorbance values from duplicate samples, with the background PNP value subtracted, were converted into the amount of PNP released by plotting against a standard PNP curve. The final value was divided by the reaction time to give the activity value.

2.5. *Isolation of nucleic acids, Southern and Northern blot hybridizations, separation of chromosomal bands*

Isolation of *T. cruzi* genomic DNA and total RNA, Southern and Northern blot hybridizations, and separation of chromosomal bands by pulsed-field gel electrophoresis were performed as previously described [14].

2.6. *Cloning of* T. cruzi *CaNB and CaNA genes*

For PCR amplification of CaNB gene, we used primers based on cDNA sequence for *T. cruzi* (G strain) CaNB (GenBank accession number AY570505): sense primer (5′-ATG AAT TCT CCC CGC AGG CTG ACA AGC G-3′) and antisense primer (5′-ATG AAT TCC CCG CGG AGT TTG CTG CGG G-3′), and for CaNA: sense primer (5′-ATG TAT TCT GAA CGA GGG CTG C-3′) and antisense primer (5′-TTA ACT CAT TTC CTC CTC TTC GC-3′), accession number XM_805398.1. *T. cruzi* genomic DNA (1 μg), 120 pmol of each primer and *Taq* recombinant polymerase kit (Invitrogen) were used to run 35 cycles of 1 min denaturing at 94 °C,