Acta Tropica 146 (2015) 141–151

Contents lists available at ScienceDirect

Acta Tropica

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

Biochemical and molecular characterization of the calcineurin in *Echinococcus granulosus* larval stages☆

María Celeste Nicolao[a,b], Andrea C. Cumino[a,b,c,*]

[a] *Laboratorio de Zoonosis Parasitarias, Departamento de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata (UNMdP), Funes 3350, Nivel Cero, 7600 Mar del Plata, Argentina*
[b] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina*
[c] *Departamento de Química, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata (UNMdP), Funes 3350, Nivel 2, 7600 Mar del Plata, Argentina*

ARTICLE INFO

Article history:
Received 10 October 2014
Received in revised form 6 March 2015
Accepted 10 March 2015
Available online 25 March 2015

Keywords:
Echinococcus
Calcineurin
Cyclosporine-A
Calcium chelators
Larval development

ABSTRACT

Calcineurin (CaN) is a Ca^{2+}-calmodulin activated serine–threonine protein phosphatase that couples the local or global calcium signals, thus controlling important cellular functions in physiological and developmental processes. The aim of this study was to characterize CaN in *Echinococcus granulosus* (Eg-CaN), a human cestode parasite of clinical importance, both functionally and molecularly. We found that the catalytic subunit isoforms have predicted sequences of 613 and 557 amino acids and are substantially similar to those of the human counterpart, except for the C-terminal end. We also found that the regulatory subunit consists of 169 amino acids which are 87% identical to the human ortholog. We cloned a cDNA encoding for one of the two catalytic subunit isoforms of CaN (Eg-can-A1) as well as the only copy of the Eg-can-B gene, both constitutively transcribed in all *Echinococcus* larval stages and responsible for generating a functionally active heterodimer. Eg-CaN native enzyme has phosphatase activity, which is enhanced by Ca^{2+}/Ni^{2+} and reduced by cyclosporine A and Ca^{2+} chelators. Participation of Eg-CaN in exocytosis was demonstrated using the FM4-64 probe and Eg-CaN-A was immunolocalized in the cytoplasm of tegumental cells, suckers and excretory bladder of protoscoleces. We also showed that the Eg-can-B transcripts were down-regulated in response to low Ca^{2+} intracellular level, in agreement with decreased enzyme activity. Confocal microscopy revealed a striking pattern of Eg-CaN-A in discrete fluorescent spots in the protoscolex posterior bladder and vesicularized protoscoleces beginning the vesicular differentiation. In contrast, Eg-CaN-A was undetectable during the pre-microcyst closing stage while a high DDX-like RNA helicase expression was evidenced. Finally, we identified and analyzed the expression of CaN-related endogenous regulators.

1. Introduction

Calcineurin (CaN), also known as protein phosphatase 2B (PP2B), is a Ca^{2+}-calmodulin activated serine–threonine protein phosphatase involved in different signaling pathways (Rusnak and Mertz, 2000). CaN plays a key role in the coupling of local or global calcium signals that control immediate cellular responses and modify gene transcription. CaN is a heterodimer composed of a catalytic A subunit (CaN-A) and a Ca^{2+}-binding regulatory B subunit (CaN-B). CaN-A contains a catalytic phosphatase domain and a regulatory domain which includes a CaN-B binding domain, a calmodulin (CaM)-binding domain and an autoinhibitory domain, which binds to the catalytic site in the absence of Ca^{+2}/CaM, thus inhibiting PP2B activity. CaN, encoded by one or more genes for each subunit, is considered an evolutionarily conserved protein in all eukaryotes (Rusnak and Mertz, 2000).

The biological functions of CaN, which involve Ca^{2+}-dependent cellular events, have been studied in many organisms, including mammals, plants, fungi, invertebrates and protozoa. When compared to other protein phosphatases (Sim et al., 2003), CaN interacts with a limited number of substrates, such as proteins involved in membrane trafficking and cytoskeletal association, ion channels, targeting and regulatory proteins and transcription factors (Rusnak

☆ Nucleotide sequence data reported in this paper are available in the GenBank database under GenBank Accession Number KC146906 (Eg-*can*-A1), HQ454284 (Eg-*can*-B) and KC146907 (Eg-*chp*).

* Corresponding author at: Laboratorio de Zoonosis Parasitarias, Departamento de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata (UNMdP), Funes 3350, Nivel Cero, 7600 Mar del Plata, Argentina.
Tel.: +54 223 4752426x450.
E-mail address: acumino@mdp.edu.ar (A.C. Cumino).

http://dx.doi.org/10.1016/j.actatropica.2015.03.016
0001-706X/

and Mertz, 2000; Li et al., 2011). Consequently, CaN encompasses the transcriptional regulation in connection with Ca^{2+} signaling in the immune, nervous, vascular, musculoskeletal and endocrine systems and controls important cell functions in physiological and developmental processes (Aramburu et al., 2004). In vertebrate secretory cells, such as neurons and immune system and pancreatic acinar cells, CaN regulates endocytosis–exocytosis through dephosphorylation of dynamin, synaptotagmin, dephosphins, actin and kinesin (Sim et al., 2003; Grybko et al., 2007; Bennett et al., 2010).

In fungi and in invertebrates, CaN has been associated with growth and developmental regulation (Chen et al., 2010; Aramburu et al., 2004; Bandyopadhyay et al., 2004), whereas in protozoan parasites it has been linked to the regulation of the life cycle, development and exocytosis, thus becoming a promising target for the development of inhibitors that block invasion and limit parasite growth (Rascher et al., 1998; Banerjee et al., 1999; Orrego et al., 2014; Singh et al., 2014). In helminth parasites, such as *Hymenolepsis microstoma*, *H. diminuta* and *Schistosoma mansoni*, it has been proved that CaN is involved in ion homeostasis (Roberts et al., 1997; Mecozzi et al., 2000). In addition, CaN has been immunolocalized in the flame cells of the *Schistosoma* excretory system, and in the cilia of *Paramecium tetraurelia*, where it seems to be involved in the regulation of exocytosis (Mecozzi et al., 2000; Fraga et al., 2010).

CaN activity is regulated by its interaction with several endogenous proteins. Among CaN inhibitors are: AKAP79, which anchors CaN to the membrane with protein kinases A or C; cain/cabin 1, which binds to CaN, blocking its interaction with physiological substrates; the CaN-B homologous protein (CHP/p22), which mimics the interaction of the regulatory subunit CaN-B with CaN-A; and a family of regulators of CaN (RCAN), which bind directly to CaN-A (Rusnak and Mertz, 2000; Mellström et al., 2008). On the other hand, calpains, cytosolic Ca^{2+}-activated cysteine proteases, activate CaN in both a direct and an indirect manner by cleavage of CaN-A autoinhibitory domain and cain/cabin 1, respectively (Kim et al., 2002). In addition to the endogenous inhibitors, the immunosuppressant drugs cyclosporine A (CsA) and FK506 (tacrolimus), both produced by soil microorganisms, inhibit CaN signaling, allowing the elucidation of its function in many eukaryotic organisms. These drugs bind to cyclophilin (CyP) or FK506-binding protein (FKBP) and the CyP-CsA and FKBP-FK506 complexes inhibit CaN allosterically (Liu et al., 1991). Similarly, the intracellular chelator BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetracetic acid) which is highly selective for Ca^{2+} over Mg^{2+} has also been widely used as an inhibitor of the calcium–calcineurin pathway (Semsarian et al., 1999; Srinivasan et al., 2010).

Human cystic echinococcosis or hydatidosis is an endemic worldwide zoonosis caused by the larval or metacestode stage of the tapeworm *Echinococcus granulosus.* The complex life cycle of *E. granulosus* involves an intermediate host (humans and domestic livestock) and a definitive one (canid). The cyst or metacestode develops in the intermediate host and produces protoscoleces from the inner germinal layer. Protoscoleces develop into adult worms in the gut of the definitive host (McManus et al., 2003). However, *E. granulosus* shows another alternative development. Protoscoleces which are released into the circulation from a ruptured cyst, located in an intermediate host, are able to asexually differentiate into secondary hydatid cysts. This reveals an evident and high developmental plasticity.

The evaluation of the action of CsA on the establishment and growth of secondary hydatid cysts of *E. granulosus* in mice has indicated that this drug is a possible candidate for future clinical application (Hurd et al., 1993). Colebrook and collaborators (Colebrook et al., 2002, 2004) demonstrated that CsA is an effective protoscolicidal agent against *E. granulosus*, assessed the dose and time-dependent susceptibility from *in vitro* treated parasites and characterized one type of CyP involved in protoscolex drug sensitivity. In our laboratory, we have previously described the synergic protoscolicidal actions during the combined therapy with rapalogs and CsA and identified the two different cellular targets: FKBP-TOR and CyP-CaN (Cumino et al., 2010). We have also identified two genes homologous to the *Homo sapiens* CaN-A gene and one to the *Taenia asiatica* CaN-B gene in the *E. multilocularis* assembled genomic contigs, as well as their corresponding Expressed Sequence Tag (EST) in the *E. granulosus Lopho* DB (Cumino et al., 2010). In the present study, we focused on the biochemical and molecular characterization of CaN in the *E. granulosus* larval stages (Eg-CaN), its involvement in protoscolex exocytic processes and the identification of its possible endogenous regulators.

2. Materials and methods

2.1. In vitro *culture of protoscoleces, metacestodes and pre-microcyst obtainment*

E. granulosus protoscoleces were removed under aseptic conditions from hydatid cysts of infected cattle presented for routine slaughter at the abattoir in the province of Buenos Aires, Argentina. Protoscolex *in vitro* culture ($n = 3000/9.5\,cm^2$), pharmacological treatment and vitality assays were performed as previously described (Cumino et al., 2010). Otherwise, *E. granulosus* metacestodes (10–20 cysts for each drug treatment) were obtained from the peritoneal cavities of CF-1 mice after intraperitoneal infection with protoscoleces (Nicolao et al., 2014a). Animal procedures and management protocols were carried out as previously described (Nicolao et al., 2014b) in accordance with National Health Service and Food Quality (SENASA) guidelines, Argentina and with the 2011 revised form of The Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. Unnecessary animal suffering was avoided throughout the study. *In vitro* protoscolex and metacestodes treatments were assayed with CsA and BAPTA at 100 μM for 24 h. For molecular experiments, parasites were washed with sterile and RNAse-free PBS and they were conserved at −80 °C until experimental use. Each experiment was assayed for three replicates and repeated three times.

Incubation of protoscoleces with insulin and fetal bovine serum (FBS) for several days induces a progressive de-differentiation toward the metacestode stage or microcyst. Microcysts represent the phase in which the protoscolex is completely transformed into a miniature cyst (loosing suckers, rostellum and hooks, and showing a laminated layer), but the success rate of protoscoleces undergoing this de-differentiation process is very low (1–3%). However, a previous differentiation stage, named pre-microcysts can be obtained more easily. The pre-microcyst, a vesicle-like structure, is a completely vesicularized protoscolex with suckers and rostellum vestiges, without a laminated layer and almost devoid of movement (Cucher et al., 2013). In order to obtain vesicularized protoscoleces and pre-microcysts, aliquots of 1500 protoscoleces were cultured in Leighton tubes in medium 199 supplemented with antibiotics (penicillin, streptomycin and gentamicin 100 μg/ml), glucose ($4\,mg\,ml^{-1}$), insulin ($1.2\,U\,ml^{-1}$) and 15% FBS. A total of 30–40 tubes per sample were cultured and the protoscoleces from the same sample develop at different rates. Cultures were maintained at 37 °C for 50 days, the medium was changed every 4–6 days and the sample was carefully recovered after of the complete decantation. The pH was monitored during the culture period by means of the pH indicator (phenol red incorporated in the 199 medium) and adjusted with 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5) to control it within the normal physiological range. Development was followed microscopically under an inverted light microscope