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Trypanosoma cruzi calmodulin: Cloning, expression and characterization

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ABSTRACT

We have cloned and expressed calmodulin (CaM) from *Trypanosoma cruzi,* for the first time, to obtain large amounts of protein. CaM is a very well conserved protein throughout evolution, sharing 100% amino acid sequence identity between different vertebrates and 99% between trypanosomatids. However, there is 89% amino acid sequence identity between *T. cruzi* and vertebrate CaMs. The results demonstrate significant differences between calmodulin from *T. cruzi* and mammals. First, a polyclonal antibody developed in an egg-yolk system to the *T. cruzi* CaM recognizes the autologous CaM but not the CaM from rat. Second, it undergoes a larger increase in the α-helix content upon binding with Ca^{2+}, when compared to CaM from vertebrates. Finally, two classic CaM antagonists, calmidazolium and trifluoperazine, capable of inhibiting the action of CaM in mammals when assayed on the plasma membrane Ca^{2+} pump, showed a significant loss of activity when assayed upon stimulation with the *T. cruzi* CaM.

1. Introduction

Calmodulin is one of the most ubiquitous proteins in the animal kingdom; its function has been associated with the control of many cellular processes, by sensing intracellular Ca^{2+} oscillations (Klee and Vanaman, 1982; Chin and Means, 2000). In mammals, CaM[1] has been described as a polyvalent protein that participates in many and varied cellular pathways. This protein has been implicated in the synthesis and degradation of cyclic nucleotides and phosphoinositides, phosphorylation/desphosphorylation of multiple proteins through several specific protein kinases and phosphatases, gene transcription, and regulation of different transport systems. Also, its role has been studied in the control of metabolism, cytoskeletal organization, cytokinesis, muscle contraction, osmotic cell volume regulation, exocytosis, intracellular communication, cell proliferation, differentiation, and apoptosis (Klee and Vanaman, 1982; Chin and Means, 2000; Benaim and Villalobo, 2002; Carafoli et al., 2001).

The tertiary structure of mammalian CaM was elucidated in the 80-s, using rat testis CaM as a model. CaM is a molecule of 65 Å divided into two similar globular clusters connected by a flexible α-helix. Each cluster contains two Ca^{2+}-binding regions with 12 important residues, principally glutamate and aspartate which coordinate Ca^{2+} association (Babu et al., 1985).

Concerning trypanosomatids, CaM has been found in *Trypanosoma brucei* (Ruben et al., 1983) and *Trypanosoma cruzi* (Benaim et al., 1991), as well as *Leishmania donovani* (Mazmuder et al., 1992), *Leishmania braziliensis*, and *Leishmania mexicana* (Benaim et al., 1987). It has been associated with many different functions in trypanosomatids, such as growth regulation in *T. brucei* (Eid and Sollner-Webb, 1991), cAMP-dependent phosphodiestherase stimulation in *T. cruzi* (Tellez-Iñon et al., 1985), CaM-dependent protein kinase in *T. cruzi* (Ogueta et al., 1994), signal transduction in the cGMP-nitric oxide pathway in *T. cruzi* (Paveto et al., 1995), and stimulation of plasma membrane Ca^{2+}-ATPase from *L. braziliensis* (Benaim and Romero, 1990), *L. mexicana* (Benaim, 1996), *T. cruzi* (Benaim et al., 1995), and *T. brucei* (Benaim et al., 1993).

Significant differences in its amino acid sequence have been found between *T. cruzi* (Chung and Swindel, 1990) and mammalian CaM. A protein sequence analysis showed 15 amino acid substitutions in *T. cruzi* CaM compared to its mammalian counterparts. This is a significant feature since no substitutions have been found

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[1] *Abbreviations used:* CaM, calmodulin; EGTA, [ethylene-bis (oxyethylenenitrilo)] tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HRP, horseradish peroxidase IPTG, isopropyl-1-thio-β-D-galactopiranoside; AP, alkaline phosphatase; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; OPD, *o*-phenylenediamine-peroxidase substrate; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane.

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among CaM sequences from vertebrates, which indicates that this is a very well conserved protein throughout evolution.

Results from our laboratory demonstrated that substitution of Tyr99 by Phe, prevents both phosphorylation and regulation of mammalian CaM (Benaim and Villalobo, 2002; Salas et al., 2005). Accordingly, *T. cruzi* and *L. mexicana* CaMs cannot be phosphorylated by the epidermal growth factor receptor (EGFR) due to the absence of Tyr99 (Benaim et al., 1998). When comparing CaM from different trypanosomatids with the protein from vertebrates, a large difference in electrophoretic mobility was observed by SDS–PAGE (Ruben et al., 1983; Benaim et al., 1991, 1998), despite the fact that both CaMs possess the same molecular mass. This difference was also observed on the typical Ca^{2+}-shift observed when CaMs were run in SDS–PAGE in the presence or absence of Ca^{2+}. These results indicate that, in fact, there are significant differences among these two proteins. Another related result was that a monoclonal antibody designed to the C-terminal region from mammalian CaM was unable to recognize purified CaM from *L. mexicana* and *T. cruzi* (Benaim et al., 1998).

In this work we cloned and expressed CaM from *T. cruzi*, thus allowing a further characterization of this protein. Our results confirmed unique biochemical characteristics for *T. cruzi* CaM, including production of very specific antibodies and singular properties when assayed in the presence of mammalian CaM classic antagonists. These important differences between CaM from trypanosomatids and vertebrates make this protein not only an excellent object of study from the therapeutic point of view, but also it could contribute to the understanding of the Ca^{2+} signaling regulation in these parasites.

2. Experimental procedures

2.1. Reagents

Plasmid pET15b was obtained from Novagen. Restriction enzymes NcoI, XhoI, and EcoRI, were provided by New England BioLabs. Wizard plasmid purification kit was purchased from Promega. Phenyl–Sepharose CL-4B, PMSF, ATP (sodium salt), Triton X-100, Tween 20, Hepes, IPTG, and HRP-goat anti-chicken were all obtained from Sigma. Nitrocellulose membranes were from Millipore. AP-goat anti-chicken was obtained from Santa Cruz Biotechnologies. Other chemicals used in this work were of analytical grade.

2.2. Cloning and expression of T. cruzi calmodulin in Escherichia coli

Cloning and expression of *T. cruzi* CaM in *E. coli* was based on the methodology proposed by Hayashi et al. (1998) and used to clone the rat CaM. The gene encoding *T. cruzi* CaM (GenBank Accession No. P18061 and GeneDB Accession No. Tc00.1047053507483.39) was amplified from *T. cruzi* genomic DNA using PCR primers designed by the aid of the computer software DNAMAN®, version 5.2.2. (Lynnon Biosoft). Forward primer was designed to contain in its 5′ end the recognition site for NcoI restriction enzyme (highlighted): CaM FW 5′-CTGGAT**CCATGG**CTGATCAACTGTCC-3′. This primer also contained the initiation codon ATG (underlined) in order to insert the PCR product in frame. For the 3′ end of the protein, we designed a reverse primer containing the recognition sequence for XhoI restriction enzyme (highlighted): CaM RV 5′-GATGATG ATGAGCAAGTGA**CTCGAG**C-3′. The PCR product was digested and cloned into pET15b (Novagen) to yield a construct (pETCaMcruzi) in such a manner that both the poly-His-tag and thrombin cleavage site were excluded from the recombinant polypeptide. By using this approach, the entire coding sequence was free of any foreign fragment resulting in a recombinant polypeptide similar to the *T. cruzi* native protein. Positive recombinant constructs were confirmed by DNA sequencing. pETCM containing the rat CaM, a generous gift from Dr. Antonio Villalobo (Instituto de Investigaciones Biomedicas, Madrid, Spain, who obtained it from Prof. Nobuhiro Hayashi (Fujita Health University, Aichi, Japan).

2.3. DNA sequencing and sequence analysis

Sequencing was performed in an ABI Prism® 310 Genetic Analyzer (Applied Biosystems). The software DNAMAN®, version 5.2.2. (Lynnon Biosoft), was used to assemble DNA sequences and performed alignment analyses.

2.4. Expression and purification of recombinant calmodulin

Escherichia coli BL21(DE3) pLysS cells, transformed with pETCaMcruzi or pETCM, were used to induce the expression of *T. cruzi* and rat CaMs, respectively, following the procedure described by Studier (2005). Briefly, a pre-culture of *E. coli* grown overnight at 37 °C in LB medium under vigorous shaking (250 rpm), supplemented with ampicilin (100 μg/ml) and chloramphenicol (34 μg/ml), was diluted 1:100 in minimal medium (25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 0.5% glucose and 0.25% aspartate). After growing cells for 4 h at 37 °C with vigorous shaking, culture medium was replaced by induction medium (1% tryptone, 0.5% yeast extract, 0.5% glycerol, 0.05% glucose, 0.2% lactose, 2 mM $MgSO_4$, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4) supplemented with ampicilin (100 μg/ml) and chloramphenicol (34 μg/ml), and further incubated for 24 h at 37 °C with vigorous agitation. Recombinant CaM species were purified as described (Hayashi et al., 1998), except that soluble bacterial protein extract was heated at 95 °C for 5 min. Heat resistant proteins remaining in the supernatant were subjected to phenyl–Sepharose chromatography as previously described (Benaim et al., 1991).

2.5. Production of T. cruzi α-calmodulin IgY antibodies in chickens

Isabrown-laying hens (16-weeks-old, 2 kg body mass) were immunized intramuscularly in the breast region at multiple sites with a total amount of 200 μg of CaM from *T. cruzi* emulsified with saline and complete Freund's adjuvant (1:1 v/v). Second and third boost doses were applied with 75 μg of protein emulsified with saline and incomplete Freund's adjuvant (IFA). Booster injections were administered every ten days. Eggs were collected daily, individually identified, and store at 4 °C.

Hens were bred, maintained and cared for in accordance with the guidelines formulated by the European Community for the Use of Experimental Animals (L358-86/609/EEC).

Isolation and purification of IgY from the yolk of preimmune and hyper-immunized eggs was done according to Polson (1990) with little modifications. Briefly, 3.5% (w/v) polyethyleneglycol (PEG 6000) was added to yolk diluted in three volumes of phosphate-saline buffer (PBS) under stirring. Supernatant containing IgY was collected by centrifugation (3000*g*) at 4 °C for 20 min, and filtered through sterile gauze. Filtrate was extracted with 20% chloroform and centrifuged at 2000*g* for 15 min. Finally, an IgY precipitation step with 8.5% PEG was made using the same conditions as above. Purified IgY pellet was resuspended in PBS and stored at −20 °C.

2.6. ELISA evaluation of anti-calmodulin IgY antibody

Microtiter plate wells were coated with 10 μg/ml *T. cruzi* or rat CaM according to Ausubel et al. (1991). Then, wells were blocked with PBS containing 0.05% Tween 20 (PBS-T) plus 5% skim milk