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Isolation and characterization of *Dictyostelium* thymidine kinase 1 as a calmodulin-binding protein ☆

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Abstract

Probing of a cDNA expression library from multicellular development of *Dictyostelium discoideum* using a recombinant radiolabelled calmodulin probe (^{35}S-VU1-CaM) led to the isolation of a cDNA encoding a putative CaM-binding protein (CaMBP). The cDNA contained an open reading frame of 951 bp encoding a 227aa polypeptide (25.5 kDa). Sequence comparisons led to highly significant matches with cytosolic thymidine kinases (TK1; EC 2.7.1.21) from a diverse number of species including humans (7e−56; 59% Identities; 75% Positives) indicating that the encoded protein is *D. discoideum* TK1 (DdTK1; *ThyB*). DdTK1 has not been previously characterized in this organism. In keeping with its sequence similarity with DdTK1, antibodies against humanTK1 recognize DdTK1, which is expressed during growth but decreases in amount after starvation. A CaM-binding domain (CaMBD; ^{20}GKTTEL IRRIKRFNFANKKC^{30}) was identified and wild type DdTK1 plus two constructs (DdTKΔC36, DdTKΔC75) possessing the domain were shown to bind CaM in vitro but only in the presence of calcium while a construct (DdTKΔN72) lacking the region failed to bind to CaM. Thus, DdTK1 is a Ca^{2+}-dependent CaMBP. Sequence alignments against TK1 from vertebrates to viruses show that CaM-binding region is highly conserved. The identified CaMBD overlaps the ATP-binding (P-loop) domain suggesting CaM might affect the activity of this kinase. Recombinant DdTK is enzymatically active and showed stimulation by CaM ($113 \pm 0.5\%$) an in vitro enhancement that was prevented by co-addition of the CaM antagonists W7 ($91.2 \pm 0.8\%$) and W13 ($96.6 \pm 0.6\%$). The discovery that TK1 from *D. discoideum*, and possibly other species including humans and a large number of human viruses, is a Ca^{2+}-dependent CaMBP opens up new avenues for research on this medically relevant protein.

Keywords: Thymidine kinase 1; Calmodulin-binding protein; *Dictyostelium discoideum*; Enzyme activity; CaMBOT; W7; W13

Thymidine kinase (TK; EC 2.7.1.21.) receives extensive biomedical attention because of its roles in apoptosis, cell proliferation, embryonic development, and antiviral drug activation. Fundamentally TKs are salvage enzymes that reutilize the degradation products from nucleic acids. Mammals produce two forms of thymidine kinases: cytosolic TK1 and mitochondrial TK2 [1]. TK1 is a cytosolic enzyme that phosphorylates deoxythymidine to produce thymidine-5′-phosphate, a precursor in the synthesis of genomic DNA. The three-dimensional structure of the TK1 family has recently been determined [2]. While TK1 expression is almost undetectable in resting cells, its level increases at the G1/S phase and peaks during the S phase of proliferating cells [3,4]. Because of this link to cycling cells, TK serves as a prognostic marker for various cancers [5,6]. The levels of TK activity during the cell cycle are regulated by both transcriptional and posttranslational

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mechanisms. Serum stimulation of human and mouse cells leads to an increase of TK at the G1/S transition that is under transcriptional control [7–9]. However, the final levels of TK activity during the cell cycle and growth stimulation are dependent upon as yet uncharacterized posttranslational mechanisms and various degradation pathways [10,11]. TK also phosphorylates many anti-neoplastic and anti-viral nucleoside analogs thus activating them for incorporation into newly synthesized DNA [12]. For these and other reasons, understanding all aspects of the regulation of TK1 has a number of implications for biomedical research.

As the major, essential Ca^{2+}-binding protein of all eukaryotes, calmodulin (CaM) is highly conserved [13–15]. CaM serves multiple roles in calcium signalling but primarily functions by binding to and regulating calmodulin-binding proteins (CaMBPs). Probing of cDNA expression libraries using the CaM binding overlay technique (CaMBOT) has been extensively used to profile, identify, and characterize CaMBPs from a diversity of organisms [16]. In *Dictyostelium*, this approach has led to the isolation and characterization of cDNAs encoding classic (e.g., calcineurin A), novel (e.g., nucleomorphin isoforms), and known proteins that were not previously identified as CaMBPs (e.g., phosphoglycerate kinase) [16–20].

In this study, CaMBOT probing in *Dictyostelium discoideum* led to the isolation of a cDNA encoding a polypeptide with a high sequence homology to mammalian TK1. Thymidine kinase has not been previously characterized in *Dictyostelium* and CaM has not previously been shown to bind to TK1 from any species. Scanning of the protein sequence of *Dictyostelium* TK1 (DdTK1) revealed a single, putative, continuous Ca^{2+}-dependent CaMBD. Full-length constructs were shown to bind CaM in vitro in a Ca^{2+}-dependent manner but those lacking the CaMBD did not bind to CaM. The identified CaMBD overlaps the P-loop of TK1 and is highly conserved across diverse species from viruses to mammals suggesting it has an as yet unidentified functional significance. Recombinant DdTK1 was enzymatically active and CaM had a stimulatory effect on its activity in vitro that may have implications for the future study of this biomedically relevant kinase.

Materials and methods

Materials. All restriction enzymes used were purchased from Amersham Pharmacia. T4 DNA ligase was purchased from New England Bio Labs. All media reagents were purchased from BioShop Canada and VWR Scientific Products. Low DNA mass ladders were purchased from Gibco-BRL. PCR molecular weight markers and the mini-complete protease inhibitor cocktail tablet were from Boehringer–Mannheim. Calmodulin–agarose beads (1 mg/mL) insolubilized on 4% beaded agarose (P4385) were obtained from Sigma–Aldrich. PCR primers and the Qiaex II Gel Extraction system were purchased from Qiagen. The pET21-b (+) vector and anti-T7·tag HRP conjugate were obtained from Novagen.

Screening of a λ ZAP cDNA expression library using ^{35}S-VU1-CaM. Titration of bacteriophage and screening of the cDNA library were essentially carried out as described [21]. Variations in protocol were implemented with respect to the use of ^{35}S-radiolabelled recombinant CaM as a probe. Screening was performed exactly as described elsewhere [17]. Clones with dissimilar restriction enzyme fragments were sequenced at the Core Molecular Biology Facility, York University. Sequences were compared with those in the GenBank Database using BLAST [22]. Protein motifs were identified, and the predicted molecular weight and isoelectric point were determined from the deduced amino acid sequences using the programs PSORT and PSORTII (Prosite and the ExPASy Molecular Biology Server; Swiss Institute of Bioinformatics) [23,24].

Polymerase chain reaction amplification of D. discoideum thymidine kinase (DdTK1). The cDNA encoding the entire open reading frame for DdTK1 was amplified by the PCR in order to express its gene product under the control of the T7 promoter in pET-21b (+). Primers were designed using the nucleotide sequence coding for DdTK1. The forward primer designated pTK-f incorporates a unique restriction site for *Eco*RI (underlined) (5′-AAAATTAAATGAATTCAATGATTGTAACTCAAATTGCTG-3′). The reverse primer designated pTK-r incorporates a unique restriction site for the restriction enzyme *Xho*I (underlined) (5′-ATTAGGATCTCTCGAGTTAATAATCATTATTTAAATG-3′). The PCR was carried out using Perkin Elmer's AmpliTaq gold in a GeneAmp PCR System 9700 for 32 cycles (each consisting of a denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 90 s). A single 717-bp PCR product was amplified. The PCR product was agarose gel-purified using the Qiagen QIAEX II gel purification kit as per the manufacturer's recommendations. Deletion constructs were amplified essentially as described above using the following primer and PCR conditions.

DdTKΔN72—Primers were designed to amplify the region encoding amino acids 44–420. *Sac*I and *Xho*I restriction sites were added to the 5′- and 3′-ends, respectively, using the primers pTK-mutA (5′-AAATTATCAAGCGAGCTCATGTTCAATTTTAGAAGATGTT-3′) and pTK-r.

DdTKΔC36—Primers were designed to amplify the region encoding amino acids 206–420. *Sac*I and *Xho*I restriction sites were added to the 5′- and 3′-ends, respectively, using the primers pTKmutB (5′-TTGGTACTCTCGAGTTAATTTTGATCACTATTGTAGC-3′) and pTK-r.

Construction and expression of pET-21b (+) DdTK1 vectors. The amplified DdTK1 cDNA sequences were digested using the restriction enzymes *Eco*RI, *Sac*I, and *Xho*I. PCR products were then ligated into the vector pET-21b (+) (Novagen) as per the manufacturer's recommendations. The ligation mixture was transformed into *Escherichia coli* strain DH5α and the recombinant plasmid was purified by way of alkaline lysis [21]. Plasmids were screened for an insert using the appropriate restriction enzymes, sequenced, and then designated pDdTK, pDdTKΔN72, and pDdTKΔC36, respectively. ΔN and ΔC indicate N- and C-terminal mutants, and the numbers specify the number of amino acids deleted from the respective ends. Plasmids with the appropriate PCR product were used to transform the *E. coli* strain BL21. The induction and expression of each plasmid was carried out essentially as described with minor modifications (Novagen). Briefly, a culture of *E. coli* BL21 carrying the pET construct was grown to saturation (~16 h) in 2 mL LB broth supplemented with 0.1 mg/mL ampicillin and 10 mM glucose at 37 °C with shaking at 300 rpm. At this time, 200 μL of the overnight culture was added to 2 mL of fresh LB broth supplemented with 0.1 mg/mL ampicillin and 10 mM glucose. Each culture was grown at 37 °C at 300 rpm until it reached an OD_{600} of ~0.6–0.8. Each culture was collected by centrifugation in sterile 1.5 mL microfuge tubes for 1 min at maximum rpm, and washed 3× in 1 mL LB broth to remove the glucose and secreted β-lactamase. Cells were resuspended in 2 mL of fresh LB broth sup-