FEBS Letters 583 (2009) 2281–2286

journal homepage: www.FEBSLetters.org

The yeast Cdc8 exhibits both deoxythymidine monophosphate and diphosphate kinase activities

Chia-Yi Chien [a], Bo-Ruei Chen [b], Chen-Kung Chou [c], Robert A. Sclafani [d], Jin-Yuan Su [b,*]

[a] Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei, Taiwan
[b] Department of Life Sciences, National Yang-Ming University, Taipei, Taiwan
[c] Department of Life Science, Chang Gung University, Tao-Yuan, Taiwan
[d] Department of Biochemistry and Molecular Genetics, University of Colorado School of Medicine, Aurora, CO 80045, USA

ARTICLE INFO

Article history:
Received 22 April 2009
Revised 10 June 2009
Accepted 11 June 2009
Available online 18 June 2009

Edited by Francesc Posas

Keywords:
Thymidylate kinase
Nucleoside diphosphate kinase
CDC8
YNK1
Thymidylate synthesis
Cell cycle regulation

ABSTRACT

The existence of multifunctional enzymes in the nucleotide biosynthesis pathways is believed to be one of the important mechanisms to facilitate the synthesis and the efficient supply of deoxyribonucleotides for DNA replication. Here, we used the bacterially expressed yeast thymidylate kinase (encoded by the *CDC8* gene) to demonstrate that the purified Cdc8 protein possessed thymidylate-specific nucleoside diphosphate kinase activity in addition to thymidylate kinase activity. The yeast endogenous nucleoside diphosphate kinase is encoded by *YNK1*, which appears to be non-essential. Our results suggest that Cdc8 has dual enzyme activities and could duplicate the function of Ynk1 in thymidylate synthesis. We also discuss the importance of the coordinated expression of *CDC8* during the cell cycle progression in yeast.

1. Introduction

Thymidylate kinase (TMPK; EC 2.7.4.9.) is an enzyme that phosphorylates dTMP to dTDP in the presence of ATP and magnesium. TMPK is important in cellular DNA synthesis because the synthesis of dTTP, either via the de novo pathway or through an exogenous supply of thymidine, requires the activity of this enzyme [1]. In the budding yeast, TMPK is encoded by the *CDC8* gene and is essential for nuclear and mitochondrial DNA replication [2–5]. A previous study has shown that expression of yeast *CDC8* peaks at G1/S transition during the cell cycle, and is coordinated with expression of other DNA synthesis genes such as *CDC9* and *CDC21*, which encode DNA ligase and thymidylate synthase, respectively [6]. The temperature-sensitive mutant allele of *cdc8-1*, which has a single amino acid change from glutamate to lysine at residue 75 of the Cdc8 protein, is defective in the TMPK activity [7,8]. *cdc8-1* mutants arrest at the Gl/S boundary of the cell cycle when incubated at the restricted temperature [9,10]. Furthermore, the mutant is hypomorphic and is partially defective in meiotic and mitochondrial functions even when grown at the permissive temperature [11,12]. It has been suggested that Cdc8 not only provides precursors for DNA synthesis but may also have a role in regulating the process of DNA replication [2,5,7,8].

Nucleoside diphosphate kinase (NDPK; EC 2.7.4.6), which acts at the next step after Cdc8 in the thymidylate synthesis pathway, is an enzyme that catalyzes the synthesis of the various nucleoside triphosphates other than ATP [13]. In the budding yeast, the *YNK1* gene encodes the only species of NDPK present in yeast and earlier studies have indicated that the yeast cell only carries one single *YNK1* gene [14]. Surprisingly, disruption of the *YNK1* locus in a haploid strain of yeast causes no obvious growth defect, even though NDPK activity in the *YNK1* null mutant is reduced to around 10% of that in wild-type cells when assayed in vitro [14]. A similar observation was also made using a *ndk1* deletion mutant, a homolog of the budding yeast *YNK1*, in the fission yeast *Schizosaccharomyces pombe* [15]. One plausible explanation is that other yeast gene products may possess NDPK activity and be able to sustain the requirement for NDPK during cell growth. However, the identity of a gene that is able to duplicate NDPK activity remains unknown.

To learn more about the role of Cdc8 in regulating DNA replication, we produced both recombinant GST-tagged wild-type protein and recombinant GST-tagged mutant Cdc8 proteins in bacteria and studied their function in vitro. Unexpectedly, the purified wild-type Cdc8 protein was found to possess NDPK activity in addition

* Corresponding author. Fax: +886 2 2823 4898.
E-mail addresses: jysu@ym.edu.tw, jinyuansu@msn.com (J.-Y. Su).

0014-5793/$36.00
doi:10.1016/j.febslet.2009.06.022

to its TMPK activity. The dual enzymatic capacity of Cdc8 is able to partially substitute for Ynk1 and sustain NDPK activity.

2. Materials and methods

2.1. Enzymes, chemicals and materials

Restriction enzymes and other molecular biology reagents were purchased from New England Biolabs. Glutathione-sepharose beads were bought from Amersham Pharmicia Biotech. Radioactive nucleotides were obtained from Moravek Biochemicals. Polyethyleneimine (PEI)-cellulose plates (Polygram Gel, 300 PEI/UV_{254}) were purchased from Macherey-Nagel, Germany. Anti-Ynk1 antiserum was raised in mice against purified yeast Ynk1 protein, which is available from Sigma–Aldrich.

2.2. Expression and purification of the Cdc8 recombinant proteins

GST-tagged Cdc8 expression vectors were constructed by subcloning DNA fragments amplified by PCR using primers designed according to the coding sequences of *CDC8* [16]. The two 661-bp *Bgl*II–*Eco*RI fragments containing either the entire *CDC8* or the entire *cdc8-1* coding region were subcloned into the plasmid pGEX-2T (Pharmacia Biotech Inc.) using the *Bam*HI and *Eco*RI sites to form pGEX2-*CDC8* or pGEX2-*cdc8-1*, respectively. The plasmids were then transformed into *Escherichia coli* strain BL21 (DE3) (Novagen) for recombinant protein expression. Expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 50 μM. The bacteria were lysed, and the GST-tagged proteins were adsorbed onto glutathione-sepharose beads as described elsewhere [17]. The proteins were eluted with 10 mM glutathione, 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM dithiothreitol; dialyzed against buffer A (25 mM Hepes, pH 7.2, 50 mM NaCl, 2 mM dithiothreitol) containing 0.05% Brij-35, 50% ethylene glycol; and finally stored at −20 °C.

Subsequent purification of protein was carried out by Mono Q anion-exchange chromatography and a Pharmacia fast protein liquid chromatography (FPLC) system. Briefly, the soluble GST-tagged protein obtained after being eluted from glutathione-sepharose beads was dialyzed against buffer A and loaded onto a Mono Q column (HR 5/5) equilibrated in buffer A containing 0.01% Brij 35. The column was developed with a 0–500 mM linear NaCl gradient at 0.5 ml/min, and 35 1-ml fractions were collected. The protein concentration was determined by SDS–polyacrylamide gel electrophoresis (PAGE), Coomassie Blue staining, and densitometry on a Molecular Dynamics computing densitometer using bovine serum albumin as a standard (A_{280} of a 1% solution = 6.5).

The *E. coli ndk* gene was cloned by PCR amplification with specific primers synthesized according to the published sequences [18]. The entire coding region of *ndk* was subcloned into a yeast expression vector, pESC-TRP (Stratagene), and transformed into the yeast strain JY170 (*MAT*a *ura3 leu2 trp1 ynk1*Δ::*URA3*) for protein expression. The yeast crude extract was prepared following standard procedures [19]. The immunoblotting of the yeast extract was performed according to an established protocol [20].

2.3. Nucleotide kinase assay

A standard nucleotide kinase assay has been described elsewhere [5]. Briefly, the reaction solution contained 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, bovine serum albumin at 0.25 mg/ml and 5 mM ATP. [^{3}H]-labeled nucleotide substrate was added to a final concentration of 0.2 mM (20 cpm/pmol). The reaction was initiated by adding the enzyme. Reactions were performed at 37 °C for the period of time indicated and terminated by adding EDTA to 20 mM. Reaction products (nucleoside diphosphates and nucleoside triphosphates) were separated by thin-layer chromatography (TLC) on PEI-cellulose plates with 0.5 M LiCl/2 M acetic acid as the solvent. The nucleotide spots were visualized and marked under the UV lamp, cut out, and the radioactivity quantified using a scintillation counter.

3. Results and discussion

3.1. Expression and analysis of the recombinant Cdc8 proteins

To study the function of the Cdc8 protein in vitro, we produced and purified the wild-type and mutant Cdc8 recombinant proteins in bacteria. The estimated molecular mass of Cdc8 protein is 24.6 kDa [16]. Both the purified wild-type and mutant GST-Cdc8 proteins, after elution from the GSH-beads, appeared as a major band with a molecular mass of 52 kDa when analyzed by SDS–PAGE (Fig. 1A). This is as expected because the molecular mass of GST is 26 kDa. The purified proteins were then analyzed for thymidylate kinase activity using [^{3}H]-dTMP as the substrate. Through-

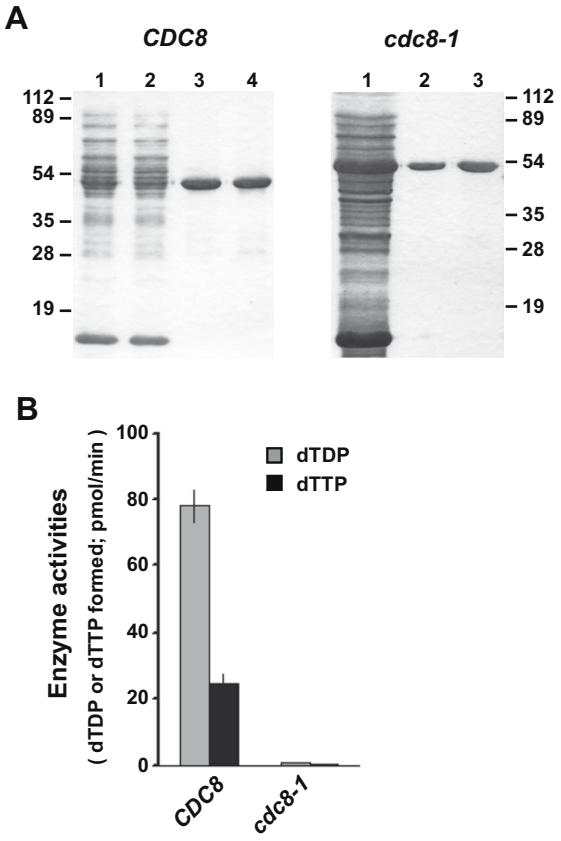

Fig. 1. Expression and enzymatic analysis of the recombinant Cdc8 proteins. (A) The expression of GST-tagged wild-type and mutant Cdc8 proteins in bacteria. Proteins collected during GSH-beads purification were analyzed by SDS–PAGE and stained with Coomassie Blue. Left: The wild-type Cdc8 protein. Lane 1: Total cell extract of GEX2-*CDC8*-expressing bacteria. Lane 2: Unbound fraction from GSH-beads. Lane 3: 1 μg and lane 4: 2 μg of purified proteins. Right: The mutant Cdc8 protein. Lane 1: Total cell extract of GEX2-*cdc8-1*-expressing bacteria. Lane 2: 1 μg and lane 3: 2 μg of purified proteins. Molecular mass markers are indicated in kilodaltons. (B) Thymidylate kinase activity of GST-Cdc8. The purified wild-type and mutant proteins obtained in (A) were used as enzymes in the nucleotide kinase assay. The reaction was initiated by adding 2 μg of the wild-type or mutant protein in a reaction mixture containing [^{3}H] dTMP as the substrate and incubated at 37 °C for 30 min. The reaction products were separated on TLC. Enzyme activity was estimated for the production of dTDP or dTTP in pmol/min from the total reaction mixture. Histograms represent mean ± range for duplicate determinations.