Cloning and expression of Polo-like kinase (CaCdc5) from *Candida albicans*

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ABSTRACT

Polo-like kinases (PLKs) are conserved regulators of multiple cell cycle events that belong to serine–threonine kinase family, with a kinase activity that is critical for various cellular functions of PLKs. A highly conserved polo-box domain present in the C-terminal non-catalytic region of polo kinases plays a crucial role in the function of these enzymes. Cdc5 and CaCdc5 are structural as well as the functional homologues of PLKs in *Saccharomyces cerevisiae* and *Candida albicans*, respectively. Morphogenesis in the fungal pathogen *Candida albicans* is an important virulence-determining factor, as a dimorphic switch between yeast and hyphal growth forms correlates with its pathogenesis. CaCdc5 has been shown to regulate this dimorphic switch as well. The present study was undertaken to clone and achieve recombinant expression of CaCdc5 in *E. coli*. CaCdc5 was successfully expressed as a GST-CaCdc5 fusion protein of ~101 kDa. The recombinant CaCdc5 can be used to study its functions *in vitro* as well as *in vivo*. **Key words:** Polo-like kinase, Serine/threonine kinase, CaCdc5 expression, *Candida albicans*

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INTRODUCTION

*Candida albicans*, a common opportunistic fungal pathogen, exists in yeast, pseudohyphal and hyphal growth forms, and can be detrimental when present in the hyphal stage¹. Several signalling pathways mediate environmentally motivated yeast hyphal transition in *Candida*², the most characterized of which include MAP kinase and cAMP-based signalling. There are several factors influencing hyphal formation in *Candida*³,⁴ and hyphal growth involves regulation beyond the level of transcription⁵, including additional regulatory networks. During hyphal growth in *C.
albicans, cell surface expansion is restricted to a small region at the hyphal tip. This apical growth zone is active during the entire hyphal growth period. Eukaryotic cell cycle is mainly controlled by Cyclin dependent kinases (CDK) and identified Polo-like Kinases (PLK). PLKs are central regulators of cell cycle, and control multiple mitotic events. PLKs belongs to Ser/Thr subfamily of protein kinases, characterized by kinase domain (KD) at the N-terminus and a conserved signature motif called polo-box domain (PBD) at the C-terminus. PLKs comprise a family of cell cycle regulators with the potential to influence hyphal morphogenesis in Candida, because they function at various stages during the initiation and progression through mitosis and are required for septation/cytokinesis. Furthermore, the PLK homologue Cdc5p in S. cerevisiae physically interacts with septins and Swe1p and can alter cell morphology by generating elongated buds upon overexpression. Interestingly, depletion of CaCDC5 in C. albicans induces cell cycle defects and changes in morphology from budding to hyphal growth. In the model fungus Saccharomyces cerevisiae, morphogenesis is tightly coordinated with cell cycle progression. Bud growth and the corresponding polarization of actin, synthesis of DNA, and duplication of spindle pole bodies occurs at the G1/S transition, whereas elongated or pseudohyphal growth is associated with a block in G2 mediated by Swe1p-dependent negative regulation of Cdc28p. A few cell cycle factors have been characterized in Candida sp.; these include the G1 cyclin CLN1, which is required for maintaining hyphal growth, and the CDC2 related kinase CRK1, which can promote hyphal formation. In addition, a transcription factor that regulates B-cyclin gene expression is required for hyphal growth in Candida sp. Therefore, understanding the mechanisms for this morphogenetic switch should provide insight into the pathogenicity of this fungus. Here, we choose to clone and express CaCdc5 in E. coli, which could be characterized in vitro, and aid in understanding its function during hyphal morphogenetic switch in C. albicans.

MATERIALS AND METHODS

Chemicals and strains
All the fine chemicals were of analytical grade, and were procured from GE Biosciences and SIGMA. Restriction enzymes and T4 DNA ligase were purchased from FermentasInc, USA. Candida albicans strain MTCC227 and the standard laboratory strains of E. coli were used for the study.

Cloning of CaCDC5 gene from Candida albicans
CaCDC5 gene was PCR amplified from Candida albicans genomic DNA using forward 26-mer (5’-AATTGAATTCCAATGTCGCCTCG-3’), bearing EcoR1 restriction site (underlined) and reverse 25-mer (5’-AGCGCTCGAGCTTAAGCTTATT-3’), bearing XhoI restriction site (underlined) using Taq polymerase and Pfu polymerase and cloned into pGEX4T2 vector at the EcoR1/XhoI cloning sites.

Screening for maximal expression of soluble CaCDC5 protein
Over expression of Candida albicans CaCdc5 was monitored by inoculating a fresh colony of the indicated expression strain bearing pCaCDC5 plasmid DNA into 5 ml HiVeg LB broth supplemented with antibiotics as described below: BL21 DE3 (ampicillin 100µg/ml); BL21CP, ampicillin (100 µg/ml) were grown at 37°C for overnight. One percent of primary culture was used to inoculate 5 ml of HiVeg LB medium supplemented with the appropriate antibiotics and
grown at 37°C. At an $A_{600}$ of ~ 0.5, an aliquot was removed as the uninduced control, and the culture was induced with the addition of IPTG to a final concentration of 0.5, and 1mM, and incubation was continued for an additional 16 h at 18°C and 4 hours at 37°C. The cells were pelleted by centrifugation (8000 rpm for 10 min), and the pellet were resuspended in buffer A (50 mMTris–HCl, pH 8, 100 mMNaCl, 5mM β-mercaptoethanol, and 1X protease inhibitors). The cells were disrupted by FastPrep®-24 instrument (Cat. # 6004-500) set at fastprep® speed 6 for fastprep® time 2x30sec. The cell suspension was centrifuged at 12,000 rpm for 15 min at 4°C. Aliquots from the pellet (resuspended in buffer A) and supernatant fractions were each mixed with SDS-PAGE loading dye, and incubated at 90°C for 10 minutes. The samples were loaded (20 µg per lane) on a 10% SDS–polyacrylamide gel. The gels were stained with Coomassie blue and inspected visually for protein expression. To assess relative CaCdc5 protein abundance in the pellet and supernatant fractions, 2µg protein was resolved by SDS–PAGE and transferred to the nitrocellulose membrane (Millipore, Immobilon P 0.45µm, 26.5cmx3.75cm). Blot was kept at 4°C overnight in blocking solution containing 5% skim milk. Membranes were then incubated with anti-GST antibodies (Abcam) overnight at 4°C. Immunoreactive proteins were detected by using anti-rabbit IgG conjugated to horseradish peroxidase (Abcam). After several washes, blots were processed for enhanced chemiluminescence (ECL) detection.

RESULTS & DISCUSSION

CaCDC5 gene cloning and confirmation

Cloning of Candida albicansCaCDC5 ORF into pGEX4T2 vector was achieved, the latter containing a T7 promoter that allows T7 RNA polymerase dependent expression of CaCDC5 along with a protease-cleavable GST tag. The resulting recombinant plasmid was transformed in E. coli DH5α and error free CaCDC5 DNA sequence was ensured by restriction analysis (Figure 1) and sequencing.

Figure1:Clone confirmation through restriction digestion. lane1,molecular marker; lane2, undigested vector;lane3, single digested vector BamH1 (cuts the gene sequence CaCdc5);lane4, single digested vector XhoI (cloning restriction enzyme); lane5, single digested
vector EcoRI (cloning restriction enzyme); lane 6, double digested vector XhoI and EcoRI (cloning restriction enzymes); lane 7, molecular marker; lane 8, undigested clone; lane 9, single digested clone BamHI (cuts the gene sequence CaCdc5, confirming presence of the gene); lane 10, single digested clone (SDC2)XhoI; lane 11, single digested clone EcoRI; lane 12, double digested XhoI and EcoRI (cloning restriction enzymes, giving a product size of \(~1.9\)kb and vector size of \(~4.9\)kb)

**Expression screening of CaCdc5 in E. coli**

Previously CaCDC5 gene was cloned and expressed in pET28a but His\(_6\)-CaCdc5 protein was not obtained in soluble fraction. Expression and purification of recombinant CaCdc5 from *E. coli* host strains were complicated by the insolubility of the expressed protein at all temperature variations and IPTG concentrations. Generally difficulties are encountered by researchers while trying overexpression of recombinant proteins in heterologous host strains. Difference in codon usage could be one of the possible reasons behind this result. Consequently, different strains were used to screen for high-level expression of CaCdc5 protein in soluble fraction. But none of the conditions resulted in soluble recombinant protein, so vector was changed and pGEX4T2 was selected. pGEX series of vector permits high levels of protein expression as compared to other vector systems\(^{15}\). The idea behind changing vector to pGEX4T2 was that the GST tag was itself big and highly soluble and when this tag was fused to gene, this construct was expressed successfully in soluble form. The two *E. coli* hosts used for the expression are BL21DE3 and BL21CP. *Candida albicans* CaCDC5 gene was PCR amplified and cloned into expression vector pGEX4T2 in-frame with the GST-tag of this vector for expression in *E. coli*. GST-CaCdc5 was found in the insoluble fraction at 37°C. Since recombinant protein overexpression in heterologous host strain is complicated by formation of inclusion bodies, but still protein expression in pellet or soluble form is not an all or none process. It depends on a variety of conditions like IPTG concentration used for induction, cell density of the culture prior to induction, host strain used for expression, expression tag used for fusion with protein, temperature at which the induction is carried out. The strategy adopted here to improve protein solubility during heterologous expression was induction at low-temperature. Accordingly, cultures were grown and induced at 18°C for 15 hours. Although a similar pattern of induction was observed in different strains, expression at 18°C resulted in soluble GST-CaCdc5 protein (Figure 2). Overall, induction at early or mid-log phase gave the best results. Among different strains used, the expression of GST-CaCdc5 was higher in BL21DE3 than the BL21CP host strain. Therefore, BL21DE3 strain expressing GST-CaCdc5 was selected and subjected to Western blotting in order to confirm the protein with specific antibody (Figure 3).
Figure 2: Analysis of expressed GST-CaCdc5 expression in *E. coli* host strains at 18°C with 1 mM IPTG. GST–CaCdc5 expression was analyzed in BL21-CP, BL21-DE3 strains of *E. coli*. Protein extracts were prepared from cell free lysates of expressed GST-CaCdc5 and separated by 10% SDS-PAGE followed by Coomassie. lane1, marker; lane2, uninduced (UI) soup in BL21 CP strain; lane3, induced (I) soup in BL21 CP strain; lane4, without any sample; lane5, induced (I) soup in BL21 DE3 strain, lane6, uninduced (UI) soup in BL21 DE3 strain. Arrows indicate the inducible expression of GST-CaCdc5 protein.

CONCLUSION

In this study, we have cloned, over expressed *Candida albicans* CaCdc5 protein from *E. coli*. The temperature at which CaCdc5 was expressed had a significant impact on its solubility. The tag used in this study affected the solubility of CaCdc5. His tag could not get the protein in soluble fraction, as compared to GST tag. Biochemical characterization of CaCdc5 kinase can be carried out for providing a platform for analyses of functional and structural aspects. Substrate identification of CaCdc5 is still a mystery to be solved hence purification of the protein and its functional activity further analysed could be used for many downstream applications.
Understanding the role of CaCdc5 during hyphal growth formation could be one of the stones to be unturned, that could lead to a conclusion on its pathogenicity.

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