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A novel type 2C protein phosphatase from the human fungal pathogen *Candida albicans*

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The reversible phosphorylation of proteins, controlled by protein kinases and protein phosphatases, is a critical mechanism by which eukaryotic organisms regulate cellular signal transduction pathways. There are two superfamilies of protein serine/threonine specific phosphatases, the PPP and PPM families, which specifically dephosphorylate serine/threonine residues [1]. The PPP family is comprised of three subtypes of phosphatases, PP1, PP2A and PP2B, which are distinguished by their associated regulatory subunits to form a variety of holoenzymes. These enzymes show considerable evolutionary sequence conservation in their catalytic domains, and the dephosphorylation activity of the PPP family is independent of divalent cations and can be inhibited by the protein inhibitors-1 and -2 as well as the tumor promoter okadaic acid. In contrast, PPM family members are monomeric enzymes and are unrelated in sequence to the PPP family, and they include type 2C protein phosphatases (PP2C) and the pyruvate dehydrogenase phosphate. Sequence analysis reveals that there are 11 conserved motifs in the members of the PP2C superfamily [2]. In addition, the dephosphorylation activity of PP2C requires metal cations, Mn^{2+} or Mg^{2+}, but its activity is not sensitive to the tumor promoter okadaic acid and other inhibitors of the PPP family [1]. In the budding yeast *Saccharomyces cerevisiae*, six PP2C-like genes, Ptc1p, Ptc2p, Ptc3p, Ptc4, Ptc5 and *YCR079w*, have been identified, and proteins encoded by the former five genes have been demonstrated to be PP2C enzymes [3]. In *Candida albicans*, an important human yeast pathogen, little is known about PP2C enzymes. In this report, we have identified and characterized the first PP2C phosphatase (CaPTC7) from the human fungal pathogen, *C. albicans*, representing a novel member of the PP2C superfamily.

To investigate the roles of PP2C phosphatases in the pathogenesis of *C. albicans*, potential PP2C homologous sequences from the *C. albicans* genomic database (http://www-sequence.stanford.edu/group/candida) were extensively searched with the six known budding yeast PP2C-like genes as queries. As a result, the six *C. albicans* homologs of the known budding yeast PP2C-like genes were identified according to their sequence similarities (Jiang et al., unpublished). Interestingly, the gene *YHR*76 was found to encode a protein of 365 amino acids (YHR76p), sharing limited sequence identity with the budding yeast Ptc1p (GenBank accession No.: AAA34920) in the catalytic domain, particularly in the two regions from N44 to K107 and from V210 to I248 where the sequence identities reach 28% and 30%, respectively (Fig. 1A). The sequence alignment of YHR76p with Ptc1p, human PP2Cβ2 (GenBank accession No.: AAG02232) and rat PP2Cδ (GenBank accession No.: AAC97497) revealed that the 250 amino acid segment of the YHR76p at the C-terminus contains the two invariant amino acids (160D and 235G) and the 11 motifs conserved in the catalytic domain of all members of the PP2C subfamily [2] (Fig. 1A), suggesting the presence of a potential PP2C catalytic domain in YHR76p. Therefore, we designated YHR76 as CaPTC7, the seventh PP2C-like gene in *C. albicans*.

To examine if CaPTC7p has phosphatase activity, the DNA fragment containing the potential catalytic domain (from A40 to K365) of CaPTC7p was amplified by PCR from the genomic DNA of *C. albicans* strain SC5314. The sense primer with a *Nde*I site is 5′-CCCCATATGGCAAGGTCCTTTGCATCC-3′, and the antisense primer with a *Xho*I site is 5′-CCCCTCGAGCTATTTAACCTTTACCAACACAACC-3′. The amplified fragment was cloned into the *Nde*I and *Xho*I sites of the pET28c vector, resulting in an in-frame N-terminally truncated and His-tagged CaPTC7p (His-ΔCaPTC7p). The construct was confirmed by DNA sequencing. The induction and purification of the recombinant protein in *Escherichia coli* was carried out as described in the 'Current Protocols in Molecular Biology'. The recombinant His-ΔCaPTC7p migrated at 47 kDa in 12% SDS–PAGE (Fig. 1B, lanes 2 and 3), and was purified to nearly a single protein band by immobilizing it on Ni^{2+}-NTA agarose beads and eluting it from the beads (Fig. 1B, lane 4). The size of His-ΔCaPTC7p in the gel is bigger than the predicted molecular weight (38 kDa), suggesting that the His-ΔCaPTC7p migrates aberrantly in SDS–PAGE.

The phosphatase activity of the recombinant His-ΔCaPTC7p was assayed using a serine/threonine phosphatase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. As shown in Fig. 1C, the dephosphorylation activity of His-ΔCaPTC7p on the peptide substrate was dependent on the presence of the cation cofactor Mg^{2+} or Mn^{2+}. The maximal phosphatase activity of His-ΔCaPTC7 was detected at the concentration of 5 mM Mn^{2+} (Fig. 1C). In the presence of Mg^{2+}, the phosphatase His-ΔCaPTC7p achieved its highest activity at a concentration of 10 mM (Fig. 1C). However, the activity of the recombinant phosphatase in this Mg^{2+} concentration was still lower than that detected in the presence of 10 mM Mn^{2+} under the same conditions (Fig. 1C), suggesting that Mn^{2+} is a relatively more potent cofactor than Mg^{2+} for the recombinant His-ΔCaPTC7p. The dephosphorylation activity of the recombinant His-ΔCaPTC7p was completely blocked by the protein serine/threonine inhibitor NaF at a concentration of 50 mM (data not shown). However, the phosphatase activity of the recombinant His-ΔCaPTC7p was neither sensitive to okadaic acid, a PP1 and PP2A inhibitor, nor to orthovanadate, a protein tyrosine phosphatase inhibitor (data not shown). Taken together, these results indicate that the CaPTC7p possesses all the characteristics of a typical PP2C phosphatase.

After searching the GenBank database, we have not observed any significant sequence homology between CaPTC7p and other known PP2C enzymes. However, CaPTC7p shows 25% and 50% sequence identity in the N-terminal 116-amino acid region and the C-terminal PP2C catalytic domain, respec-

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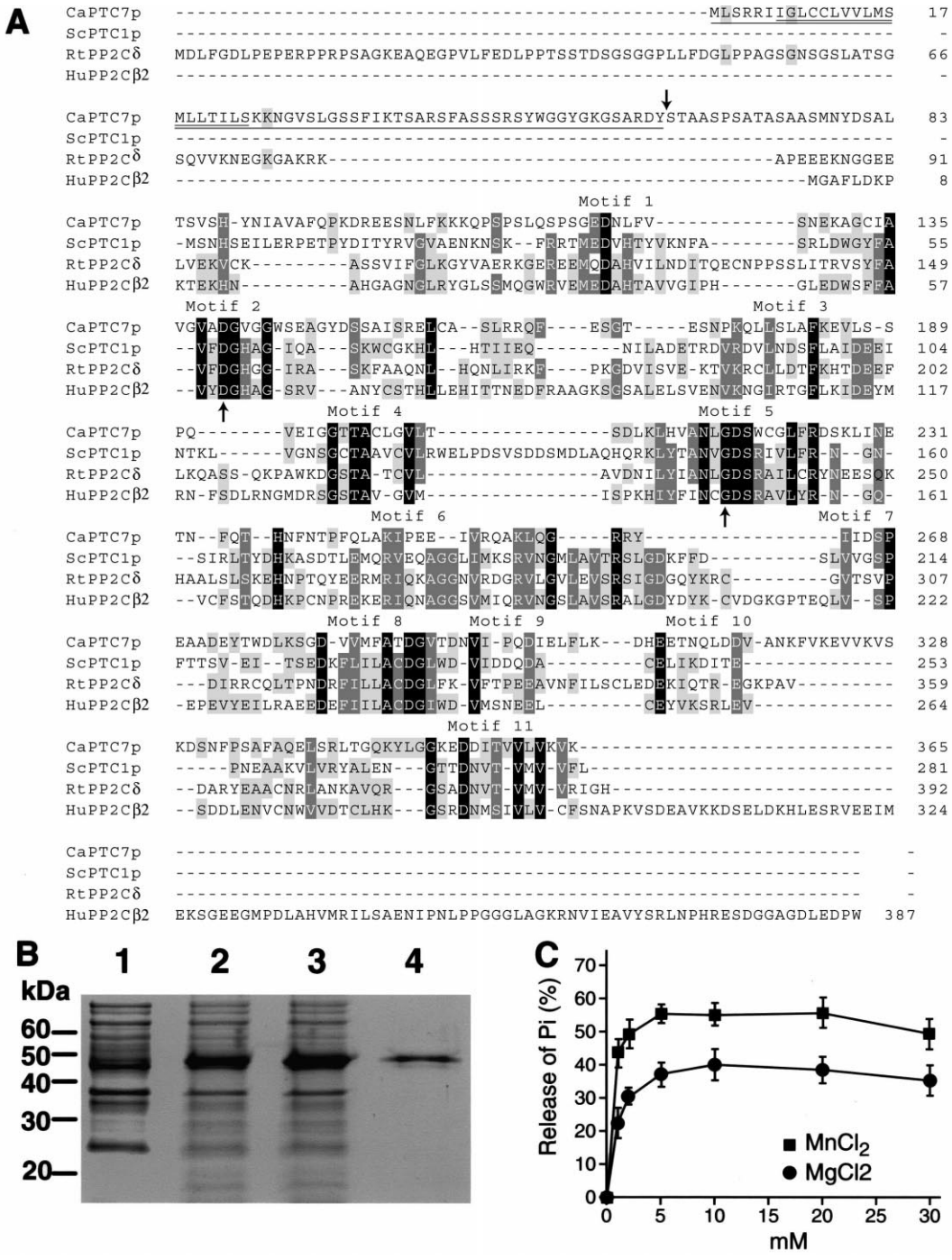

Fig. 1. A: The alignment of CaPTC7p with Ptc1p, rat PP2Cσ, and human PP2Cβ2. The two invariant amino acids, D and G, are indicated by upward arrows and the 11 conserved motifs found in the PP2C superfamily [2] are marked above the sequences. Residues highly conserved within PP2C members are shaded. The potential mitochondrion-targeting sequence of CaPTC7p is underlined, within which, the putative transmembrane domain (I7 to S24) is double underlined, and the putative cleavage site within the R-3 motif (ARDY$_{1}$S63) for the mitochondrial presequence [4] is indicated with the downward arrowhead. B: SDS–PAGE analysis of recombinant His-ΔCaPTC7p. Lanes 1–3: Total bacterial lysates prepared from the cells expressing the His-ΔCaPTC7p by 1 mM IPTG-induction for 0, 1 and 2 h, respectively. Lane 4: Purified His-ΔCaPTC7p (1 μg). The gel was stained with Coommasie blue. Molecular mass (kDa) is indicated to the left of the gel. C: The effects of the cation cofactors Mg^{2+} and Mn^{2+} on the dephosphorylation activity of the recombinant His-ΔCaPTC7p. The concentrations of $MgCl_2$ and $MnCl_2$ are indicated.

tively, with YHR076wp, a potential budding yeast homolog (GenBank accession No.: NP_011943). The N-terminal region of CaPTC7p contains a potential mitochondrion-targeting signal and a transmembrane domain (Fig. 1A), suggesting that it likely resides in the mitochondrion where it might be involved in the regulation of metabolic pathways [4]. In addition, the catalytic domain of CaPTC7p displays 35, 36, 33 and 33% sequence identities with the CG15035 gene product of *Drosophila melanogaster* (GenBank accession No.: AAF47506), the hypothetical protein W09D10.4 of *Caenorhabditis elegans*

(GenBank accession No.: CAB07860), the At5g66720 gene product of *Arabidopsis thaliana* (GenBank accession No.: NP_201473) and the azr1 protein of *Schizosaccharomyces pombe* [5], respectively. The gene azr1$^+$ was originally isolated as a multi-copy suppressor of the 5-azacytidine sensitivity of G2 checkpoint and DNA repair-deficient *S. pombe* strains, but the nature of the azr1 protein was unknown [5]. We observed a potential PP2C catalytic domain in each of these proteins from the four eukaryotic model organisms (data not shown), indicating that the CaPTC7 phosphatase is highly conserved in eukaryotes.

In summary, we describe the identification and characterization of the CaPTC7p, the first PP2C phosphatase in *C. albicans*, encoded by the *YHR*76 gene. Our results suggest that the CaPTC7p possesses the characteristics of classical PP2C enzymes. The interesting structural features of CaPTC7p and its high conservation in the eukaryotic organisms make it a novel member of the PP2C superfamily.

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