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The *YHR076w* gene encodes a type 2C protein phosphatase and represents the seventh PP2C gene in budding yeast

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The reversible phosphorylation of proteins is a critical mechanism by which eukaryotic organisms regulate cellular processes. Protein kinases, which add phosphate groups, and protein phosphatases, which remove phosphate groups, serve to control the phosphorylation status of their substrates. 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 includes three subtypes of phosphatases, PP1, PP2A and PP2B, which contain distinct regulatory subunits forming a variety of holoenzymes; these enzymes show strong sequence conservation in their catalytic domains. 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 (OA). The other class of phosphatases, members of the PPM family, are monomeric enzymes and are unrelated in sequence to the PPP family; they include primarily the type 2C protein phosphatases (PP2C). 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 OA and other inhibitors of the PPP family.

In the budding yeast *Saccharomyces cerevisiae*, six PP2C-like genes have been identified, *PTC1*, *PTC2*, *PTC3*, *PTC4*, *PTC5* and *YCR079w*, five of which encode proteins with PP2C phosphatase activity. The protein encoded by the PP2C-like gene *YCR079w*, when expressed in the GST-fusion form in bacterial cells, fails to show any phosphatase activity in the presence of Mg^{2+} or Mn^{2+} [3]. We previously identified and characterized a novel PP2C phosphatase (CaPtc7p) from the human fungal pathogen *Candida albicans* [4]. The *C. albicans* gene, encoding CaPtc7p, has a potential homologue, *YHR076w*, in *S. cerevisiae*. The protein encoded by *YHR076w* was previously identified as an antigen that cross-reacted with the antiserum generated against a protein preparation enriched for chicken smooth muscle caldesmon, and was localized in the mitochondrion [5]. However, its identity and function remained unknown. Sequence analysis shows that Yhr076wp contains the two invariant amino acids (140D and 223G) 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 Yhr076wp. Therefore, we designate *YHR076w* as *ScPTC7*, the seventh PP2C-like gene in *S. cerevisiae*.

To demonstrate that ScPTC7p has PP2C phosphatase activity, we expressed in bacteria an N-terminally truncated (excluding the transmembrane domain) and GST-fused recombinant ScPTC7p (GST-ΔScPTC7p), which was purified to near homogeneity (Fig. 1B). The construction of the GST-ΔScPTC7p was described previously [5]. The induction and purification of the recombinant protein in *E. coli* were carried out according to standard procedures [6]. The phosphatase activity of the recombinant GST-ΔScPTC7p was assayed using a serine/threonine phosphatase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. This purified recombinant protein, but not the GST alone, exhibited Mg^{2+} or Mn^{2+} dependent dephosphorylation activity on a synthetic phosphopeptide {RRA(pT)VA} (Fig. 1C). The dephosphorylation activity of the recombinant GST-ΔScPTC7p was significantly inhibited by the non-selective protein serine/threonine inhibitor NaF at a concentration of 50 mM, but was not sensitive to OA, a PP1 and PP2A inhibitor (Fig. 1D). Taken together, these results indicate that the GST-ΔScPTC7p indeed possesses the characteristics of a typical PP2C phosphatase.

As was also noted for CaPtc7p, the PSORT motif prediction program (http://psort.nibb.ac.jp/) predicted the presence of a transmembrane domain (L17 to F33) and a potential mitochondrial-targeting sequence at the N terminus of ScPtc7p, within which the putative cleavage site in the R-2 motif (SRG|PL) for the mitochondrial presequence was observed (Fig. 1A). This structural feature is supported by the previous observation on the mitochondrial localization of ScPtc7p [5]. In line with these observations, we have also demonstrated that *C. albicans* CaPtc7p is localized in the mitochondrion (Jiang et al., unpublished results).

ScPtc7p shares 25% and 50% sequence identity at its N-terminal extension and the C-terminal PP2C catalytic domain, respectively, with CaPtc7p (Fig. 1A; [4]). In addition, the PP2C catalytic domain of ScPtc7p shows 33%, 33%, 32% and 31% sequence identities with the Azr1 protein of *Schizosaccharomyces pombe* (GenBank accession no.: X98329), the CG15035 gene product of *Drosophila melanogaster* (GenBank accession no.: AAF47506), the hypothetical protein W09D10.4 of *Caenorhabditis elegans* (GenBank accession no.: CAB07860) and the At5g66720 gene product of *Arabidopsis thaliana* (GenBank accession no.: NC_003075). The PSORT motif prediction program also predicts the presence of the putative cleavage site for the mitochondrial presequence with the R-2 motif (L14RG|KD18 for At4G16580p, V26RA|VF30 for CeW09D10.4p and A28RY|VH32 for SpAZR1p), but not for DmCG15035p. Taken together, these observations indicate that ScPtc7p is highly conserved in eukaryotic organisms.

In conclusion, we have characterized a new PP2C phosphatase in *S. cerevisiae*, which is encoded by the seventh PP2C gene *YHR076w* that is designated as *PTC7*. The mitochondrial localization of Ptc7p and its conservation in eukaryotic model organisms have significant implications in the regulation of mitochondrial physiology by protein phosphorylation in higher eukaryotic cells. The powerful genetic and molecular tools available in *S. cerevisiae* would provide an opportunity

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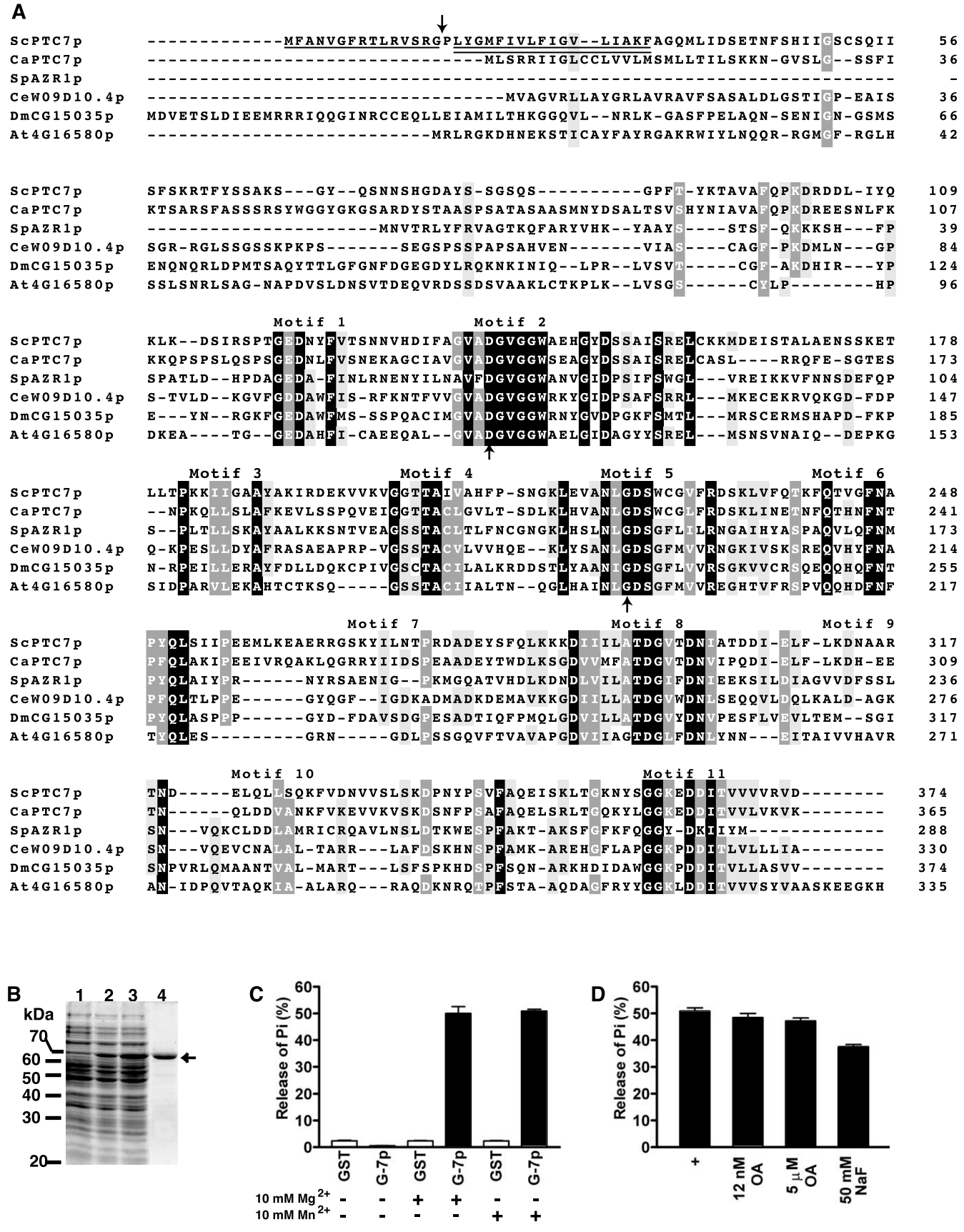
A
ScPTC7p
CaPTC7p
SpAZR1p
CeW09D10.4p
DmCG15035p
At4G16580p
Motif 1
Motif 2
Motif 3
Motif 4
Motif 5
Motif 6
Motif 7
Motif 8
Motif 9
Motif 10
Motif 11
B
1 2 3 4
kDa
70
60
50
40
30
20
C
Release of Pi (%)
60
50
40
30
20
10
0
GST
G-7p
GST
G-7p
GST
G-7p
10 mM Mg2+ - - + + - -
10 mM Mn2+ - - - - + +
D
Release of Pi (%)
60
50
40
30
20
10
0
+
12 nM OA
5 μM OA
50 mM NaF

to uncover the molecular function of this interesting new phosphatase.

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Fig. 1. A: Sequence alignment of the deduced amino acids of ScPTC7p, CaPTC7p, SpAZR1p, CeW09D10.4p, DmCG15035p and At4G16580p. The amino acids are numbered on the right. The two invariant amino acids (140D and 223G) 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 these proteins are shadowed. The potential mitochondrion-targeting sequence at the N terminus of ScPTC7p is underlined, within which the putative cleavage site in the R-2 motif (SRG|PL) for the mitochondrial presequence is indicated with the downward arrowhead. The putative transmembrane domain (L17 to F33) is double underlined. B: SDS–PAGE analysis of recombinant GST-ΔScPTC7p. Lanes 1–3, total bacterial lysates prepared from the cells expressing the GST-ΔScPTC7p by 0.5 mM IPTG induction for 0 h, 1 h and 2 h, respectively. Lane 4, 2 μg of purified GST-ΔScPTC7p, the position of which is indicated with an arrow. The gel was stained with Coomassie blue and the molecular mass is indicated to the left of the gel. C: Dependence of the phosphatase activity of GST-ΔScPTC7p on cation Mg^{2+} or Mn^{2+}. G-7p represents GST-ΔScPTC7p. D: Effects of phosphatase inhibitors on the dephosphorylation activity of GST-ΔScPTC7p in the presence of 10 mM Mn^{2+}. The phosphatase activity was determined with purified GST-ΔScPTC7p (1 μg) at 30°C for 30 min in the absence (+) or presence of OA or NaF as indicated.