MINIREVIEWS

Type 2C Protein Phosphatases in Fungi

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Type 2C Ser/Thr phosphatases are a remarkable class of protein phosphatases, which are conserved in eukaryotes and involved in a large variety of functional processes. Unlike in other Ser/Thr phosphatases, the catalytic polypeptide is not usually associated with regulatory subunits, and functional specificity is achieved by encoding multiple isoforms. For fungi, most information comes from the study of type 2C protein phosphatase (PP2C) enzymes in Saccharomyces cerevisiae, where seven PP2C-encoding genes (PTC1 to -7) with diverse functions can be found. More recently, data on several Candida albicans PP2C proteins became available, suggesting that some of them can be involved in virulence. In this work we review the available literature on fungal PP2C and explore sequence databases to provide a comprehensive overview of these enzymes in fungi.

Reversible phosphorylation of proteins is a major mechanism regulating many biological processes such as metabolism, gene transcription, or cell cycle. It is an extremely common event in signal transduction, and it is considered the main mechanism of posttranslational modification leading, for instance, to a change in enzyme activity. The phosphorylation state of a protein results from the balance of protein kinases and protein phosphatases activities. Alterations in the phosphorylation state of proteins are a cause of various diseases, such as cancer, diabetes, rheumatoid arthritis, or hypertension. The importance of this regulatory mechanism is evident when it is considered that it affects around 30% of the proteome of eukaryotes. The understanding of the catalytic process for PP2C came from the determination of the crystal structure of the human isoform PP2Cα (12), which revealed key Asp residues required for coordination of Mg$^{2+}$ or Mn$^{2+}$ ions (mutation of these residues is usually performed to generate catalytically inactive forms of PP2C). In sharp contrast with PPP enzymes (particularly with PP1 phosphatases), in which the catalytic polypeptide interacts with a large number of very different regulatory subunits that provide functional specificity, PP2Cs are normally monomeric enzymes. The myriad of functions performed by PP2C enzymes is possibly the result of the expression of a large number of catalytic isoforms. For instance, at least 14 genes are found in humans, and probably more than 20 different polyptides are produced by splicing mechanisms. A stunning situation is found in the model plant Arabidopsis thaliana, in which near 80 PP2C proteins have been identified or predicted (46, 113). In many cases, the different isoforms have characteristic amino- or carboxyl-terminal extensions (see below), which could provide the structural basis for functional specificity, although little about this aspect is known.

Although the reader is directed to recent reviews (48, 55, 83) of the role of PP2C enzymes in these organisms, a brief overview is provided here for comparative purposes. Human PP2Cs have been implicated in many processes, such as regulation of mitogen-activated protein kinase (MAPK) cascades (p38 or Jun N-terminal protein kinase [JNK]), cell cycle progression, ubiquitination and degradation of proteins, and mechanisms for cell death and survival (see references 48, 55, and 100 for this review; and the catalytically Asp-based subfamily, represented by HAD and FCP/SCP (see reference 89 for a review). Although PP2C proteins are distantly related in primary sequence to PPP enzymes, their tridimensional structure and catalytic mechanisms appear to be quite similar. Type 2C phosphatases are widely represented in bacteria, fungi, plants, insects, and mammals (83), suggesting that they are involved in key cellular processes, such as proliferation, metabolism, and cell survival (11, 48). The understanding of the catalytic process for PP2C came from the determination of the crystal structure of the human isoform PP2Cα (12), which revealed key Asp residues required for coordination of Mg$^{2+}$ or Mn$^{2+}$ ions (mutation of these residues is usually performed to generate catalytically inactive forms of PP2C). In sharp contrast with PPP enzymes (particularly with PP1 phosphatases), in which the catalytic polypeptide interacts with a large number of very different regulatory subunits that provide functional specificity, PP2Cs are normally monomeric enzymes. The myriad of functions performed by PP2C enzymes is possibly the result of the expression of a large number of catalytic isoforms. For instance, at least 14 genes are found in humans, and probably more than 20 different polyptides are produced by splicing mechanisms. A stunning situation is found in the model plant Arabidopsis thaliana, in which near 80 PP2C proteins have been identified or predicted (46, 113). In many cases, the different isoforms have characteristic amino- or carboxyl-terminal extensions (see below), which could provide the structural basis for functional specificity, although little about this aspect is known.

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Their target, protein phosphatases in general and PP2C enzymes in particular do not exhibit significant site specificity. This prevents a simple, sequence-based approach for the determination of their protein and amino acid targets. In addition, PP2C-focused research has been hampered by the lack of specific inhibitors against these enzymes.

**PP2C IN SACCHAROMYCES CEREVISIAE**

Even in a relatively simple eukaryotic organism such as the yeast *Saccharomyces cerevisiae*, there are several PP2C-encoding genes. Initially, the family comprised five isoforms called Ptc1 to -5 (for phosphatase two C) (8), although in the following years two more members were added, Ptc6 (77) and Ptc7 (40) (Fig. 1). All these proteins share a conserved PP2C domain, which can be accompanied by amino-terminal (Ptc5 and Ptc7) or carboxyl-terminal (Ptc2 and Ptc3) extensions. As is the case in higher eukaryotes, yeast PP2Cs were initially involved in the regulation of cell growth and stress signaling. For instance, several PP2C isoforms were initially associated with the negative regulation of the HOG (high-osmolarity glycerol) signaling pathway, inducible after osmotic stress, by dephosphorylating and inactivating the HOG1 MAPK.

**The Ptc1 phosphatase.** The Ptc1 isoform is by far the best characterized PP2C in yeasts. Although it shares some of its functions with other members of the family, recent work has shown that the transcriptional profile of *ptc1* mutants growing under standard conditions is markedly different from that of *ptc2, -3, -4, and -5* strains, in a way that could not be anticipated from primary structure comparisons (29). This observation reinforces the notion that Ptc1 plays specific functional roles that are not shared by other family members.

(i) **The link between Ptc1 and the HOG pathway.** *PTC1* was first identified in 1993 in a search for mutations that exacerbate the growth defect of strains lacking the tyrosine phosphatase...
Ptp1 or Ptp2 (57). Lack of PTC1 drastically affects growth of a ptp2 mutant under normal conditions, which suggested that both phosphatases were acting on various substrates with redundant functions or on a single substrate phosphorylated at Ser/Thr and Tyr residues (as in the case of a MAPK). In this same study it was shown that the gene product had phosphatase activity \textit{in vitro} that required Mn\textsuperscript{2+} or Mg\textsuperscript{2+} ions. One year later it was determined that the overexpression of PTC1 is able to rescue the lethal phenotype of an sln1 mutant, which is due to hyperactivation of the HOG signaling pathway, and it was suggested that Ptc1 negatively affects Pbs2, the MAPK kinase upstream of Hog1 (58). In an almost parallel work it was shown that while the ptc1 mutant is tolerant to the effects of killer toxin K1, the pbs2 ptc1 double mutant is hypersensitive (similar to the case for pbs2 cells), thus establishing an epistatic relationship between PTC1 and PBS2 (39). These authors also showed that in cells lacking PTC1 intracellular glycerol levels are higher than in the wild type and that this phenotype is also suppressed by the deletion of PBS2, suggesting that the ptc1 mutation may increase the activity of the HOG pathway (Fig. 2a).

Overall, these results provided genetic evidence placing Ptc1 as a negative regulator of the HOG signaling pathway, a notion that was reinforced by experiments with the fission yeast \textit{Schizosaccharomyces pombe}, in which the orthologue \textit{ptc1} gene was found to play a role in osmoregulation by counteracting the effect of the Pbs2 orthologue MAPK kinase Wis1 (see below) (90, 91). Later it was shown that the severe growth defect of a ptc1 ptp2 mutant is indeed due to hyperactivation of the HOG pathway and that this phenotype disappears when PBS2 or HOG1 is deleted (38). Four years later it could be demonstrated that, \textit{in vitro}, Ptc1 dephosphorylates Hog1 at Thr-174. This residue and Tyr-176 are located in the activation loop, and their phosphorylation is indispensable for the activation of the MAPK. It was also shown that the phosphatase, which is present in the nucleus and the cytoplasm, is necessary to regulate the Hog1 phosphorylation state \textit{in vivo}, both at the basal level and during adaptation to stress (108).

Large-scale experiments using the two-hybrid method revealed that Ptc1 physically interacts with Nbp2, a protein that contains an SH3 (Src homology 3) domain (37, 105). Remarkably, Pbs2, which acts as a scaffold for various elements of the HOG pathway such as Hog1, Sho1, Ste11, and Ssk2/22 (70, 101), had been copurified with Nbp2 and Ptc1. Thus, it was demonstrated that Ptc1 performs its functions on the HOG pathway through the adaptor protein Nbp2, whose N-terminal domain is necessary for interaction with the phosphatase, while the SH3 domain is responsible for binding to Pbs2 (61). As far as we know, this is the only example of the existence of a targeting regulatory protein for a PP2C in \textit{S. cerevisiae} (Fig. 2a).

\textbf{(ii) Ptc1 and the CWI pathway.} In addition to the relatively well characterized role of Ptc1 in the regulation of the Hog1 MAPK pathway, there is evidence linking this phosphatase to another MAPK-regulated route, the cell wall integrity (CWI) pathway. Schematically, this pathway is composed of several membrane sensors that signal through the small GTPase Rho1 to the Pkcl kinase, which is the upstream component of the

\begin{figure}
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\caption{Functional role of PP2C enzymes in yeast MAPK pathway regulation and organelle inheritance. (a) Regulation of MAPK pathways. Different \textit{S. cerevisiae} and \textit{S. pombe} pathways are depicted, indicating the triggering stimuli and the transcription factors involved in the response. The three layers defining the MAPK pathways are shaded in gray. Discontinuous lines denote suspected or still poorly characterized interactions. See text for details. (b) The central role of Ptc1 and the different proteins presumed to mediate the phosphatase function on organelle inheritance. Note that only endoplasmic reticulum and mitochondrial inheritances seem to require Slt2 MAPK intervention.}
\end{figure}
MAPK cascade that activates the Slt2/Mpk1 MAPK (see references 21 and 52 for reviews of this pathway). A search for mutations that were able to suppress the thermosensitive phenotype and the high rate of recombination of strains carrying the hypooactive pck1-4 allele yielded the KCS2 gene (for Pck1-4 suppressor), which turned out to be allelic with PTC1 (35). Since the lack of PTC1 compensates for reduced Pck1 kinase activity, it could be proposed that Ptc1 might regulate the dephosphorylation of specific targets of Pck1 or any member of the signaling pathway to which Pck1 belongs (Fig. 2a). It must be noted that the ptc1 slt2 double mutant has been described as synthetic lethal (35), suggesting that Ptc1 might have additional substrates that are relevant for cell wall integrity. However, this synthetic phenotype is largely strain dependent. For instance, while the lethality of the slt2 ptc1 mutation was reported not to be alleviated by the addition of sorbitol to the culture medium (35), in a different genetic background commonly used in our laboratory (JA100), addition of 1 M sorbitol allows survival (29). Furthermore, in both the S288C and its derived BY4741 genetic backgrounds, the slt2 ptc1 strain is viable even in the absence of osmotic support (14, 29), although it is extremely sensitive to cell wall-damaging agents (29).

The relationship between Ptc1 and the CWI pathway is reinforced by a number of findings. For instance, one of the alleles of the PTC1 gene is CWH47 (for calcinoflor white hypersensitive), revealing that the gene deletion causes hypersensitivity to this chitin antagonist (73). Ptc1-deficient cells also display a phenotype characteristic of some strains with mutations of genes involved in cell wall synthesis: they are tolerant to the K1 killer toxin (39, 67). This might be due to a decrease in the amount of β-1,6-glucans, probably because of unusually high levels of Exgl exo-β-glucanase activity (39). Furthermore, the ptc1 mutant is sensitive to caspofungin (62), caffeine (27), and alkaline pH (29, 87, 88), circumstances that activate the CWI pathway, and the ptc1 mutation is synthetically lethal with different mutations in genes important for cell wall construction, such as FKS1, GAS1, or SM1 (51, 102). Furthermore, it has been shown that cells lacking Ptc1 have larger amounts of active Slt2 (14, 29). In addition, mutation of PTC1 results in increased expression of several genes normally induced by cell wall damage, such as YKL161c, CRH1, or SED1, and this response is dependent on both the Slt2 MAPK module and the downstream transcription factor Rim1 (29). All these data reinforce the notion that lack of phosphatase mimics a situation of cell wall damage. Although data collected in recent years reveal the existence of a interaction between the Slt2 and HOG pathways (4, 24, 25, 32), it must be noted that the hypersensitivity of the ptc1 strain to cell wall-damaging agents is not attributable to a hyperactivation of Hog1, since a ptc1 hog1 double mutant still displays a strong sensitivity to cell wall-damaging agents (29). Similarly, a lack of Nbp2 does not improve tolerance of the ptc1 strain to calcofluor white or caffeine (29).

(iii) Ptc1 and cation homeostasis. It has been described that ptc1 cells are highly sensitive to calcium ions (29, 31, 35) and that this is most probably due to hyperactivation of the phosphatase calcineurin, because sensitivity is largely abolished by deletion of the CNB1 gene (29). Similarly, the ptc1 mutant is also sensitive to amiodarone, an antifungal agent that causes a dramatic increase in cytoplasmic calcium concentrations (31). In addition, deletion of PTC1 renders cells sensitive to metals such as zinc and cesium (29) and, as mentioned above, to alkaline pH. It is worth noting that many of these traits are found in certain mutants with impaired vacuolar function. Remarkably, the ptc1 mutant displays fragmented vacuoles, mimicking those of class B vps (vacuolar protein-sorting) mutants (5, 29, 96). Several genome-wide studies have identified Ptc1 as a protein required for maintaining the structure and function of the vacuole (5, 81, 85), although there is some controversy about the ability of ptc1 mutants to correctly process carboxypeptidase Y (CPY). While some reports propose that processing is not correct (5, 85), other studies have shown normal CPY processing (3, 29). It must be noted that vacuolar malfunction does not always result in deficient CPY processing (85). A further link between Ptc1 and vacuolar function came from the finding that overexpression of VPS73, a gene of unknown function involved in vacuolar protein sorting, largely rescues not only vacuolar fragmentation but also sensitivity to cell wall-damaging agents, high calcium levels, and alkaline pH, as well as other ptc1-specific phenotypes (29). These findings prompted the authors to hypothesize that lack of Ptc1 would primarily cause vacuolar malfunction, from which other phenotypes of the mutant would derive.

Deletion of PTC1 confers a lithium (but not sodium)-sensitive phenotype that is not shared by other members of the PP2C family (78). A ptc1 mutant is less effective in extruding Li+ and accumulates higher concentrations of this cation, as a consequence of decreased expression of the Na+-ATPase ENA1 gene. This effect is not attributable to the role of Ptc1 in Hog1 regulation, but instead a number of observations suggest that Ptc1 may regulate the Hal3/Ppz1,2 pathway (13). For instance, lack of Hal3 provokes a decrease in ENA1 expression similar to that produced by mutation of PTC1, and the effects are not additive. Moreover, blocking the mechanism for Ppz-mediated activation of ENA1 (i.e., simultaneous deletion of the Trk1,2 potassium transporters and chemical inhibition of calcineurin) does not further increase sensitivity of the ptc1 strain to lithium (78). How Ptc1 may interact with the Hal3/Ppz system is still unknown. It has been recently reported that the protein kinase Hal5, which is involved in regulation of potassium influx, is a multicopy suppressor of the lithium-sensitive phenotype of the ptc1 mutant, but the molecular basis of this effect remains to be elucidated (116).

(iv) Ptc1 and the inheritance of cellular organelles. Work in the past few years has focused on an intriguing role of Ptc1: the inheritance of different organelles, such as mitochondria, vacuoles, and the endoplasmic reticulum (ER) (Fig. 2b). Cells lacking Ptc1 exhibit a marked defect in cortical ER inheritance caused by a delay in the last step of this process (14), and the ptc1 mutation displays strong genetic interactions with mutations in SEC3 or AUX1/SWA2, encoding proteins required for cortical ER inheritance (the ptc1 sec3 double mutant shows a severe growth defect, whereas ptc1 aux1 strains are not viable).

The absence of the adaptor protein Nbp2 leads to a phenotype similar to that of the ptc1 mutation, suggesting that both proteins together regulate the inheritance of this organelle. Remarkably, although this phenotype seems to be unrelated to the HOG pathway (14), both the addition of sorbitol and the inactivation of Slt2 solve the defect in ER inheritance in the
ptc1 mutant, indicating that activation of the CWI pathway is probably the cause of this defect in ptc1 cells (14). In this regard, recent studies have shown that Ptc1 is necessary to inactivate the pool of Slt2 associated with the bud tip that promotes the cortical distribution of the ER in daughter cells (53). Moreover, deletion of SPA2, a component of the polariosome that is required for recruitment of Slt2 to the bud tip, suppresses the ER inheritance defect of ptc1 cells (53). This defect is also suppressed by mutation of Mss4, a phosphatidylinositol-4-phosphate 5-kinase that plays a predominant role in the heat shock-induced activation of Slt2. This effect is probably due to the drastically reduced Slt2 phosphorylation levels observed in the ptc1 mss4-102 mutant (53).

In wild-type strains, mitochondrial inheritance and bud emergence are concomitant processes. However, Roeder and colleagues found that cells lacking Ptc1 produce buds that are initially devoid of the mitochondrial compartment (76). These same authors ruled out an involvement of the HOG pathway. However, a recent report shows that the mitochondrial inheritance defect of ptc1 cells is substantially suppressed in the ptc1 sit2 double mutant (53), indicating a role of the Sit2 kinase in this process. It should be noted that, unlike what happens to the ER inheritance, in the case of mitochondrial inheritance the relevant pool of Slt2 is not located at the bud tip and is apparently independent of Mss4 function (Fig. 2b). This distinction makes sense in light of the different stages at which inheritance of the ER and mitochondria are blocked in ptc1 cells. Further links between Ptc1 and mitochondrial function come from the observations that in a gem3 background (lacking a protein of the Rho family needed to maintain mitochondrial tubular morphology), deletion of PTC1 causes significant growth defects (20). Mitochondrial instability of the ptc1 mutant has been proposed as the reason for its sensitivity to the antitumor drug doxorubicin (111).

A recently published study has shown that Ptc1 complements the defects of a vac10-1 mutant, which was identified after a search for strains defective in vacuolar inheritance (43). This strain turned out to contain a mutation that eliminates the catalytic activity of Ptc1, indicating that Ptc1 phosphatase activity is necessary to ensure proper vacuolar inheritance. In light of the available genetic data, it seems that the mechanism by which Ptc1 controls the movement of the vacuoles from the mother to the daughter cell does not involve the HOG pathway or the CWI pathway (14, 43).

Besides affecting vacuole, mitochondrion, and cortical ER inheritance, the lack of PTC1 alters the inheritance of other organelles such as peroxisomes and secretory vesicles (43). All these organelles have a common characteristic: they are transported by Myo2 or Myo4, the twin engines of class V myosin. The ability of these proteins to direct multiple organelles to different cellular locations is based primarily on the existence of specific receptors for each organelle. In this regard, it has been proposed (Fig. 2b) that Ptc1 affects the stability of several of these receptors, such as Vac17, Inp2, and Mmr1, which link Myo2 to the vacuole, peroxisomes, and mitochondria, respectively (43). This would explain a number of phenotypes associated with the absence of PTC1, placing this phosphatase as a key element in controlling the distribution of cellular organelles (16, 43).

(v) Other phenotypes caused by the mutation of PTC1. In addition to the phenotypes described so far, the Ptc1 phosphatase has been implicated in several processes, although in most cases the mechanism of action remains unclear. Years ago, a search for mutants that were unable to correctly process tRNAs allowed the identification of the TPD1 gene (for tRNA processing defective), which was later found to be allelic to PTC1, thus implicating the phosphatase in tRNA splicing (75). This same work also revealed other alterations in ptc1 cells; for instance, they are unable to grow in media containing nonfermentable compounds such as lactate, ethanol, or glycerol as a carbon source (this was subsequently confirmed in an independent study [76]). This defect may be related to the vacuolar defects described above. In addition, ptc1 cells show decreased efficiency of sporulation and exhibit major defects in cell separation in diploids (especially at 37°C). More recently, it has been shown that haploid ptc1 cells also display cell separation defects and a random budding pattern at 37°C (29).

The early observations that cells lacking Ptc1 are sensitive to rapamycin and caffeine (29, 68, 77, 112) pointed to the possible existence of a functional connection between Ptc1 and the TOR pathway. In this regard, it has been recently shown that Ptc1 is required for normal TOR signaling, possibly by regulating a step upstream of the type 2A-related Ser/Thr phosphatase Sit4 in a way independent of the HOG pathway (28). This finding provides the first evidence that a PP2C is linked to this important and conserved pathway. Remarkably, mutations in components of the Sit2 MAPK pathway alleviate to a certain extent the rapamycin-sensitive phenotype of ptc1 cells (our unpublished observations). This result suggests that it would be worth revisiting the link between the TOR and CWI pathways.

Work recently published by Malleshaiah and coworkers highlights the role of Ptc1 in the regulation of the mechanism that controls the switch-like mating decision (59). It is well known that pheromones activate a MAPK cascade that leads to phosphorylated (active) Fus3, which dissociates from Ste5 and phosphorylates downstream targets to mediate mating (for a review, see reference 6). Interestingly, deletion of PTC1 substantially prevents shmooing and reduces activation of Fus3, whereas the overexpression of PTC1 has the opposite effect. The authors also demonstrated that Ptc1 is responsible for the dephosphorylation of four sites on Ste5, a requisite for full relief and dissociation of the Fus3-Ste5 complex, which leads to the mating response (Fig. 2a).

Deletion of PTC1 increases sensitivity to a number of agents, such as sorbate, a weak organic acid used to preserve food (64), or oleate (54). Since sensitivity to oleate is typical (though not exclusive) of cells with impaired peroxisomal function, it could be explained by the defects in peroxisome inheritance mentioned above. A ptc1 mutant is also slightly sensitive to linoleic acid hydroperoxide, an oxidizing compound produced by lipid peroxidation that causes a delay in the G1 phase of the cell cycle. It has been speculated that the mechanism by which PTC1 may be involved in the tolerance to this agent would involve Mns22, a protein required for resistance to ionizing radiation, although a possible role for the HOG pathway has not been discarded (19). Furthermore, the toxicity of oxalate increases in cells lacking PTC1. In this case, it has been postulated that the HOG pathway could be involved in transduction of signals caused by oxalate stress (9). Interestingly, PTC1...
is a weak multicopy suppressor of the lethal phenotype of a fcl1 flc2 double mutant. Flc1 and Fcl2 are two flavin carriers needed to import flavin adenine dinucleotide (FAD) into the endoplasmic reticulum. The reason why PTC1 overexpression improves growth in a fcl1 fcl2 strain could be attributed to the link between Ptc1 and the CWI route (71). Finally, a genome-wide screen for mutants with affected telomere length revealed that ptc1 cells display shorter telomeres than the corresponding wild-type strain (1).

The Ptc2, Ptc3, and Ptc4 phosphatases. Ptc2 and Ptc3 are two proteins that are 62% identical (77% similar) (8, 57) and whose origin could be due to a genomic duplication event (110). These two proteins differ structurally from Ptc1 because they possess a carboxyl-terminal extension of around 170 residues (Fig. 1) that, in the case of Ptc2, is necessary to achieve maximum activity (114). Ptc2 and Ptc3 are unable to fulfill the role of Ptc1 in the HOG pathway (57). However, these two phosphatases are also involved in the regulation of the Hog1 MAPK (Fig. 2a), but with a slightly different role than Ptc1, since in this case they serve to limit the maximum of activation of the HOG pathway after stress (114). Unlike Ptc1, Ptc2 and Ptc3 do not interact with Nbp2 (61).

Ptc2 also regulates negatively the unfolded-protein response in the endoplasmic reticulum through dephosphorylation of the Ser/Thr protein kinase Ire1 (109), which is necessary to induce the appropriate transcriptional response. Ptc2 physically interacts with Ire1 through a region, located in the central part of the phosphatase, comprising part of the catalytic and carboxyl-terminal segments (between amino acids 174 and 355). Ptc2 is also necessary to preserve cell viability in the face of agents that compromise the integrity of DNA (63). In the presence of such agents or under replicative stress, DNA checkpoints are activated to maintain genomic stability. Specifically, the S-phase checkpoint generates a cellular response that halts the cell cycle through a mechanism involving phosphorylation and activation of the protein kinase Rad53. PTC2 overexpression confers sensitivity to genotoxic agents such as hydroxyurea or UV radiation (63) and allows growth of strains carrying a dominant lethal allele of Rad53. Similarly, ptc2 mutants are sensitive to selenite, a compound that induces the oxidative decarboxylation of pyruvate to form acetyl coenzyme A (acetyl-CoA) (26, 47). This justifies Ppp1 (PDH protein phosphatase 1) as an alias for Ptc5.

It was presumed for many years that the protein encoded by YCR079w was a type 2C phosphatase (94), although the first attempt to demonstrate the phosphatase activity of the recombinant protein was unsuccessful (8). It was only recently that Ruan and colleagues could demonstrate that Ycr079w displays the typical characteristics of a PP2C, and they renamed the protein Ptc6 (77). Ptc6 is located both in the intermembrane space and in the mitochondrial matrix (26, 99) and, as mentioned above for Ptc5, regulates the phosphorylation state of Pda1 (and consequently received the alias of Ppp2) (26). It has been shown that Ptc6 is necessary for survival of stationary-phase cells, and it has recently been found to be involved, probably through the Rtg3 transcription factor, in the mitochondrial degradation process known as mitophagy. The latter phenotype justifies its alias of Aup1 (autophagy-related protein phosphatase) (44, 99). It is worth noting that although ptc5 and ptc6 phenotypes largely overlap (our unpublished results), a lack of Ptc6 (but not of Ptc5) results in sensitivity to rapamycin (28, 77), suggesting distinctive functions for Ptc6.

The biochemical characteristics of Ptc7 were determined in 2002 (40). Similarly to Ptc5 and Ptc6, this phosphatase was initially located in the mitochondria (74). According to these authors, strains lacking Ptc7 do not differ from the wild type in growth rate or morphological characteristics. However, overexpression of PTC7 improves growth when ethanol or galactose is used as a carbon source in a low-oxygen environment. It is noteworthy that the expression of PTC7 increases after osmotic stress in a Hog1-dependent manner (79). The existence of a functional intron in the PTC7 gene has recently been discovered (45). As a result, depending on the processing of the intron, the gene can produce two mRNAs that encode two different proteins (Fig. 1). Remarkably, they are located in different cellular compartments and play different roles. The
protein translated from the smaller RNA, called Ptc7s, is located in mitochondria, and its expression depends on the source of carbon in the growth medium. On the other hand, the translation of the full mRNA leads to the Ptc7u protein, which contains a transmembrane domain, is located in the nuclear envelope, and mediates the effects caused by the toxin latrunculin A, a compound that affects actin polymerization (45).

PP2Cs IN OTHER FUNGI

Although early work on the fission yeast Schizosaccharomyces pombe shed light on the functional role of PP2C in fungi (see below), most information in the last 15 years came from the investigation of these enzymes in budding yeast. However, in the last few years, several laboratories have undertaken the study of type 2C phosphatases in organisms other than S. cerevisiae. Before initiating a review of the data available in the literature, we would like to take advantage of the large amount of genomic data currently accessible to provide an overview of the existing PP2C enzymes in fungi. A total of 144 sequences from 22 different fungi were selected after a search for similarity to the seven PP2C protein sequences from S. cerevisiae at the NCBI database. The classification of these proteins according to their primary structure shows that there are five major groups of PP2Cs in fungi (Fig. 3a; see Fig. S1 in the supplemental material). One group is formed by the family of proteins related to S. cerevisiae Ptc1, which is present in all analyzed organisms (Fig. 3b). A second group includes the related Ptc2/Ptc3 and Ptc4 proteins. Interestingly, Ptc4 proteins are present in most species of the Saccharomycotina subphylum but are absent in the rest of the organisms analyzed (Fig. 3b). Another group includes Ptc5-related proteins, which are present in all organisms studied. The fourth group contains a large family of proteins related to S. cerevisiae Ptc6. This includes two subfamilies. One corresponds to the typical Ptc6, which is found in all members of the Saccharomycotina subphylum investigated here. Therefore, this distribution almost overlaps with that of Ptc4, with the exception of Yarrowia lipolytica. The second is a branch of Ptc6-related proteins that is absent only in Saccharomycotina. A relevant member of this family is the Ptc6-related protein of S. pombe named Ptc4 (no. 76 in Fig. S1 in the supplemental material) (see below). It is worth noting that almost all analyzed species that contain a Ptc4 protein also contain a member of the “classical” Ptc6. Furthermore, all species in which the “classical” Ptc6 is absent do contain one or more representatives of the Ptc6-related family. Finally, a large group of proteins with similarity to S. cerevisiae Ptc7 can be found, and these include two families of proteins: a family of “typical” Ptc7s, present in all organisms analyzed, and a group of proteins, here named the Ptc7-related proteins, that is absent in Saccharomycotina except in those species belonging to the CTG clade (organisms that translate the CTG codon as serine instead of leucine). This group includes the recently described Ptc8 protein from Candida albicans (no. 121) (17). Interestingly, many Ptc7 (but not Ptc7-related) proteins seem to include a transmembrane domain at the N terminus.

From this analysis it is worthwhile to highlight the following ideas. (i) Yarrowia lipolytica seems to encode two distinct Ptc1 proteins (no. 1 and 15 in Fig. S1 in the supplemental material).
One of them (no. 1) is a protein of 864 amino acids that possesses a long (over 400-amino-acid) carboxyl-terminal extension. In this regard, it is worth noting that within the Saccharomyces subphylum, *Yarrowia lipolytica* is an early-diverging lineage. This may also explain why this species also lacks *Ptc4*, which is common to other members of Saccharomyces.

(ii) Whereas *Ptc7* is present in all organisms analyzed, within the Saccharomyces, only *Yarrowia lipolytica* and the species included in the CTG clade contain *Ptc7*-related proteines. The latter are also missing in *Ustilago maydis* and *Schizosaccharomyces pombe*.

(iii) Whereas *Ptc6* is a family of proteins found exclusively in species included in the Saccharomyces lineage, *Ptc6*-related proteines are absent in this lineage.

(iv) As a conclusion, members of the *Ptc1*, *Ptc2/3*, *Ptc5*, and *Ptc7* families are present in all analyzed organisms.

**PP2C in Schizosaccharomyces pombe**. In 1994, Shiozaki and coworkers (90) isolated the *ptc1* gene (no. 2 in Fig. S1 in the supplemental material) as a multicopy suppressor of *swo1-26*, a temperature-sensitive mutation of a gene encoding a closely related homologue to the heat shock protein Hsp90. The *ptc1* gene product is a 40-kDa protein that shares 35% identity with *S. cerevisiae* *Ptc1*. Biochemical analyses showed that it displays Mg$^{2+}$-dependent phosphatase activity using casein as substrate. The *ptc1* mutant has a normal growth rate and conjugates and sporulates normally, although it is sensitive to elevated temperatures. Furthermore, *ptc1* mRNA levels increase 5- to 10-fold during heat shock (35°C), suggesting that *Ptc1* activity is important for survival under this condition (90).

The authors demonstrated that a strain lacking *ptc1* has nearly normal levels of PP2C activity, thus suggesting the presence of multiple PP2C genes in fission yeast. In fact, 1 year later the same group (91) isolated and characterized two additional PP2C genes from *S. pombe*: *ptc2* and *ptc3* (no. 25 and 24, respectively). Together, *Ptc1*, *Ptc2*, and *Ptc3* account for most of the PP2C activity (approximately 90%) in *S. pombe* cells. The *ptc2* and *ptc3* mutants are viable and no obvious phenotypes are observed upon deletion of these genes. However, a *ptc1 ptc3* double mutant displays aberrant cell morphology (an increased number of swollen and deformed cells) and tends to lyse at elevated temperatures, defects that are exacerbated by deletion of *ptc2*. Remarkably, these phenotypes are almost completely suppressed by addition of osmotic stabilizers such as sorbitol or inactivation of components of the stress-activated protein kinase (SAPK) pathway, such as *Wis1* or *Spcl/Styl*. *Wis1* is the MAPK kinase that phosphorylates Thr-171/Tyr-173 of the MAPK Spc1/Styl, whose orthologues in *S. cerevisiae* are *Pbs2* and *Hog1*, respectively (Fig. 2a). These results reinforced the notion that PP2Cs negatively regulate the *Wis1*-Spcl kinase cascade, possibly via direct dephosphorylation of *Wis1* and/or *Spcl* (91). Furthermore, Gaits and coworkers reported that the *ptc1 ptc3* double mutation shows a synthetic lethal phenotype with *pypl*. *Pypl* is a tyrosine phosphatase that dephosphorylates Tyr-173 of the Spcl kinase. Interestingly, lethality is rescued by the *wis1* mutation (23). Subsequently, it was shown that *Ptc1* and *Ptc3* (but probably not *Ptc2*) dephosphorylate Thr-171 in Spcl both *in vivo* and *in vitro* (65), thus reinforcing the role of PP2C activity in attenuating the heat shock-activated Spcl kinase. It should be noted, however, that Gaits and coworkers (23) presented several pieces of evidence indicating that PP2Cs also regulate events downstream from the MAPK Spc1 and/or additional MAPK signaling pathways.

Although *ptc1 ptc3* or *ptc1 ptc2 ptc3* mutants display no apparent significant defect in cell wall composition or structure (91), it has been reported that Ptc1 (but not Ptc2 or Ptc3) controls the activity of the MAPK Pmk1/Spm1 (56). Pmk1 is a structural homologue to Slt2 from *S. cerevisiae*, and it is involved in the maintenance of cell integrity, as well as regulation of morphogenesis, cytokinesis, and ion homeostasis. Pmk1 becomes phosphorylated and subsequently activated under multiple stresses, including cell wall-damaging compounds, hyper- or hypotonic conditions, glucose deprivation, and oxidative stress (Fig. 2a). Ptc1 copurifies with Pmk1 and dephosphorylates the active protein kinase both *in vivo* and *in vitro*. An additional link between type 2C phosphatases and the CWI pathway regulation was established when Takada and coworkers (23) presented that *Ptc1* and *Ptc3* modulate Pmk1 phosphorylation levels induced by the cell wall-damaging agent miacafungin (97). These authors also showed that Pmk1 regulates these PP2Cs at the transcriptional level through Atf1, a transcription factor downstream of the Spc1 MAPK pathway. From the data described so far, it is evident that despite the fact that budding and fission yeasts largely diverge in evolution, Ptc1 and/or *Ptc2/3* phosphatases have maintained an important role in the regulation of diverse MAPK signaling pathways in both organisms.

As mentioned above, the *S. pombe ptc1 ptc2 ptc3* mutant is viable and retains approximately 10% of the PP2C activity measured in extracts from wild-type cells (91). In an effort to identify new PP2C genes, Gaits and Russell performed a BLAST search on the portion of the *S. pombe* genome sequenced at that time, using as bait the sequences of *Ptc1*, *Ptc2*, and *Ptc3* (22). The analysis yielded a gene that was named *ptc4*, encoding a protein with an estimated molecular mass of 42 kDa that displays the classical Mg$^{2+}$-dependent phosphatase activity *in vitro*. It must be noted that this protein does not correspond to the *S. cerevisiae* *Ptc4* isoform, but it belongs to the Ptc6-related branch (no. 76 in Fig. S1 in the supplemental material). Cells lacking *ptc4* are viable but grow slowly on minimal medium and undergo premature growth arrest in response to nitrogen starvation. The phenotype of *ptc4* cells was reminiscent of the growth delay and sterility observed in the autophagy-defective mutants of *S. cerevisiae* (104). Interestingly, when grown in minimal medium, the *ptc4* strain displays a large number of highly fragmented vacuoles, closely related to the class B mutants (2). Furthermore, *ptc4* cells are unable to undergo vacuole fusion in response to hypotonic stress or nutrient starvation. Since vacuolar function is required for the process of autophagy, the vacuolar defect of *ptc4* mutants might explain why these cells undergo premature growth arrest in response to nitrogen starvation. It is remarkable that *Ptc4* localizes in vacuolar membranes, which suggests that this phosphatase regulates vacuole fusion by dephosphorylation of one or more proteins in the vacuole membrane. Conversely, *Ptc4* overexpression appears to induce vacuole fusion (22).

**PP2C in Candida albicans**. Relatively little is known regarding PP2C enzymes in the human opportunistic pathogenic yeast *Candida albicans*, and most available information has been accumulated in the last 2 years. The first evidence of *C. albicans* PP2Cs dates from the early 2000s, when Jiang and
coworkers (41) searched for potential homologous sequences from the public *Candida* genome database using the known *S. cerevisiae* PP2C genes as queries. According to their sequence similarities, they identified six sequences related to budding yeast PP2C: *C. albicans* PTC1 (CaPTC1), CaPTC2/3, CaPTC4, CaPTC5, CaPTC6, and CaPTC7. A seventh member, CaPTC8, has recently been added to the list (17).

The first *C. albicans* PP2C characterized in some detail was CaPtc7. Jiang and coworkers (41) demonstrated that CaPtc7 has the biochemical characteristics of classical PP2C enzymes (i.e., Mn²⁺- and Mg²⁺-dependent in vitro phosphatase activity blocked by NaF but not sensitive to okadaic acid). The N-terminal region of CaPtc7 contains a transmembrane domain and a potential mitochondrion-targeting signal that justifies its presence in this organelle (41, 107). Homologues of CaPtc7 can be found in *S. cerevisiae* (Fig. 1), *S. pombe*, *Caenorhabditis elegans*, and *A. thaliana*, indicating that this phosphatase is conserved in eukaryotes. In addition, the catalytic domain of CaPtc7 displays an overall 33% sequence identity with the Azr1 protein of *Schizosaccharomyces pombe* (41) (no. 144 in Fig. S1 in the supplemental material). The azr1⁺ gene was originally isolated as a multicopy suppressor of the 5-azacytidine sensitivity of G₂ checkpoint- and DNA repair-deficient *S. pombe* strains (69), but the phosphatase activity of Azr1 has never been demonstrated. The expression of the CaPTC7 gene is developmentally regulated at both the transcriptional and protein levels during serum-induced morphogenesis, suggesting that CaPtc7 might be an important regulator of morphogenesis of this organism. However, disruption of CaPTC7 does not affect vegetative growth or filamentous development in *C. albicans* (107). Therefore, the biological function of this phosphatase still remains unknown.

The CaPTC1 gene (no. 10 in Fig. S1 in the supplemental material) encodes a putative protein phosphatase (to our knowledge, the activity of the protein has never been determined) which shares 52% identity with the *S. cerevisiae* Ptc1 protein (34). The homozygous null mutant is viable, but, in contrast to what is known for the *S. cerevisiae ptc1* strain, it is not sensitive to calcofluor white, Congo red (indeed, it shows greater tolerance to Congo red than the wild-type strain), alkaline pH, or high temperature. On the other hand, it shows increased sensitivity to the echinocandin-derived antifungals caspofungin and micafungin, which interfere with the synthesis of glucan at the cell wall (34). Interestingly, CaPtc1 may contribute to the pathogenicity of *C. albicans*, since deletion of the CaPTC1 gene significantly reduces the virulence of this organism in both silkworm and mouse models of disseminated candidiasis (34).

CaPtc4 (no. 57 in Fig. S1 in the supplemental material) shares 31% sequence identity with *S. cerevisiae* Ptc4 (ScPtc4), and it has only recently been characterized (117). Bioinformatic analyses indicate that CaPtc4 has a potential myristoylation site at the N terminus which is conserved in its sequence homologues of other fungi (i.e., *S. cerevisiae*, *Candida dublinensis*, *Pichia stipitis*, and *Kluyveromyces lactis*). The purified PP2C-like catalytic domain of CaPtc4 exhibits a typical PP2C activity toward synthetic peptides phosphorylated on threonine residues (117). Although CaPtc4 lacks a mitochondrion-targeting sequence, Zhao and coworkers showed that a CaPtc4-green fluorescent protein (GFP) fusion is present mostly in this organelle. The homozygous *Captcha4* mutant displays sensitivity to sodium and potassium ions and to antifungal drugs such as fluconazole and ketoconazole (117).

In a recent report, it has been demonstrated that the catalytic domain of CaPtc6 (no. 70 in Fig. S1 in the supplemental material) displays phosphatase activity (115). The protein is located in mitochondria, in accordance with the presence of a mitochondrion-targeting sequence at the N terminus. Although the CaPtc6 mutant is not sensitive to rapamycin or caffeine, the introduction of CaPtc6 into the *S. cerevisiae* BY4743 ptc6/pbtc6 diploid strain significantly reverses the sensitivity to both compounds, suggesting that CaPtc6 could be a functional homologue of ScPtc6 (115).

A bioinformatic search in the public *C. albicans* database using the CaPtc7 protein sequence as a query revealed the existence of a protein that shows 38% sequence identity to CaPtc7. This protein contains an established PP2C domain that exhibits a low degree of identity (14 to 19%) to other members of the *C. albicans* PP2C family (17). The protein was therefore designated CaPtc8 (no. 121 in Fig. S1 in the supplemental material). As indicated by these authors, no evident sequence homologue of CaPtc8 exists in *S. cerevisiae*. According to our similarity analysis, CaPtc8 could be included in the Ptc7-related subfamily (see above). However, CaPTC8-like genes appear to be evolutionarily conserved, since homologues in *Arabidopsis thaliana*, *Oriza sativa*, or *Drosophila melanogaster* can be found (17). The transcription of CaPTC8 is induced by several stress conditions, such as high osmolarity, temperature, and serum stimulation, whereas deletion of CaPTC8 causes cells to be defective in hypha formation, indicating that CaPTC8 is required for the yeast-hypha transition (17).

The last member of the *C. albicans* PP2C family that has been characterized is the product of CaPTC2 (no. 37 in Fig. S1 in the supplemental material), whose phosphatase activity has been confirmed (18). CaPtc2 is predominantly cytosolic, although it is also associated with the mitochondria, and it has a potential myristoylation site located at the N terminus, suggesting a possible anchorage to membranes. The expression of CaPTC2 is repressed during the early steps of hypha formation and then induced in mature hyphae. Deletion of CaPTC2 yields *C. albicans* cells that are sensitive to azole antifungals such as fluconazole and ketoconazole and to the detergent SDS. A Captc2 mutant also displays hypersensitivity to the genotoxic stress-inducing agents methyl methanesulfonate and hydroxyurea, suggesting that CaPtc2 could share a functional role with *S. cerevisiae* Ptc2 and Ptc3. However, expression of ScPTC2 or ScPTC3 from its own promoter was not able to complement the hypersensitivity of the Captc2 mutant to the mentioned genotoxic drugs.

The product of the CaPTC5 gene, which contains a predicted transmembrane domain between residues 64 and 86, remains uncharacterized to date.

**PP2C in Fusarium graminearum.** *Fusarium graminearum* (also known as *Gibberella zeae*) is the major causal agent of *Fusarium* head blast disease on wheat and barley, which causes yield losses and affects the quality of grains. Using ScPtc1 as a query, Jiang et al. identified a single homologue sequence, encoded by a gene named *FgPTC1*, in the genome of *F. graminearum* (42). FgPtc1 (no. 16 in Fig. S1 in the supplemental
material) displays PP2C activity in vitro on a synthetic phosphopeptide, and deletion of FgPTC1 causes the mycelium of *F. graminearum* to become sensitive to 0.2 M LiCl but not to 1 M KCl or 1 M NaCl. These results are consistent with the role of *S. cerevisiae* PTC1 in lithium toxicity (78). In addition, FgPTC1 complemented the function of ScPTC1 in lithium toxicity as well as tolerance to Congo red, CaCl₂, and ZnCl₂, albeit not that to alkaline pH. Therefore, FgPtc1 can be considered a functional homologue of ScPtc1. Remarkably, deletion of FgPTC1 attenuates the virulence of *F. graminearum* on wheat, suggesting that FgPTC1 plays an important role in regulating the hyphal growth and virulence of *F. graminearum*.

**PP2C in Aspergillus nidulans.** Our knowledge about PP2C isoforms in *Aspergillus nidulans* is very scarce. Recently, Son and Osmani carried out a systematic gene deletion analysis of 28 identified phosphatase catalytic subunit-encoding genes (including six predicted PP2C-encoding genes) and showed that each single PP2C mutant is viable (93), although no further characterization was carried out. It must be noted that according to our analysis (see above), this organism does not contain two Ptc2 isoforms, as proposed by these authors. Instead, the reported Ptc2-Ppml1 (AN2472) could be classified as Ptc6 related (no. 83 in Fig. S1 in the supplemental material). In addition, our analysis has revealed an additional PP2C member in *A. nidulans* (AN0308.2), which can be classified as a Ptc7-related protein (no. 114).

**CONCLUDING REMARKS AND OUTLOOK**

In spite of the growing body of knowledge on PP2C enzymes, a large number of important questions remain open. From one side, it seems evident that common cellular targets can be identified for specific PP2C isoforms across different fungi. For instance, Ptc1 and Ptc2/3 isoforms are regulators of diverse MAPK signaling pathways in budding and fission yeasts, usually with deactivation of these pathways. This role seems to be conserved across evolution, as has been also documented for animals and plants. Similarly, a role for PP2C enzymes in cell cycle progression and response to DNA damage has been identified in both yeasts and mammals. On the other hand, a given PP2C isoform can exhibit distinctive cellular roles. For instance, a lack of Ptc1 in budding yeast results in a huge variety of phenotypes compared with those for other members of the PPF2C family. In this regard, Ptc1 poses an intriguing question: are most phenotypes secondary to the loss of a major primary function (as has been suggested for vacuolar malfunction), or do they reflect a large variety of different cellular targets?

A distinctive feature of PP2C catalytic polypeptides is that they are usually not associated with regulatory or targeting subunits. The interaction of Ptc1 with Nbp2 in budding yeast appears to be an exception rather than a rule. However, the recent identification of a family of proteins (RCAR/PYR) in the plant *Arabidopsis thaliana* that interact with and regulate several PP2C isoforms (36) may reopen this issue, although it must be noted that this specific family of plant regulatory subunits does not seem to be present in fungi. An additional relevant issue relates to the possible role of specific isoforms (such as Ptc1) in fungal virulence. Examples have been described here for *C. albicans* and *F. graminearum*. However, the molecular bases for this virulence are still unknown. Exploitation of hypothetical differences between fungal and mammalian or plant PP2C may help in the development of efficient antifungal drugs.

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