**Saccharomyces cerevisiae** Homologs of Mammalian B and B’ Subunits of Protein Phosphatase 2A Direct the Enzyme to Distinct Cellular Functions*

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Protein phosphatase 2A (PP2A) is a major cellular serine/threonine protein phosphatase, present in the cell in a variety of heterotrimeric forms that differ in their associated regulatory B-subunit. Cloning of the mammalian B’ subunit has allowed the identification of a highly homologous *Saccharomyces cerevisiae* gene, RTS1. Disruption of the gene results in a temperature-sensitive growth defect that can be suppressed by expression of rabbit B’α or B’γ isofoms. The B’α subunit is much more effective in restoring normal growth at 37 °C than B’γ. Immunoprecipitated Rts1p was found associated with type 2A-specific protein phosphatase activity that is sensitive to 2 nM okadaic acid, but not to 100 nM phosphatase inhibitor-2, and to be phosphorylated in *vivo*. However, overexpression of RTS1 was unable to suppress the cold sensitivity, defective cytokinesis, and abnormal cell morphology resulting from defects in the *CDC55* gene, which encodes the yeast homolog of a different B subunit of another form of 2A phosphatase, PP2Aβ. These results indicate that Rts1p is a yeast homolog of the mammalian B’ subunit and that the various regulatory B-subunits of PP2A are not functionally redundant but direct the enzyme to distinct cellular functions.

Serine/threonine protein phosphatases are ubiquitous enzymes that participate in many diverse biological processes. The enzymes are broadly classified as type 1 (PP1), type 2A (PP2A), 2B (PP2B), and 2C (PP2C), based on substrate specificity, catalytic properties, metal ion requirements, and sensitivity to various inhibitors (1–3).

The 2A protein phosphatases are heterotrimeric complexes consisting of a core dimer, composed of a catalytic (C2) and a regulatory (A) subunit, associated with one of the variable regulatory B-subunits (4–6). Two mammalian C2 (7–9) subunit isoforms have been identified which are highly conserved in the living kingdom. Three genes, *PPH21*, *PPH22*, and *PPH3*, encoding homologs of mammalian C2, have also been found in the budding yeast, *Saccharomyces cerevisiae* (10–12). Disruption of any one of these genes has no significant effect, whereas disruption of *PPH21* and *PPH22* causes a severe growth defect (12). However, the double mutants can survive, provided that the *PPH3* gene is intact (10, 11). The PP2A catalytic subunits have been shown to be essential for normal cell cycle progression in *S. cerevisiae* and for the organization of the actin cytoskeleton (13), as well as for the maintenance of normal cellular morphology (11). Other reports implicate the phosphatase in regulation of glycerogen metabolism in yeast (14). The regulatory A subunit has been identified in cells of diverse origin. Two isoforms of the A subunits have been characterized in mammalian cells (15) and one in Drosophila (16). In *S. cerevisiae*, the *TPD3* gene encodes a homolog of the mammalian subunit (17). Mutations in this gene greatly diminish cell growth at 14 and 37 °C. At the low temperature, cytokinesis appears to be affected, whereas at 37 °C, transcription by RNA polymerase III is impaired.

In contrast to the relative paucity of the A and C2 subunit isoforms, the B-subunits of PP2A show great diversity. Three families, B, B’, and B”, have been found associated with the PP2Aα, PP2Aβ, and PCSM, respectively (18–21). Three isoforms of B subunit have been identified in mammals (21–23), and cDNAs encoding some 13 isoforms of the B’ subunit have been isolated recently (24–26). Cloning of the cDNA encoding the B’ polypeptide suggests the existence of two alternatively spliced variants (27). Even though all of the B-subunits associate with the same dimeric C2A core, no obvious homology has been found among the B, B’, and B” forms. The abundance of the diverse B-subunit isoforms and their association with the relatively invariant set of C2 and A subunits has led to the suggestion that the B-subunits are responsible for targeting specific phosphatase 2A holoenzymes to distinct cellular locales and/or for conferring specificity toward appropriate substrates (4, 5, 28).

Recent cloning of the mammalian B’ subunits (24) allowed identification in GenBank of a highly homologous, 53–56% identical, yeast gene, RTS1 (accession number U06630), whose function is not clearly understood. This gene is unrelated to *CDC55*, the yeast homolog of the B subunit, whose disruption results in a cold-sensitive phenotype and morphologically aberrant cells (22). RTS1 was isolated independently by two laboratories, using different screening approaches. Evangelista et al. (29) isolated RTS1 as a multicopy suppressor of a ROX3
gene mutation. The ROX3 gene encodes an essential nuclear protein that functions in the global stress response pathway, controlling the level of CYC7 transcription. Shu and Hallberg (30) have isolated the same gene as a high copy suppressor of hsp60-ts mutant alleles. Disruption of RTS1 results in a temperature-sensitive phenotype and reduction of the mRNA levels of the mitochondrial chaperone Hsp60p, Cpn10p, and Mge1p at the restrictive temperature (30), whereas CYC7 mRNA levels were increased (29).

In this paper, we present evidence that RTS1 encodes the S. cerevisiae homolog of the mammalian B subunit of PP2A. The mammalian Bα and to a lesser extent the γ isoforms are able to suppress the temperature-sensitive phenotype associated with the RTS1 disruption. Moreover, Rts1p was found associated with type 2A-specific protein phosphatase activity and was phosphorylated in vivo. However, the RTS1 gene could not restore normal growth or morphology to a CDC55-disrupted strain. These observations indicate that S. cerevisiae, like mammalian cells, possesses multiple forms of B-subunit. Most importantly, our results demonstrate that the various B-subunits of PP2A are not functionally interchangeable but direct the enzyme to distinct cellular functions.

EXPERIMENTAL PROCEDURES

Strains—The following yeast strains were used: RZ53-6 (MATα ura3–52 trp1–289 ade1 leu2–3, 112; RZ33–6αrt1), identical to RZ53-6, except for the replacement of the wild-type RTS1 allele with the rts1–1::URA3 disruption (29), and AHY86 (MATα leu2–3, 112 ura3–52cdc55::URA3; 22). The cells were grown either in YPD (1% yeast extract, 2% Bacto-peptone, 2% glucose) or in synthetic complete (SC) medium (31) lacking the selective nutrient. Solid media contained 2% agar.

Plasmid Constructions—Oligonucleotide primers were synthesized by the Biochemistry Biotechnology Facility at Indiana University School of Medicine on an Applied Biosystems synthesizer model 394. Restriction endonucleases were obtained from New England Biolabs or from Life Technologies, Inc. All recombinant DNA manipulations followed standard procedures (32). Plasmid pDB20, containing the URA3-selective marker, was obtained from Kelly Tatchell (Louisiana State University, Shreveport). It was derived from the Escherichia coli plasmid pBR322 (33) by insertion of a 2-kb alcohol dehydrogenase (ADH1) promoter/terminator fragment (34) into the multiple cloning site of the vector. To construct the pDB21 vector with the LEU2-selective marker, the ADH1 promoter/terminator region was excised from pDB20 with EcoRI and PstI and ligated into the Yeplac181 vector (35). In both pDB20 and pDB21, the HindIII site was eliminated from the multiple cloning region such that a HindIII site between the ADH1 promoter and terminator was unique.

For expression in yeast, the complete Bα subunit coding sequence, tagged at the NH2 terminus with a hexahistidine sequence (His-tag) was excised with NcoI and BamHI as a 1.74-kb fragment from plasmid B′-αpET-15b (24), blunt-end, and subcloned into the blunt-ended unique HindIII site of pDB21 to yield pDB21(His-Bα). The complete Bγ subunit coding sequence was assembled by ligating the 1.481-bp AflII/EcoRI fragment from clone Br6-2 with the 533-bp EcoRI-PvuII fragment from clone Br6-2 (24) at the EcoRI site. The resulting 2,014-bp fragment was subcloned into the blunt-ended BamHI site of pTIZ18U (S. U. Biochemical Corp.) to yield pTIZ18U(Bγ). The hemagglutinin epitope tag YPY-DVPDYA (HA-tag) was introduced between the first and second codon of the open reading frame by polymerase chain reaction amplification of a 300-bp Nde1-HindIII fragment. The resulting fragment was ligated to the HindIII-SnaI 1,570-bp fragment of Bγ cDNA excised from pTIZ18U(Bγ). The 1,870-bp tagged Bγ DNA was blunt-end and inserted into the blunt-ended HindIII site of pDB21. This procedure yielded the plasmid pDB21(HA-Bγ).

Plasmids pDB20(HA-RTS1) and pDB21(HA-RTS1) were constructed by inserting the HA-tag/epitope tag between the first and second codon of the open reading frame of RTS1 using YEp195RTS1 (29) as template for polymerase chain reaction amplification of a 672-bp Nde1-SpeI fragment. This fragment was then ligated at the SpeI site of the remaining 2.07-kb SpeI-BamHI portion of RTS1 excised from YEp195RTS1. The resulting 2.75-kb fragment was subcloned into the Nde1-BamHI sites of the pET-15b vector (Novagen). The 2.45-kb Nde1-BstI B fragment obtained by partial digestion of pET15-b-RTS1 was then blunt-ended and subcloned into the blunt-ended HindIII site of pDB20 or pDB21.

Plasmids pDB20(HA-CDC55) and pDB21(HA-CDC55) were constructed as follows. The plasmid pTSV31(CDC55) containing the CDC55 gene on a 6-kb SacI-HpaI fragment (22) was used as template for PCR to generate a 550-bp fragment containing an HA-tag followed by the complete coding sequence and ~800 bp of 3′-untranslated sequence, was blunt-end and ligated into the filled-in HindIII site of pDB20 or pDB21. All polymerase chain reaction products were verified by dideoxynucleotide sequencing (36).

Western Blot Analyses—Yeast cells, transformed by the procedure of Ito et al. (37) or of Eble (38), were grown with vigorous shaking at 30 °C in liquid synthetic medium. The cultures were harvested at late log-}

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**Footnotes:**

- **5** Labeling of Yeast Cells—RZ53–6αrt1 cells transformed with the pDB21(HA-RTS1) were grown in liquid synthetic medium without leucine to a density of 7.5 × 10⁸ cells/ml. The cultures were then collected by centrifugation and suspended to a density of 6 × 10⁶ cells/ml in synthetic low phosphate medium (4). Two 25-ml cultures were incubated with shaking for 3 h at 30 °C to deplete intracellular phosphate pools, after which time one flask received 4 ml of 32P (DuPont NEN), and the incubation continued for 2 h. The labeled and unlabeled cells were processed as above for preparation of cell extracts and im.
Mammalian B’ subunit isoforms of PP2Aα rescue the mutant phenotypes of rts1. Wild-type (a–d) and rts1 mutant (e–h) cells were transformed with plasmids a and e, pDB21; b and f, pDB21(His-B’α); c and g, pDB21(HA-β’γ); d and h, pDB21(HA-RTS1). Transformants were grown on SC-leucine plates at 30 °C for 2 days, then replicated and grown for 2 additional days at 30 °C or 3 days at 37 °C.

The difference in the ability of the individual subunits to rescue the mutant strain became readily apparent when transformed cells were incubated at 37 °C (Fig. 1). Under these conditions, pDB21(His-B’α) and pDB21(HA-RTS1) restored normal growth to the mutant, whereas pDB21(HA-β’γ) was less effective. The approximate generation times at 37 °C for the cells transformed with vector only, B’α, B’γ, and RTS1 were 32, 9.0, 23, and 8.0 h, respectively, compared with 6.7 h for the parent strain. These results indicate that the cDNAs encoding the mammalian B’ subunits can functionally replace the yeast RTS1 gene even though the α and γ isoforms are not equally efficient. Analysis of cell morphology revealed that, unlike B’α, B’γ subunit expression resulted in abnormal multiple and elongated cells (Fig. 2), a phenotype somewhat reminiscent of that observed in cdc55 mutants (22).

The ability of the mammalian B’ cDNAs to rescue the temperature sensitivity of the yeast mutant was paralleled by the level of expression of the proteins. As shown in Fig. 3, B’α, B’γ, and Rts1p polypeptides could be detected by Western blotting of yeast extracts. Although the majority of each species was found in the insoluble fraction, a significant amount was present in the Triton X-100-soluble fraction. The apparent molecular weight of the proteins on SDS-PAGE, 55,000 for His-B’α and 90–95,000 for HA-Rts1p, deviates from the predicted 60,000 and 86,000, respectively, suggestive either of post-translational modifications or abnormal mobility caused by intrinsic properties of the polypeptides. The observation that B’α expressed in E. coli (24) migrates with an apparent molecular weight similar to that seen in yeast implies that the abnormal mobility of this isoform is probably not due to phosphorylation. Western blots of extracts prepared from rts1 mutants transformed with pDB21(HA-RTS1) revealed the presence of two or three discrete bands between 92 and 95 kDa (Fig. 3C and Fig. 4). When cell extracts were prepared in the presence of the serine/threonine phosphatase inhibitors NaF and potassium phosphate, an upward shift in the mobility of the lower band of the Rts1p was observed (Fig. 4A). Consistent with this initial observation, 32P labeling of yeast cells and immunoprecipitation of Rts1p showed that Rts1p is phosphorylated in vivo (Fig. 4C). Three bands were detected by immunoblotting, and all three were labeled with 32P. The slowest migrating species contained the highest ratio of 32P to protein, suggesting that it was the most heavily phosphorylated.

Rts1p Is Associated with PP2A Activity—Since the mammalian B’ subunit isoforms were able to rescue the rts1 phenotype, an important question was whether Rts1p was associated with PP2A activity. Measurements of phosphorylase phosphatase activity in immunoprecipitates from extracts of yeast expressing HA-Rts1p indicated the presence of phosphatase activity that was insensitive to 100 mM inhibitor-2, a protein phosphatase 1-specific inhibitor, but almost completely inhibited by...
FIG. 3. Expression of B′-subunit proteins in rts1 mutant. Extracts were prepared as described under “Experimental Procedures” from RZ53–6Δrts1 cells transformed with pDB21(His-B′α) (panel A), pDB21(HA-B′γ) (panel B), or pDB21(HA-RTS1) (panel C). Fifty μg of protein of the soluble cellular fraction (S – T), the corresponding volume of the Triton-soluble (S + T) and the insoluble fraction (P) were separated on a 9% SDS-polyacrylamide gel (panels A and B) or 6% SDS-polyacrylamide gel (panel C) and subjected to Western blotting. In panel A the probe was affinity-purified anti-B′α antibody, and 100 ng of recombinant B′α protein was included as a positive control. In panels B and C the probe was the12CA5 monoclonal antibody. Note the presence of two Rts1p bands in panel C. Extracts from cells transformed with pDB21 vector served as negative controls for each subunit. A protein band of ~48 kDa was observed in all cell extracts with the 12CA5 antibody. The nature of this cross-reacting species is unknown. The migration of molecular mass markers, expressed in kDa, is indicated to the right of each panel.

FIG. 4. Electrophoretic mobility of Rts1p and in vivo 32P labeling. Panel A, extracts from an rts1 mutant transformed with pDB21 vector or pDB21(HA-RTS1) were prepared in the absence (−) or the presence (+) of phosphatase inhibitors (50 mM NaF and 10 mM potassium phosphate) as described under “Experimental Procedures,” resolved by 6% SDS-PAGE, and analyzed by Western blotting. The blots were probed with 12CA5 antibody followed by autoradiography for 5, 18, and 72 h. The migration of glycogen phosphorylase (94 kDa) is indicated on the right. Panels B and C, yeast cell were 32P labeled, and Ha-tagged Rts1p was immunoprecipitated as described under “Experimental Procedures.” The immunoprecipitates from unlabelled and labelled cells were subjected to Western analysis. The immune complexes were detected colorimetrically (panel B), and the polystyrene diffusor membrane was subjected to autoradiography (panel C). Lanes 1 and 2, 12 μl of immunoprecipitate from 32P-labeled and unlabelled samples, respectively.

2 mM okadaic acid (Table I), a concentration that preferentially inhibits PP2A. Analysis by immunoblotting indicated that the monoclonal antibody 12CA5 almost quantitatively immunoprecipitated the HA-Rts1p (Fig. 5). Determination of phosphatase activity in extracts from rts1 mutant cells indicated that ~30% of the activity could be inhibited by 2 mM okadaic acid (data not shown). However, we do not know what proportion of this activity can associate with the yeast B′ subunit homolog.

Rts1p Expressed in cdc55 Strains Does Not Suppress the Mutant Phenotype—Cdc55p is highly homologous to the mammalian B subunit of PP2A, (22). Disruption of CDC55 resulted in a cold-sensitive growth phenotype and abnormal multiple elongated cells at the restrictive temperature. Since the various B-subunits of PP2A associate with the same C2 complex, they must be involved in 

Attempts to transform RZ53–6 and rts1 mutant strains with the pDB21(HA-CDC55) plasmid did not yield transformants. These strains would not tolerate the presence of a high copy CDC55 plasmid, suggesting that high level of expression of Cdc55p might cause lethality in these strains. However, we do not believe this to be the case. More likely, the inability to transform these strains with pDB21(HA-CDC55) is due to the presence of the ade1 allele. Other yeast strains, such as F808 and DBY745, which harbor the same ade1 allele, could not be transformed by the multicy copy CDC55 plasmid. In contrast, two unrelated strains that did not carry the ade1 allele, YNN27 and JC482, generated large numbers of transformed colonies with pDB21(HA-CDC55). Thus, it appears as though the ade1 mutation and the high copy CDC55 plasmid are unable to coexist for reasons that at this time are not understood. Perhaps high levels of Cdc55p and accumulation of the adenine precursor, phosphoribosylaminoimidazole carboxylate, combine to have a toxic effect on the cell.

The expression of Cdc55p and Rts1p in the cdc55 disruption mutant transformed with pDB20(HA-CDC55) or pDB20(HA-RTS1) was then investigated. Western blot analysis revealed that both HA-Cdc55p and HA-Rts1p were expressed in comparable amounts (Fig. 6). Cells overexpressing Cdc55p or Rts1p were grown at the permissive 30 °C temperature and then transferred to 14 °C. After 3 days, the cells were examined microscopically. The results demonstrated that although pDB20(HA-CDC55) restored normal morphology to the mutant cells (Fig. 7), pDB20(HA-RTS1) had no effect on the cdc55 phenotype. Therefore, even though both Rts1p and Cdc55p associate with the same C2-A complex, they must be involved in
The yeast homolog of the B subunit of PP2A0, Rts1p, and the other mammalian B subunit isoforms are able to rescue the temperature-sensitive growth defect of the \( \text{cdc55} \) deletion strain (Fig. 1). Third, Rts1p coimmunoprecipitates with protein phosphatase activity that is insensitive to okadaic acid (Fig. 5 and Table I) at a concentration of 2 nM ineffective against PP1. The presence of at least three phosphorylated species of the protein to the nucleus (49, 50). Indeed, recently it has been reported (46) that the mammalian B'γ, but not B’α, polyepitope contains in its COOH-terminal region a putative bipartite nuclear localization signal that could direct the protein to the nucleus (49, 50). The potential nuclear localization of the B'γ, which would increase PP2A activity in the nucleus, may also be responsible for the abnormal morphology of the cells overexpressing the protein (Fig. 2), either by affecting nuclear functions or by redistributing other pools of the enzyme.

A clearer functional distinction exists between Cdc55p, the B subunit isoforms may perform somewhat different cellular roles. The two isoforms are 70% homologous; and interestingly the B'γ, but not the B’α, polyepitope contains in its COOH-terminal region a putative bipartite nuclear localization signal that could direct the protein to the nucleus (49, 50). The potential nuclear localization of the B'γ, which would increase PP2A activity in the nucleus, may also be responsible for the abnormal morphology of the cells overexpressing the protein (Fig. 2), either by affecting nuclear functions or by depleting other pools of the enzyme.

A clearer functional distinction exists between Cdc55p, the B subunit isoforms are able to rescue the temperature-sensitive growth defect of the \( \text{cdc55} \) mutant at both 30 and 37 °C. B’α restores growth completely, up to the wild-type level, whereas B’γ only provides partial complementation. It is unlikely that this is the result of a different degree of expression of B’γ protein, since Western analysis indicated that the polypeptide is present at a level comparable to that of Rts1p (Fig. 3). These results suggest that the two mammalian B' subunit isoforms may perform somewhat different cellular roles. The two isoforms are 70% homologous; and interestingly the B'γ, but not the B’α, polyepitope contains in its COOH-terminal region a putative bipartite nuclear localization signal that could direct the protein to the nucleus (49, 50). The potential nuclear localization of the B'γ, which would increase PP2A activity in the nucleus, may also be responsible for the abnormal morphology of the cells overexpressing the protein (Fig. 2), either by affecting nuclear functions or by depleting other pools of the enzyme.
subunit of PP2Aα, and Rts1p. Overexpression of Rts1p cannot suppress the cold-sensitive phenotype of a cdc55 mutant. The cells grown at 14 °C remain defective in septation and cytokinesis (Fig. 7). Therefore, in keeping with the idea of spatial or functional targeting, the B- and C subunits of PP2A must channel the same phosphatase toward different cellular substrates or locales. Furthermore, the somewhat slower growth observed in wild-type R253–6 cells overexpressing RTS1 may be suggestive of enrichment of the PP2A holoenzyme containing Rts1p at the expense of other essential pools.

Both the mammalian and yeast PP2A may be involved in cell cycle control. Carboxyl methylation of the catalytic subunit of PP2A has been reported to correlate with cell cycle progression (51), and the activity of a microtubule-associated PP2A fluctuates as the cells traverse the cell cycle (28). The B subunit of Drosophila PP2A was shown to be required for correct chromatid migration in the anaphase (52). Mutations in both the yeast catalytic subunit gene (13) and B subunit gene (22) result in cell cycle defects, although the two display dissimilar morphological aberrations: multiple elongated buds in the cdc55 mutant at the restrictive temperature and small, deformed buds in the pph21 mutant. The rts1 strain cells at either 30 or 37 °C appear to be significantly enlarged, again indicating a distinct function of the B subunit.

Mutations in the CDC55 and RTS1 genes result in phenotypes similar to those displayed by A subunit defects. Mutations in the yeast TPD3 gene, encoding the regulatory A subunit of PP2A, result in both cold- and temperature-sensitive phenotypes (17). At 13 °C, the cells resemble cdc55 mutants in that they show elongated buds, consistent with defective cytokinesis. The same mutation does not allow growth at 37 °C, a phenotype analogous to that of the rts1 mutant. The ability of one mutation to confer a dual phenotype is consistent with the regulatory A subunit being a component of different phosphatase holoenzymes containing either the Cdc55p or the Rts1p.

Several lines of evidence point toward an involvement of RTS1 in modulating cellular responses to stress conditions. First, overexpression of RTS1 suppresses a roX3 temperature mutation (29) and several hsp60-ts alleles (30), both genes known to respond to stress. Hsp60 resides in mitochondria, and RoX3p is localized in the nucleus but affects the level of expression of a mitochondrially located protein, iso-2-cytchrome c. The fact that RTS1 is required for expression of the mitochondrial chaperonins Cpn10p and Mge1p (30), which are defective in the hsp60-ts strains, suggests that this protein phosphatase subunit plays a role in regulating the expression of genes whose products are destined for mitochondria and/or involved in stress responses. Supporting this notion is the observation that, although RTS1 is not an essential gene, its disruption not only impairs growth at elevated temperature but also decreases utilization of nonfermentable carbon sources, a feature consistent with impaired mitochondrial function.

Second, disruption of the RTS1 gene itself confers temperature sensitivity and results in elevated expression of the CYC7 gene in response to osmotic stress and heat shock (29). These responses may occur through a common stress response element (53). The osmotic stress response is mediated, at least in part, by the activation of the Hog1p protein kinase pathway (54–56). Thus, we infer that the protein kinases and PP2Aα may share common substrates or that they may be targets for each other. Other protein phosphatases, PTP2 and PP2C, have also been implicated in the control of the osmosensing mitogen-activated protein kinase pathways in S. cerevisiae (56) and in Schizosaccharomyces pombe (57).

In conclusion, the present study demonstrates that the RTS1 gene encodes the S. cerevisiae homolog of the mammalian B subunits of PP2Aα, and that the Rts1p is phosphorylated in vivo. Mammalian Bα and, to a lesser extent Bγ, are able to complement the temperature-sensitive defect of rts1, but RTS1 cannot replace CDC55, the yeast homolog of the B subunit of PP2A. Thus, the B subunits are not redundant but confer functional specificity to the various forms of PP2A.
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