

A highly specific phosphatase that acts on ADP-ribose 1''-phosphate, a metabolite of tRNA splicing in *Saccharomyces cerevisiae*

Neil P. Shull, Sherry L. Spinelli and Eric M. Phizicky*

Department of Biochemistry and Biophysics, University of Rochester School of Medicine, 601 Elmwood Avenue, Rochester, NY 14642, USA

Received December 6, 2004; Revised and Accepted January 7, 2005

ABSTRACT

One molecule of ADP-ribose 1'',2''-cyclic phosphate (Appr>p) is formed during each of the approximately 500 000 tRNA splicing events per *Saccharomyces cerevisiae* generation. The metabolism of Appr>p remains poorly defined. A cyclic phosphodiesterase (Cpd1p) has been shown to convert Appr>p to ADP-ribose-1''-phosphate (Appr1p). We used a biochemical genomics approach to identify two yeast phosphatases that can convert Appr1p to ADP-ribose: the product of ORF YBR022w (now *Poa1p*), which is completely unrelated to other known phosphatases; and *Hal2p*, a known 3'-phosphatase of 5',3'-pAp. *Poa1p* is highly specific for Appr1p, and thus likely acts on this molecule *in vivo*. *Poa1* has a relatively low K_M for Appr1p (2.8 μM) and a modest k_{cat} (1.7 min^{-1}), but no detectable activity on several other substrates. Furthermore, *Poa1p* is strongly inhibited by ADP-ribose (K_i , 17 μM), modestly inhibited by other nucleotides containing an ADP-ribose moiety and not inhibited at all by other tested molecules. In contrast, *Hal2p* is much more active on pAp than on Appr1p, and several other tested molecules were *Hal2p* substrates or inhibitors. *poa1*- Δ mutants have no obvious growth defect at different temperatures in rich media, and analysis of yeast extracts suggests that ~90% of Appr1p processing activity originates from *Poa1p*.

INTRODUCTION

tRNA splicing in *Saccharomyces cerevisiae* occurs in three essential steps and produces the by-product adenosine diphosphate ribose 1'',2''-cyclic phosphate (Appr>p). In the first step

of splicing, tRNA endonuclease recognizes the pre-tRNA and removes its intron, leaving a 5' tRNA half-molecule with a 2',3' cyclic phosphate on its 3' end and a 3' half-molecule with a hydroxyl on its 5' end (1–3). tRNA ligase opens the 2',3' cyclic phosphate to a 2'-phosphate, phosphorylates the hydroxyl on the 3' half-molecule and joins the two half-molecules to leave a mature length tRNA with a 2'-phosphate at the splice junction (4–6). This 2'-phosphate is removed by tRNA 2'-phosphotransferase in two distinguishable steps: ADP-ribosylation of the 2'-phosphate with the cofactor NAD, followed by resolution of the intermediate to form mature length spliced tRNA and Appr>p (7–9). Hence, the cyclic phosphate of Appr>p originates as a backbone phosphate of the pre-tRNA, and Appr>p is produced in equimolar yield with spliced tRNA.

Determination of the pathway by which Appr>p is metabolized is of interest for two reasons. First, Appr>p is produced in appreciable quantities. Based on the number of tRNA molecules made per generation (10), and the fraction of tRNA genes with introns (11,12), it is estimated that ~500 000 molecules of tRNA are spliced per generation. In the absence of a metabolic breakdown pathway, Appr>p could reach a concentration of 10–40 μM in the cell. Second, Appr>p could act as a regulatory molecule to signal the status of tRNA splicing in the cell, since it is produced every time a tRNA is spliced, and tRNA splicing is likely the only significant source of the molecule (8,13). Appr>p may also be produced during *HAC1* mRNA splicing in the unfolded protein response, since tRNA ligase is involved in the joining step (14–16), and the resulting 2'-phosphate would likely have to be removed by Tpt1p. However, if so, the amount of Appr>p produced by *HAC1* splicing would be negligible compared with that produced by tRNA splicing, based on the number of *HAC1* mRNA molecules likely to be made (17,18).

A likely first step in Appr>p metabolism in yeast extracts is the conversion of Appr>p to adenosine diphosphate ribose-1''-phosphate (Appr1p) by Cpd1p (19). A highly specific cyclic phosphodiesterase was originally identified in yeast extracts

*To whom correspondence should be addressed. Tel: +1 585 275 7268; Fax: +1 585 271 2683; Email: Eric_Phizicky@urmc.rochester.edu

(19). Subsequently, this activity was attributed to *CPD1* (20,21), Cpd1p was shown to encode cyclic phosphodiesterase (21), and extracts from *cpd1-Δ* yeast strains were shown to have no detectable CPDase activity (21). These results suggest that Cpd1p acts on Appr>p *in vivo* to produce Appr1p, although there is no evidence that explicitly addresses this role.

To determine the next step in Appr>p metabolism, we extended a previous search of a yeast genomic library of purified glutathione *S*-transferase–open reading frame (GST–ORF) fusion proteins to find proteins that act on Appr1p (20). We report here that ORF YBR022w (now named *POA1*) and *HAL2* each encode phosphatases that can convert Appr1p to ADP-ribose *in vitro*. Poa1p is unusual because it is substantially more specific and slower than most phosphatases that act on small molecules; of a number of substrates tested, only Appr1p is appreciably dephosphorylated by Poa1p. Hal2p was previously described as a 3'-phosphatase acting on pAp produced during methionine biosynthesis in yeast (22). Although Hal2p prefers pAp as a substrate to other common nucleotides (22), Hal2p has appreciable activity on Appr1p in our *in vitro* studies. Although either protein could catalyze a phosphatase reaction after Cpd1p converts Appr>p to Appr1p, our evidence suggests that Poa1p is responsible for ~90% of Appr1p processing activity in yeast extracts.

MATERIALS AND METHODS

Yeast and *Escherichia coli* strains, plasmids and growth

Strains NSY32 (*hal2::kan*, clone ID#1756) and NSY34 (*poa1::kan*, clone ID#3159) are derivatives of strain NSY60 (BY4741, *MATa*, *his3-Δ1*, *leu2-Δ0*, *ura3-Δ0*, *met15-Δ0*, *lys2-Δ0*), and were purchased from Open Biosystems. The *kan* marker in NSY34 was exchanged for a *nat* marker by transformation of EcoRI cut p4339 containing the *nat* marker into NSY34 as described previously (23) to form NSY49 (*poa1::nat*). The *poa1*, *hal2* double mutants were made by PCR amplification of the *hal2::kan* region of NSY32 with primers 5'-GACGCACCAATGTATGGTGCAG-3' and 5'-CGATTGCTTTCGAATTTTCAACGGG-3', and transformation of the PCR product into NSY49 to form NSY58 (*MATa*, *hal2::kan*, *poa1::nat*).

Both *POA1* and *HAL2* were expressed in *E. coli* as N-terminal His₆-ORF fusion proteins under control of the *P_{lac}*-T7 polymerase. *POA1* was PCR amplified from yeast genomic DNA using primers 5'-GAACTCGAGGAATTCATGTCTAACATCACTTAT-3' and 5'-GAACTCGAGGAATTCTTACAGCTGATATACTG-3', XhoI treated, ligated into the vector pET15b (Novagen) and transformed to create strain NPS1-14. *HAL2* was PCR amplified from yeast genomic DNA using primers 5'-GAACTCGAGCATATGGCATTGGAAAGAGAATTA-3' and 5'-GAACTCGAGTTAGGCGTTTCTTGACTGAAT-3', treated with NdeI and XhoI, ligated into pET15b and transformed to create strain NPS51-2. Plasmids containing sequenced ORFs were transformed into *E. coli* BL21(DE3) pLysS cells, grown at 37°C in 500 ml cultures of Luria–Bertani (LB) containing 100 μg/ml ampicillin to an OD₆₀₀ = 0.4, induced with 1 mM isopropyl

β-D-thiogalactopyranoside for 30 min at 37°C, harvested, quick frozen in dry ice and stored at –70°C.

Purification of proteins expressed in *E. coli*

Purification of His₆-Poa1p and His₆-Hal2p was performed as described previously (24), using TALON resin (Clontech) for immobilized metal-ion affinity chromatography (IMAC), except that purified proteins were dialyzed into buffer containing 20 mM Tris–HCl, pH 7.5, 4 mM MgCl₂, 55 mM NaCl, 1 mM DTT and 50% glycerol.

Preparation of substrates

NAD labeled with ³²P on the phosphate adjacent to the adenine (Ap*_pN) was prepared based on the method of Culver *et al.* (19). An aliquot of 40 μCi [α³²-P]ATP (3000 Ci/mmol, Amersham Biosciences) and 1 mM NMN (Sigma) were treated with partially purified His₆-Nma1p (encoding yeast NMN pyrophosphorylase, prepared from *E. coli* strain NPS52-3) in a 20 μl reaction mixture containing 100 mM Tris–HCl, pH 7.5 and 10 mM MgCl₂. Reactions were incubated overnight at 30°C, and the product Ap*_pN was separated from other reactants on silica TLC plates developed in solvent containing 100% EtOH/1 M NH₄OAc, pH 7.2 (7:3 v/v), eluted in ddH₂O and dried to completion.

Ap*_{pr}1p was prepared from Ap*_pN, using Tpt1p to convert labeled NAD to Ap*_{pr}>p and Cpd1p to open the cyclic phosphate and generate Ap*_{pr}1p. Reaction mixtures of 10 μl contained 5 × 10⁶ c.p.m. Ap*_pN, 100 μM unlabeled NAD (Sigma), 100 μM Tpt1p trimer substrate A_pA_pA_p and 0.03 μg His₆-Tpt1p (derived from strain EMP1199) in buffer containing 20 mM Tris–HCl, pH 7.5, 5 mM MgCl₂, 2.5 mM spermidine, 100 μM DTT and 0.4% Triton X-100, and were incubated for 60 min at 30°C. Then, 0.04 μg His₆-Cpd1p (from strain NPS9-12) was added for 90 min to convert Ap*_{pr}>p to Ap*_{pr}1p, products were separated on silica TLC plates developed in solvent containing 1-propanol/NH₄OH/ddH₂O(55:35:10, v/v/v), and Ap*_{pr}1p was eluted in water and filtered with a Spin-X centrifuge tube filter (Corning Incorporated).

Unlabeled Appr1p was prepared by a preparative reaction of chemically synthesized Appr>p [a gift from W. Filipowicz, Friedrich-Meiescher-Institute, Basel, Switzerland (25)] with 2 μg His₆-Cpd1p under the conditions described above for 4–8 h. Products were isolated on silica TLC plates developed in solvent containing 100% EtOH/1 M NH₄OAc, pH 7.2 (7:5, v/v), excised, eluted in water, filtered and quantified based on A₂₆₀.

Radioactively labeled p*_pAp was made from 3' AMP and polynucleotide kinase as described previously (26), followed by separation on silica TLC plates developed in solvent containing 1-propanol/NH₄OH/ddH₂O (55:35:10, v/v/v). Unlabeled pAp was purchased from Sigma.

Labeled ribose-1-p* was made by incubation of 10 mM inosine and 100 μCi [³²P]H₃PO₄ with 0.75 U nucleoside phosphorylase (Sigma) in a 10 μl reaction mixture containing 10 mM Tris–HCl, pH 7.5. After 20 min at 37°C, products were isolated on polyethyleneimine (PEI)–cellulose TLC plates developed in 0.75 M LiCl. Product spots were excised and eluted in 0.5 M NH₄HCO₃, pH 8.0 for 15 min at room temperature, dried to completion and resuspended in water.

Assay for Appr1p phosphatase and pAp phosphatase activities

Appr1p phosphatase assays were performed at 30°C in reaction mixtures containing crude extract, GST-ORF fusion proteins or purified Poa1p and Hal2p as indicated, in buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 2.5 mM spermidine, 100 μM DTT, 0.4% Triton X-100, 5–25 nM Ap*pr1p and excess unlabeled Appr1p to adjust substrate concentration as noted. Reaction products were separated on PEI-cellulose TLC plates developed with either 1 or 2 M sodium formate, pH 3.5, and quantified by PhosphorImager (MolecularDynamics). Initial rates of Appr1p hydrolysis by Poa1p (21 nM) or Hal2p (173 nM) were determined in time courses that led to <15% conversion of substrate to product. Steady-state kinetic parameters were obtained by fitting the resulting initial velocities to the Michaelis-Menten equation using KaleidaGraph software version 3.0. The kinetic parameters for pAp phosphatase activity by Hal2p were determined in an analogous way using trace amounts of labeled p*Ap with increasing amounts of unlabeled pAp (0–1500 nM). Hal2p was added at a concentration of 26 fM.

Inhibition reactions

All Appr1p phosphatase inhibition assays were performed in the presence of 34 nM Poa1p or 104 nM Hal2p, 5 or 5000 nM Appr1p and 10 mM of potential inhibitor, for a period of time that converts ~50% of substrate to product under non-inhibited conditions. IC₅₀ values were obtained by titrating the inhibitor concentration under the same conditions.

K_I for ADP-ribose

A K_I for ADP-ribose was determined by the method of Dixon (27). Initial rates of Appr1p hydrolysis were determined using the previously described Ap*pr1p assay, with 43 nM Poa1p and varied concentrations of Appr1p (1–25 μM) and ADP-ribose (0–300 μM). The inverse of the initial rates determined at each different fixed concentration of substrate was plotted as a function of [ADP-ribose]. The resulting slopes are linearly dependent on 1/[Appr1p]; the K_I for ADP-ribose was determined from the slope of this second plot since slope = K_M/(V_{max}K_I), and K_M and V_{max} are known for Appr1p. All fits were performed by linear regression with KaleidaGraph software.

Determination of pH maximum for Poa1p

Time-course reactions were performed in reaction mixtures containing 25 nM Ap*pr1p and 5 nM Poa1p in buffer containing 5 mM MgCl₂, 2.5 mM spermidine, 100 μM DTT and 0.4% Triton X-100, as well as 20 mM of one of the following buffers: NaOAc, pH 4.0, 4.5, 4.7 and 5.0; Homo-PIPES, pH 4.7 and 5.0; MES, pH 5.4, 5.8, 6.0 and 6.2; Bis-Tris, pH 6.0, 6.6 and 7.0; HEPES, pH 7.0 and 7.5; and Tris-HCl, pH 7.5 and 8.0. Several time-course reactions were performed at the same pH in two separate buffers to rule out buffer specific effects. Rates were plotted and pK_as were fit to the curve using KaleidaGraph software using the equation $(v/E)_{\text{obs}} = [(v/E)_{\text{low pH}} + (v/E)_{\text{max}} 10^{(\text{pH}-\text{pK}_{\text{a1}})}] / [1 + 10^{(\text{pH}-\text{pK}_{\text{a1}})} + 10^{(\text{pK}_{\text{a2}}-\text{pH})}]$.

Test of other substrates at pH 6.0

Potential substrates of Poa1p were assayed at pH 6.0 by incubation of 5.3 μM Poa1p (or an equivalent amount of a mock purification) with test substrates for 16–24 h at 30°C. Ribose-1-p* was tested at 10 nM or 3 μM, and phosphate product was resolved from reactant on PEI-cellulose TLC plates developed in 1 M sodium formate, pH 3.5. All other substrates were tested at 1 mM, and phosphate product was measured using a malachite green assay (28).

Assay of yeast crude extracts

Yeast crude extracts were made as described with glass beads (29) from cells that were grown to A₆₀₀ of 1.0, harvested, washed in cold ddH₂O and stored at –70°C, and then assayed for Ap*pr1p processing activity for 1 h at 30°C with 17 nM Ap*pr1p, and 5-fold serial dilutions of extract, starting with 16 μg wild-type extract, 29 μg *poa1::nat* extract, 23 μg *hal2::kan* extract or 21 μg *poa1::nat*, *hal2::kan* extract.

RESULTS

Identification of two phosphatases with activity on Appr1p

A previous survey of a genomic collection of purified *S.cerevisiae* GST-ORF fusion proteins indicated that ORF YBR022w co-purifies with an Appr1p processing activity (20). This collection is derived from a library of 6144 yeast strains, each expressing a unique GST-ORF fusion protein (30). Assignment of Appr1p processing activity to this ORF involved purification of 64 pools of GST-ORF fusion proteins, each derived from 96 strains, assays of the pools and deconvolution of the active pool 6 to show that ORF YBR022w is associated with the activity.

Another screen of this same library, using Appr1p labeled with ³²P on the phosphate adjacent to the adenosine (denoted Ap*pr1p, see Materials and Methods), and longer incubation times for increased sensitivity, revealed pool 6, as expected, as well as pool 49, as having apparently identical activity on Appr1p (Figure 1A). In each case, it appears that three products are formed during the reaction, labeled I, II and III. Deconvolution of pool 49 (Figure 1B) shows that Appr1p processing activity co-purifies with the previously described pAp phosphatase, Hal2p (Met22p).

The products of ORF YBR022w and Hal2p could each have catalytic activity, could each be associated with separate activities or could both be part of the same complex. To examine the activity of the proteins separately from other yeast components, each protein was expressed in *E.coli* and purified. As shown in Figure 2A, crude extracts from cells expressing each protein produce a prominent polypeptide of the expected size (27 kDa for the product of ORF YBR022w and 45 kDa for Hal2p) that is absent in control extracts (cf. lanes a and b and lanes e and f). Each protein was then purified by immobilized metal-ion chromatography (see Materials and Methods), resulting in substantially purified preparations, as determined by SDS-PAGE analysis (Figure 2A, lanes c, d, g and h). Examination of Appr1p processing activity demonstrates that YBR022w protein and Hal2p have similar activities, each producing substantial amounts of products I and II,

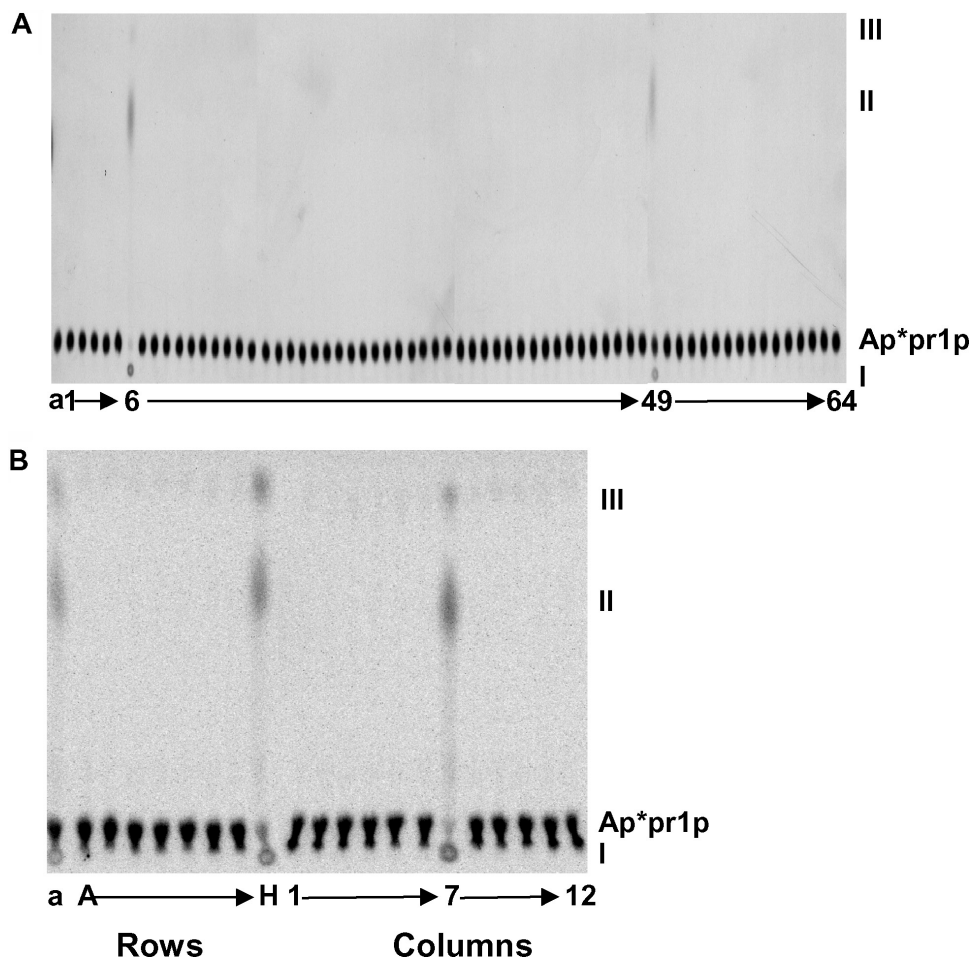


Figure 1. Identification of two yeast ORFs associated with activity on Appr1p. (A) Assay of a genomic collection of pools of purified yeast GST-ORF fusion proteins. Pools of GST-ORF fusion proteins were assayed for Appr1p processing activity at 30°C for 4 h with 40 nM Ap*pr1p and products were resolved on PEI-cellulose TLC plates developed in 1 M sodium formate. a, buffer control. Products I, II and III are indicated. (B) Assay of sub-pools of plate 49 for activity on Appr1p. Sub-pools of GST-ORF fusion proteins from rows A–H and columns 1–12 of plate 49 were assayed with 3 nM Ap*pr1p at 30°C for 1 h, as indicated, and resolved as in (A). a, GST-ORF pool of plate 49.

but much less of product III, which co-migrates with AMP (see Figure 2B, lanes c–g and lanes h–l).

Since Hal2p is a known phosphatase acting on the 3'-phosphate of pAp, it seems likely that both YBR022w protein and Hal2p are phosphatases acting on the 1''-phosphate of Appr1p, although the appearance of two products from each protein was unexpected. To further characterize the products observed upon reaction with these proteins, we compared the activities of YBR022w protein and Hal2p with that of calf intestinal phosphatase (CIP). As shown in Figure 2B, incubation of Appr1p with CIP yields the same characteristic two products I and II as YBR022w protein and Hal2p, both of which co-migrate with ADP-ribose from a commercially obtained standard (cf. lanes c–g and h–l with lanes m–q). We have confirmed that CIP and YBR022w protein produce the same products from Appr1p with several other TLC systems, although in most of these systems ADP-ribose migrates as a single spot (data not shown). This result demonstrates that YBR022w protein and Hal2p are phosphatases that remove the 1''-phosphate from Appr1p, and therefore we assigned the name *POA1* to ORF YBR022w (phosphatase of ADP-ribose 1''-p).

A kinetic characterization of Poa1p and Hal2p reveals differing catalytic efficiencies for Appr1p

To evaluate the possible contribution of each protein to Appr1p metabolism, we compared the steady-state kinetic parameters of Poa1p and Hal2p with Appr1p and with pAp, the previously known substrate of Hal2p. We used labeled Appr1p and pAp to monitor phosphatase activity and measured the reaction progress as a function of increasing concentration of either substrate. Both spots that have been identified as ADP-ribose (I and II) were summed to equal the total amount of products in each reaction and all reactions were linear over the time measured. Some representative time courses for the hydrolysis of Appr1p with Poa1p are shown in Figure 3A. The resulting initial rates were plotted as a function of substrate concentration and were fit to the Michaelis–Menten equation to yield the steady-state k_{cat} and K_M for each enzyme and substrate combination (Figure 3B; data not shown). The data were well described by this equation and are summarized in Table 1.

The results of the kinetic characterizations indicate that Poa1p is a more efficient enzyme for hydrolysis of Appr1p than Hal2p, although both act relatively slowly on this

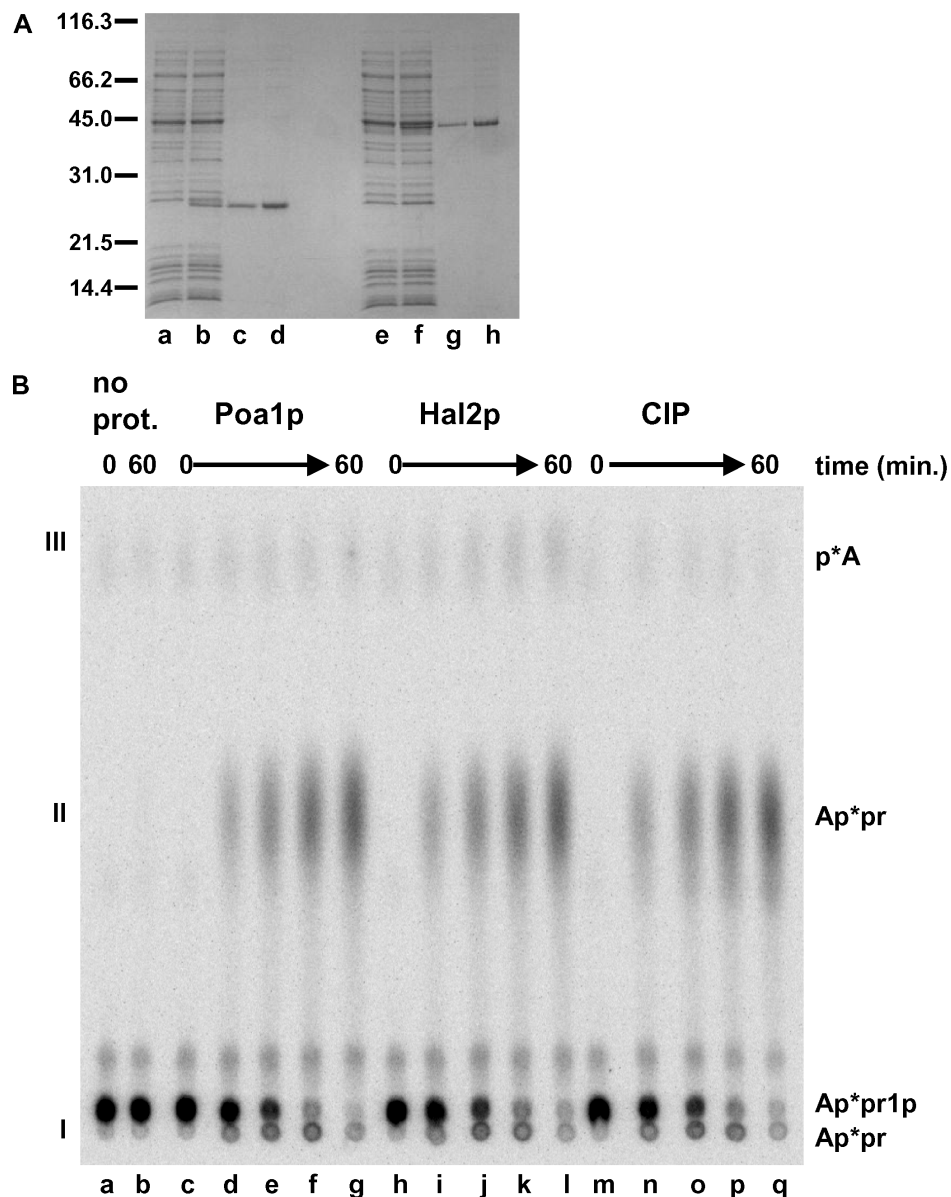


Figure 2. Overexpression and purification of Poa1p and Hal2p from *E. coli*. (A) His₆-YBR022w protein and Hal2p were purified as described in Materials and Methods from extracts of strains expressing His₆-YBR022w protein (a–d) and His₆-Hal2p (e–h), and analyzed by SDS–PAGE. a and e, 40 μg crude extract from strain expressing vector; b and f, 40 μg crude extract from strain expressing His₆-YBR022w protein (b) or His₆-Hal2p (f); c, d, g and h, 5 and 8 μg purified His₆-YBR022w protein (c and d) or His₆-Hal2p (g and h). (B) His₆-YBR022w protein, Hal2p and CIP all act as a phosphatase on Appr1p. Reactions mixtures containing 19 nM Ap*pr1p substrate and 210 nM purified His₆-YBR022w protein, 520 nM purified Hal2p or 56 fM CIP were incubated at 30°C, and reactions were stopped by spotting 2 μl to PEI–cellulose plates that were then resolved as in Figure 1. a and b, buffer control for 0 and 60 min; c–l, reactions containing YBR022w protein (c–g), Hal2p (h–l), or CIP (m–q) incubated for 0, 15, 30, 45 and 60 min. Migration of AMP (pA), ADP-ribose (Appr), and products I, II and III are indicated.

substrate (Table 1). With Appr1p, both enzymes have similar k_{cat} values (Poa1p, $k_{\text{cat}} = 1.7 \text{ min}^{-1}$, Hal2p, $k_{\text{cat}} = 3.2 \text{ min}^{-1}$), but Poa1p has an ~25-fold lower K_M (Poa1p, $K_M = 2.8 \text{ μM}$, Hal2p, $K_M = 74 \text{ μM}$), resulting in a k_{cat}/K_M difference of 14-fold in favor of Poa1p. Thus, this data suggest that either enzyme could work on Appr1p *in vivo* in yeast.

Hal2p greatly prefers its known substrate pAp, whereas Poa1p appears to not recognize this substrate. Consistent with previously published values (26,31), we find that Hal2p has a much lower K_M (0.1 μM) and a much higher k_{cat} (54 min^{-1}) for pAp than is observed for Appr1p. In contrast, Poa1p has no detectable activity on pAp, under

conditions where a turnover of 0.005 min^{-1} (0.3% of the activity with Appr1p) should be easily observed (data not shown). Thus, Hal2p prefers the substrate pAp by 10⁴-fold relative to Appr1p, as measured by k_{cat}/K_M values, whereas Poa1p prefers Appr1p at least 340-fold relative to pAp, as measured by observed rates.

A number of experiments confirmed that the slow rate of Appr1p hydrolysis by Poa1p was likely not an artifact.

First, we showed that the position or the presence of the purification tag did not affect the activity. Titration experiments of purified proteins demonstrate that Poa1p with an N-terminal His₆ tag is equally as active as Poa1p with a

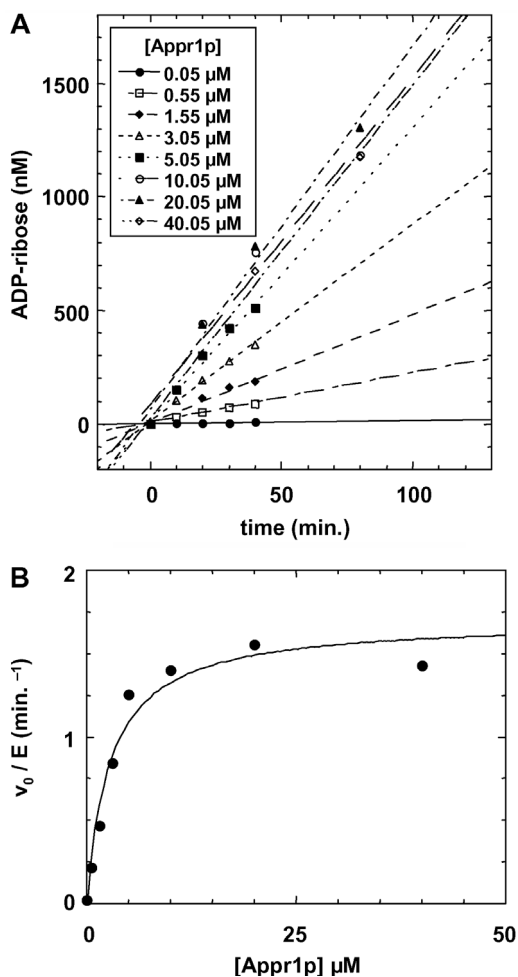


Figure 3. Kinetic parameters of Poa1p phosphatase for Appr1p. (A) Time courses of conversion of Appr1p to ADP-ribose at different substrate concentrations. Appr1p phosphatase activity was measured at 30°C for the times indicated, in reaction mixtures containing 21 nM Poa1p, 5–25 nM labeled Ap*pr1p and unlabeled Appr1p. Products were resolved as in Figure 2 and quantified, and data were plotted using KaleidaGraph software. (B) A Michaelis–Menten plot of the Appr1p phosphatase activity of Poa1p. Data from (A) were fit to a Michaelis–Menten curve using KaleidaGraph software.

Table 1. Kinetic parameters for Poa1p with Appr1p, and Hal2p with Appr1p and pAp

Enzyme	Substrate	K_M (μM)	k_{cat} (min ⁻¹)	k_{cat}/K_M (M ⁻¹ s ⁻¹)
Poa1p	Appr1p	2.8	1.7	1.0×10^4
Hal2p	Appr1p	74.0	3.2	7.2×10^2
Hal2p	pAp	0.1	54.0	1.0×10^7

C-terminal His₆ tag, and titrations of extracts demonstrate that the His₆-Poa1p has the same activity as Poa1p with a different N-terminal tag, or Poa1p with no tag at all (data not shown).

Second, the activity of Poa1p is not stimulated by altering salt concentrations. Indeed, as the salt was raised from 4 to 104 mM, the rate of hydrolysis was reduced by 21% under k_{cat} conditions, and by 72% under k_{cat}/K_M conditions.

Third, the activity of Poa1p was not significantly affected by pH under k_{cat}/K_M or k_{cat} conditions. We determined the rate

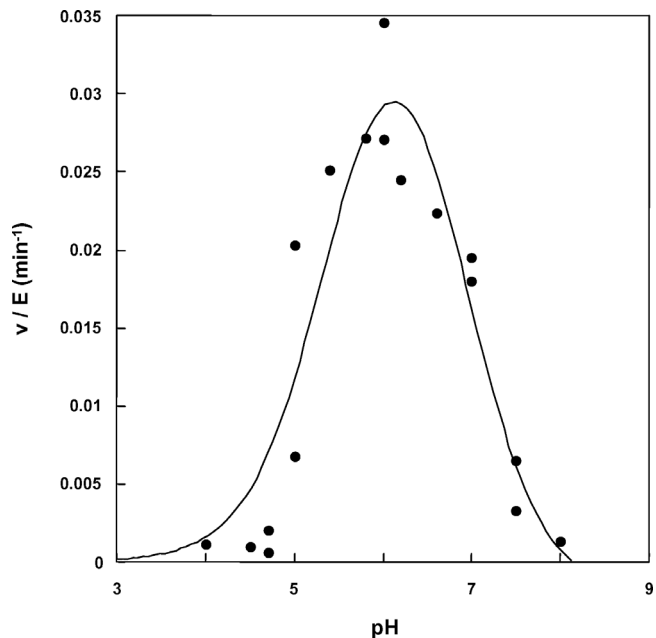


Figure 4. The pH activity profile for Poa1p under k_{cat}/K_M conditions. Poa1p Appr1p phosphatase activity was measured at 30°C with 5 nM Poa1p and 25 nM Ap*pr1p at different pH values over a suitable time course, and rates were determined, as described in Materials and Methods. The following buffers were used: NaOAc, pH 4.0, 4.5, 4.7 and 5.0; Homo-PIPES, pH 4.7 and 5.0; MES, pH 5.4, 5.8, 6.0 and 6.2; Bis-Tris, pH 6.0, 6.6 and 7.0; HEPES, pH 7.0 and 7.5; and Tris-HCl, pH 7.5 and 8.0.

of hydrolysis of Appr1p by Poa1p at different pH values ranging from pH 4.0 to pH 8.0, using a concentration of Appr1p well below the K_M under standard conditions (25 nM). As shown in Figure 4, a bell-shaped curve is observed under k_{cat}/K_M conditions, with pKa values at pH 5.0 and pH 7.0, and a maximum at ~pH 6.0. However, the rate of hydrolysis at pH 6.0 is only ~3.5-fold greater than that at pH 7.5. Experiments with different buffers at several pH values ensure that these differences are not due to buffer effects. A similar small difference was observed under k_{cat} conditions. At Appr1p concentrations of 40 μM, well above the K_M at pH 7.5, the observed rate of Appr1p hydrolysis varied only ~2-fold as the pH was varied from 7.5 to 6.2 (data not shown). Thus, Poa1p phosphatase activity is not affected significantly at either high or low concentrations of Appr1p within this pH range.

Poa1p is highly specific for Appr1p

Although Poa1p is a relatively slow phosphatase when acting on Appr1p, it appears to be highly specific for this substrate. We examined a number of other small molecules as possible substrates for Poa1p, but did not find any other molecule that was subject to Poa1p-catalyzed dephosphorylation. Ribose-1-p was a likely candidate, because Poa1p removes the 1' phosphate from the ribose moiety of Appr1p; however, no significant activity ($<5 \times 10^{-5}$ min⁻¹) was found from an overnight incubation of 5.3 μM Poa1p with ribose-1-p*, when assayed at both low and high concentrations of the substrate. Similarly, Poa1p had no detectable activity above background (<0.004 – 0.01 min⁻¹) with 1 mM concentrations of other potential substrates, including pAp, NADP, ribose-5-p,

Table 2. Inhibitors of Appr1p phosphatase activity of Poa1p^a

Competitor	IC ₅₀
ADPR	30 μ M
AppA	1 mM
NAD	1.5 mM
NADP	2 mM
ADP	>10 mM
3'pA	>10 mM
2'pC	>10 mM
5'3'pAp	ND ^b
5'pA	ND
2'pA	ND
2'pG	ND
3'pG	ND
3'pC	ND
ribose-1p	ND
ribose-5p	ND
glucose-1p	ND
fructose-6p	ND
glyceraldehyde-3p	ND

^aFor surveys at 10 mM competitor, 8 nM Poa1p, 26 nM Appr1p, 1 h; and 35 nM Poa1p, 5 μ M Appr1p, 2 h; for IC₅₀ values, 35 nM Poa1p, 5 μ M Appr1p, 2 h.

^bND, no detected inhibition in survey at 10 mM.

fructose-6-p, Gp, G^p, Ap, A^p, pA, U^p, C^p and 5'/2' pAp using a sensitive colorimetric assay (see Materials and Methods), and assays at either the optimum pH or in many cases also at pH 7.5. In contrast, CIP exhibited phosphatase activity on all of the substrates.

Poa1p specifically recognizes the ADP-ribose moiety

Of a number of molecules examined as potential inhibitors of Poa1p, only those with an ADP-ribose moiety have any significant effect. We surveyed the effect of 10 mM concentrations of various nucleotides and sugar phosphates on the activity of Poa1p measured at Appr1p concentrations well below K_M (26 nM), or at $1.8 K_M$ (5 μ M), and then determined IC₅₀ values of those that inhibited. Poa1p phosphatase activity was strongly inhibited by ADP-ribose (IC₅₀, 30 μ M), moderately inhibited by other molecules that contained an ADP-ribose moiety (AppA, NAD and NADP, with IC₅₀ values in the range 1–2 mM), marginally inhibited by ADP, Ap and C^p (with IC₅₀ values > 10 mM) and not inhibited at all by any other tested nucleotide or sugar phosphate (Table 2). This result underscores the suggestion that Poa1p is specific for Appr1p, and suggests further that recognition involves the ADP-ribose moiety. In contrast, Appr1p phosphatase activity of Hal2p (monitored at concentrations of Appr1p well below K_M) is inhibited by a variety of molecules including ADP-ribose, NADP, Ap, pAp, G^p and ribose 5-phosphate, as might be expected of a phosphatase with several substrates (data not shown).

To determine the mode of inhibition of ADP-ribose, the strongest of the Poa1p inhibitors, initial rates were determined at varied concentrations of both Appr1p (0.031–25 μ M) and ADP-ribose (0–300 μ M). The resulting inverse initial rates for each substrate concentration were plotted as a function of inhibitor concentration (Figure 5A), yielding plots with slopes that vary as a function of [S], and converge on the same y-intercept, indicative of classical competitive inhibition. A Dixon plot of the resulting slopes for each line versus 1/[Appr1p] yields the $K_{I,ADP-ribose}$ of 17 μ M (Figure 5B).

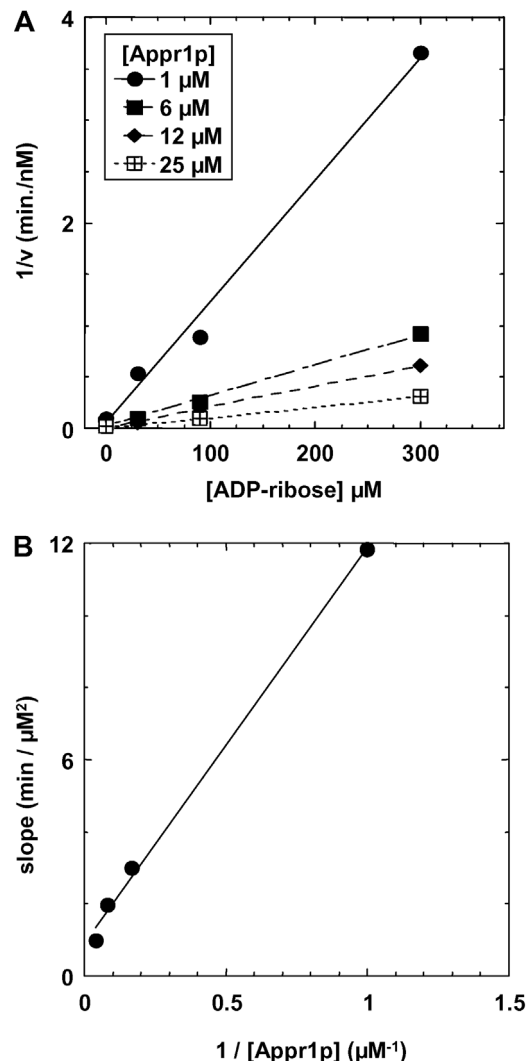


Figure 5. Determination of K_I of ADP-ribose for Poa1p. (A) Plot of $1/v$ versus [ADP-ribose] for each of four Appr1p concentrations. Poa1p Appr1p phosphatase activity was measured at the indicated concentrations of Appr1p and ADP-ribose for a suitable time course as described in Figure 3, and rates were determined and plotted as indicated. (B) Plot of slope versus $1/[Appr1p]$. Slopes were obtained for each curve in (A) above, and plotted versus $1/[Appr1p]$ as indicated. The slope of this plot ($K_M/V_{max} K_I$) was used to determine the K_I of ADP-ribose (17 μ M), as described in Materials and Methods.

Therefore, Poa1p is strongly inhibited by the product of its own reaction.

Extracts of *poa1-Δ* mutants have little activity on Appr1p *in vitro*

Examination of Appr1p processing in yeast extracts suggests that Poa1p is responsible for most of the observed Appr1p phosphatase activity. We compared the activity of crude extracts from a wild-type control haploid strain with those from *poa1-Δ* mutants, *hal2-Δ* mutants and *poa1-Δ*, *hal2-Δ* double mutants (see Materials and Methods). As shown in Figure 6, high concentrations of a wild-type extract (lane a) produce predominantly Pi, whereas lower concentrations (lanes b and c) produce primarily AMP. Presumably, ADP-ribose is formed initially, and then rapidly cleaved by a

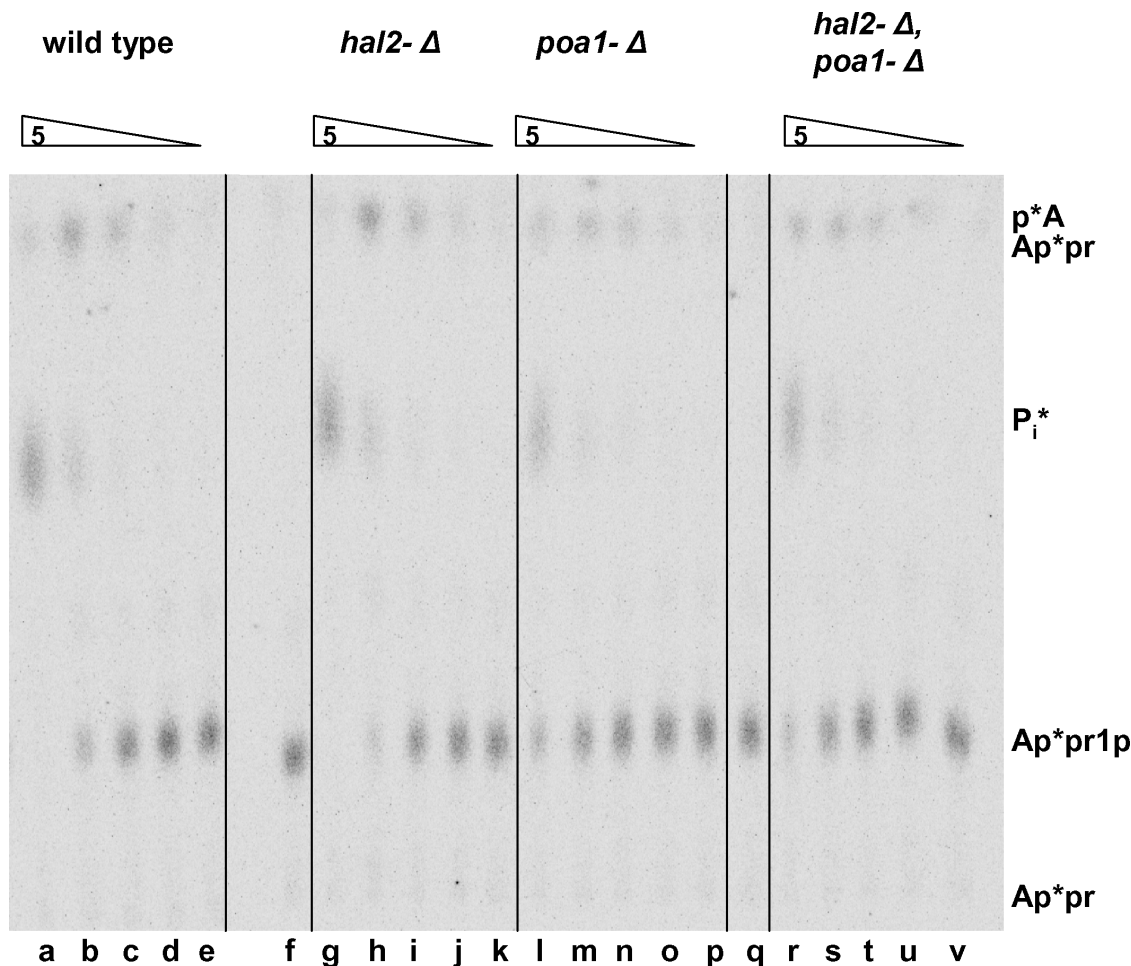


Figure 6. Comparison of Appr1p processing activity of crude extracts from wild-type cells and cells lacking Poa1p and/or Hal2p. Crude extracts derived from wild-type cells and *poa1*- Δ mutant, *hal2*- Δ mutant, or *poa1*- Δ , *hal2*- Δ double mutant cells were serially diluted 5-fold and assayed for Appr1p processing activity with 17 nM Ap*pr1p for 1 h at 30°C, as described in Materials and Methods. a–e, 16–0.04 μ g wild-type extract; g–k, 23–0.04 μ g *hal2*- Δ extract; l–p, 29 μ g to 0.05 μ g *poa1*- Δ extract; r–v, 21–0.03 μ g *poa1*- Δ , *hal2*- Δ extract; f and q, buffer controls. Reactions were treated with phenol:chloroform and resolved by TLC as in Figure 1.

pyrophosphatase to produce AMP, which is then hydrolyzed by another phosphatase to yield Pi. Although ADP-ribose co-migrates with AMP in the TLC shown in Figure 6, resolution of the products in a different TLC system demonstrates that little ADP-ribose is evident in the products (data not shown). Quantification of the titrations shows that Appr1p is 50% processed by 1.8 μ g wild-type extract, 1.2 μ g *hal2*- Δ extract, 33 μ g *poa1*- Δ extract or 20 μ g *poa1*- Δ , *hal2*- Δ double mutant extract. Thus, in crude extracts under these conditions, Poa1p is responsible for ~90% of the phosphatase activity on Appr1p, while Hal2p has little detectable activity on this molecule. The phosphatase activity that is remaining in a *poa1*- Δ extract is likely due to one or more non-specific phosphatases, since it is inhibited by Gp, an unrelated substrate (data not shown).

Poa1p has no significant homologies by blast search

Comparison of the sequence of Poa1p with other proteins in available databases reveals little sequence homology to other proteins as determined by BLAST (32) and, in particular, no

homology to any known phosphatase. Poa1p is a member of a family that is highly conserved in *Candida glabrata* (e^{-53}) and *Candida albicans* (e^{-22}), as well as four other fungal species (e^{-20} to e^{-6}), but only poorly conserved in other organisms. The best other BLAST score is 1×10^{-5} , with an Enterobacteria phage RB69 sequence for a homologous protein of unknown function (accession nos AAP76015.1 and GI:32350416). The role of these homologs in their respective organisms is unknown. The entire Poa1p sequence has also been defined as a domain in the SMART database (http://smart.embl-heidelberg.de/smart/do_annotation.pl?DOMAIN=A1pp). This domain, A1pp, occurs in 301 proteins from organisms in all three major kingdoms. Notably, the A1pp domain occurs three times in human poly(ADP-ribose) polymerase 14, in the C-terminal region of the macrohistone H2A family, and in the non-structural proteins of some single-stranded RNA viruses. We note that Poa1p does not appear in any of the physical interaction, genetic interaction or localization data sets, as summarized at the *Saccharomyces* Genome Database (<http://www.yeastgenome.org/>); thus, there is no additional information about its function.

DISCUSSION

We have identified two phosphatases that can act on Appr1p, a probable downstream product of the tRNA splicing pathway in *S.cerevisiae*, to produce ADP-ribose: Poa1p, a previously undescribed protein; and Hal2p, a known phosphatase that acts on pAp produced during methionine biosynthesis (22). The kinetic data suggest that either enzyme could act on Appr1p *in vivo*. Although Poa1p has a k_{cat}/K_M for Appr1p that is ~ 14 -fold higher than that of Hal2p for this molecule (1.0×10^4 versus 7.2×10^2), this preference is partially offset by the 6.3-fold increased cellular concentration of Hal2p relative to Poa1p (7330 versus 1170 molecules) (33).

Two lines of evidence suggest that Poa1p is responsible for Appr1p phosphatase activity in the cell. First, Poa1p is highly specific for Appr1p. A survey of a number of small molecules yielded no other potential substrate of Poa1p, under conditions where 0.3% of the activity observed with Appr1p could be detected. Furthermore, among a variety of molecules tested, significant inhibition of Poa1p activity is only observed from dinucleoside pyrophosphates bearing an ADP-ribose moiety (Table 2 and Figure 5), suggesting that this moiety is a major determinant of specificity. In contrast, Hal2p is a much less specific phosphatase for Appr1p; indeed, Hal2p is known to remove the 3'-phosphate of pAp and the 2'-phosphate of certain nucleotides (22), in addition to its activity described here on the phosphate at the 1 position of ribose. Second, $\sim 90\%$ of the activity observed in cell extracts appears to be due to Poa1p, as determined by the analysis of extracts from mutant cells (Figure 6). Furthermore, the remainder is likely due to a phosphatase other than Hal2p, based on the lack of observed difference in Appr1p phosphatase activity between wild-type and *hal2*- Δ extracts, and between *poa1*- Δ and *poa1*- Δ , *hal2*- Δ extracts; this minor phosphatase was not observed in our genomic survey, perhaps because the proteome examined is not complete (34). Thus, our results suggest that Poa1p acts on Appr1p in the cell.

Poa1p is an unusual phosphatase in two respects. First, it lacks homology with any other phosphatase. Other known small molecule phosphatases in yeast are members of a yeast phosphatase family or have close orthologs in other organisms (BLAST scores of e^{-30} or less). For example, among yeast phosphatases Pho3p, Pho5p, Pho11p, Pho12p and Dia3p form a highly homologous family, with $>75\%$ identity among them, and Dog1p, Dog2p, Gpp1p and Gpp2p align with $>35\%$ identity. Similarly, two other yeast phosphatases, His2p and Ser2p, are highly conserved phosphatases from species to species, with roles in amino acid biosynthesis, and Hal2p shares a conserved domain with fructose 1,6 bisphosphatase according to the cDART database (<http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>). In contrast, Poa1p is not closely related to any known phosphatase by these search methods. The only connection to a protein of known function is the presence of the A1pp domain of Poa1p in poly(ADP-ribose) polymerase 14 and in the macro-histone H2A family, as defined by the SMART database. This A1pp domain in other proteins may have a similar phosphatase activity or a similar ADP-ribose binding activity to that of Poa1p.

Second, Poa1p is highly specific for its substrate Appr1p. A large number of acid and alkali phosphatases found in *E.coli*

and eukaryotes remove phosphates from a wide range of small molecules (35–39); indeed, even metabolic phosphatases, such as glucose-6-phosphatase, have significant activity on several other substrates (40). In contrast, the high specificity of Poa1p for its substrate Appr1p is much more similar to the high specificity of regulatory phosphatases, such as fructose 2,6 bisphosphatase (41).

The results described here illuminate the likely last steps of a unique metabolic pathway, in which Appr>p is formed during tRNA splicing and then broken down to more common metabolites after splicing. Formation requires the sequential action of tRNA ligase to generate ligated tRNA with a splice junction 2'-phosphate, and Tpt1p to transfer the phosphate to NAD to form Appr>p. Breakdown of Appr>p is likely initiated by Cpd1p, generating Appr1p (19–21,42). This is inferred from the lack of Appr>p cyclic phosphodiesterase activity in extracts lacking Cpd1p (21), and the specificity of Cpd1p for Appr>p (19) (N. P. Shull and E. M. Phizicky, unpublished data), although others have found that Cpd1p is only modestly specific for Appr>p (21). Then, based on the results described here, Appr1p is processed by Poa1p, and possibly another minor phosphatase, to generate ADP-ribose. Finally, it seems likely that a pyrophosphatase converts ADP-ribose to AMP, based on the observed production of pA and subsequently Pi in extracts. Since *poa1*- Δ extracts lack most of the Appr1p phosphatase activity, and produce much less pA and Pi than wild-type extracts, we conclude that the pyrophosphatase acts primarily on ADP-ribose and not on Appr1p.

The recent remarkable findings of Schwer *et al.* (43) demonstrate that the otherwise essential ligase/phosphotransferase pathway of tRNA splicing (5,13,44) can be replaced by T4 enzymes that ligate tRNAs without producing splice junction 2'-phosphates or Appr>p, and that cells expressing the corresponding T4 genes have no obvious growth defect. We speculated previously that Appr>p might have a regulatory role because it is formed in equimolar yield during tRNA splicing (8,13). Although the results of Schwer *et al.* (43) suggest that the production of Appr>p is not essential, the accumulation of Appr>p could have cellular consequences.

We note that the cellular population of Poa1p is not quite enough to clear all of the Appr1p generated by tRNA splicing and the subsequent action of Cpd1p, based on our estimate of 500 000 tRNAs spliced per generation, the concentration of Poa1p in the cell (33) and the kinetic parameters of Poa1p. Experiments described above establish that the low hydrolysis rate of Poa1p is not due to factors, such as salt concentration, pH or the presence of the purification tag on the protein. Rather, the slow turnover might be inherent to the protein, perhaps due to the slow release of ADP-ribose. Thus, there may be a basal concentration of Appr1p in the cell, unless Poa1p is stimulated by other cellular factors, or there is another source of Appr1p processing activity. We note that Cpd1p does not stimulate or inhibit Poa1p under k_{cat}/K_M or k_{cat} conditions. It is possible that the slow turnover of Poa1p is tolerated due to the low metabolic demand for Appr1p processing and the lack of physiological consequence of Appr1p in the cell. In this connection, we note that *poa1*- Δ mutants, which might be expected to accumulate Appr1p, have no obvious growth phenotype in rich media at different temperatures, and that *poa1*- Δ *hal2*- Δ double mutants do not have any noticeable growth defect relative to single mutants. Alternatively, it is

conceivable that the speculated regulatory role of Appr^{>p} (8) is exerted through Appr1p, since it is uniquely generated through the same pathway that produces Appr^{>p}.

ACKNOWLEDGEMENTS

We are grateful to Jane Jackman for advice on the kinetic experiments, to Witold Filipowicz (Friedrich-Miescher-Institute, Basel, Switzerland) for the generous gift of chemically synthesized Appr^{>p}, to Andrei Alexandrov for advice on strain construction, and to Jane Jackman, Elizabeth Grayhack and Andrei Alexandrov for comments on the manuscript. This research was supported by NIH grant GM 52347 to E.M.P. Funding to pay the Open Access publication charges for this article was provided by NIH grant GM 52347.

REFERENCES

- Peebles, C.L., Gegenheimer, P. and Abelson, J. (1983) Precise excision of intervening sequences from precursor tRNAs by a membrane-associated yeast endonuclease. *Cell*, **32**, 525–536.
- Ho, C.K., Rauhut, R., Vijayraghavan, U. and Abelson, J. (1990) Accumulation of pre-tRNA splicing '2/3' intermediates in a *Saccharomyces cerevisiae* mutant. *EMBO J.*, **9**, 1245–1252.
- Trotta, C.R., Miao, F., Arn, E.A., Stevens, S.W., Ho, C.K., Rauhut, R. and Abelson, J.N. (1997) The yeast tRNA splicing endonuclease: a tetrameric enzyme with two active site subunits homologous to the archaeal tRNA endonucleases. *Cell*, **89**, 849–858.
- Phizicky, E.M., Schwartz, R.C. and Abelson, J. (1986) *Saccharomyces cerevisiae* tRNA ligase. Purification of the protein and isolation of the structural gene. *J. Biol. Chem.*, **261**, 2978–2986.
- Phizicky, E.M., Consaul, S.A., Nehrke, K.W. and Abelson, J. (1992) Yeast tRNA ligase mutants are nonviable and accumulate tRNA splicing intermediates. *J. Biol. Chem.*, **267**, 4577–4582.
- Greer, C.L., Peebles, C.L., Gegenheimer, P. and Abelson, J. (1983) Mechanism of action of a yeast RNA ligase in tRNA splicing. *Cell*, **32**, 537–546.
- McCraith, S.M. and Phizicky, E.M. (1991) An enzyme from *Saccharomyces cerevisiae* uses NAD⁺ to transfer the splice junction 2'-phosphate from ligated tRNA to an acceptor molecule. *J. Biol. Chem.*, **266**, 11986–11992.
- Culver, G.M., McCraith, S.M., Zillmann, M., Kierzek, R., Michaud, N., LaReau, R.D., Turner, D.H. and Phizicky, E.M. (1993) An NAD derivative produced during transfer RNA splicing: ADP-ribose 1''-2'' cyclic phosphate. *Science*, **261**, 206–208.
- Spinelli, S.L., Kierzek, R., Turner, D.H. and Phizicky, E.M. (1999) Transient ADP-ribosylation of a 2'-phosphate implicated in its removal from ligated tRNA during splicing in yeast. *J. Biol. Chem.*, **274**, 2637–2644.
- Waldron, C. and Lacroute, F. (1975) Effect of growth rate on the amounts of ribosomal and transfer ribonucleic acids in yeast. *J. Bacteriol.*, **122**, 855–865.
- Abelson, J., Trotta, C.R. and Li, H. (1998) tRNA splicing. *J. Biol. Chem.*, **273**, 12685–12688. Review.
- Hani, J. and Feldmann, H. (1998) tRNA genes and retroelements in the yeast genome. *Nucleic Acids Res.*, **26**, 689–696.
- Culver, G.M., McCraith, S.M., Consaul, S.A., Stanford, D.R. and Phizicky, E.M. (1997) A 2'-phosphotransferase implicated in tRNA splicing is essential in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **272**, 13203–13210.
- Sidrauski, C., Cox, J.S. and Walter, P. (1996) tRNA ligase is required for regulated mRNA splicing in the unfolded protein response. *Cell*, **87**, 405–413.
- Sidrauski, C. and Walter, P. (1997) The transmembrane kinase Ire1p is a site-specific endonuclease that initiates mRNA splicing in the unfolded protein response. *Cell*, **90**, 1031–1039.
- Calfon, M., Zeng, H., Urano, F., Till, J.H., Hubbard, S.R., Harding, H.P., Clark, S.G. and Ron, D. (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature*, **415**, 92–96.
- Iyer, V. and Struhl, K. (1996) Absolute mRNA levels and transcriptional initiation rates in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **93**, 5208–5212.
- Velculescu, V.E., Zhang, L., Zhou, W., Vogelstein, J., Basrai, M.A., Bassett, D.E., Jr, Hieter, P., Vogelstein, B. and Kinzler, K.W. (1997) Characterization of the yeast transcriptome. *Cell*, **88**, 243–251.
- Culver, G.M., Consaul, S.A., Tycowski, K.T., Filipowicz, W. and Phizicky, E.M. (1994) tRNA splicing in yeast and wheat germ. A cyclic phosphodiesterase implicated in the metabolism of Adp-ribose 1'',2''-cyclic phosphate. *J. Biol. Chem.*, **269**, 24928–24934.
- Martzen, M.R., McCraith, S.M., Spinelli, S.L., Torres, F.M., Fields, S., Grayhack, E.J. and Phizicky, E.M. (1999) A biochemical genomics approach for identifying genes by the activity of their products. *Science*, **286**, 1153–1155.
- Nasr, F. and Filipowicz, W. (2000) Characterization of the *Saccharomyces cerevisiae* cyclic nucleotide phosphodiesterase involved in the metabolism of ADP-ribose 1'',2''-cyclic phosphate. *Nucleic Acids Res.*, **28**, 1676–1683.
- Murguia, J.R., Belles, J.M. and Serrano, R. (1995) A salt-sensitive 3'(2'),5'-bisphosphate nucleotidase involved in sulfate activation. *Science*, **267**, 232–234.
- Tong, A.H., Evangelista, M., Parsons, A.B., Xu, H., Bader, G.D., Page, N., Robinson, M., Raghibizadeh, S., Hogue, C.W., Bussey, H. et al. (2001) Systematic genetic analysis with ordered arrays of yeast deletion mutants. *Science*, **294**, 2364–2368.
- Steiger, M.A., Kierzek, R., Turner, D.H. and Phizicky, E.M. (2001) Substrate recognition by a yeast 2'-phosphotransferase involved in tRNA splicing and by its *Escherichia coli* homolog. *Biochemistry*, **40**, 14098–14105.
- Hall, J., Genschik, P. and Filipowicz, W. (1996) Synthesis and characterization of the (5'→5'') ester of adenosine 5'-diphosphate with α-D-ribofuranose cyclic 1'',2''-phosphate: a NAD derivative produced during tRNA splicing. *Helv. Chim. Acta*, **79**, 1005–1010.
- Spiegelberg, B.D., Xiong, J.-P., Smith, J.J., Gu, R.F. and York, J.D. (1999) Cloning and characterization of a mammalian lithium-sensitive bisphosphate 3'-nucleotidase inhibited by inositol 1,4-bisphosphate. *J. Biol. Chem.*, **274**, 13619–13628.
- Dixon, M. (1953) The determination of enzyme inhibitor constants. *Biochem. J.*, **55**, 170.
- Van Veldhoven, P.P. and Mannaerts, G.P. (1987) Inorganic and organic phosphate measurements in the nanomolar range. *Anal. Biochem.*, **161**, 45–48.
- McCraith, S.M. and Phizicky, E.M. (1990) A highly specific phosphatase from *Saccharomyces cerevisiae* implicated in tRNA splicing. *Mol. Cell. Biol.*, **10**, 1049–1055.
- Grayhack, E.J. and Phizicky, E.M. (2001) Genomic analysis of biochemical function. *Curr. Opin. Chem. Biol.*, **5**, 34–39.
- Albert, A., Yenush, L., Gil-Mascarell, M.R., Rodriguez, P.L., Patel, S., Martinez-Ripoll, M., Blundell, T.L. and Serrano, R. (2000) X-ray structure of yeast Hal2p, a major target of lithium and sodium toxicity, and identification of framework interactions determining cation sensitivity. *J. Mol. Biol.*, **295**, 927–938.
- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.*, **25**, 3389–3402.
- Ghaemmaghami, S., Huh, W.-K., Bower, K., Howson, R., Belle, A., Dephoure, N., O'Shea, E. and Weissman, J. (2003) Global analysis of protein expression in yeast. *Nature*, **425**, 737–741.
- Phizicky, E.M., Martzen, M.R., McCraith, S.M., Spinelli, S.L., Xing, F., Shull, N.P., Van Slyke, C., Montagne, R.K., Torres, F.M., Fields, S. et al. (2002) Biochemical genomics approach to map activities to genes. *Methods Enzymol.*, **350**, 546–559.
- Heppel, L.A., Harkness, D.R. and Hilmore, R.J. (1962) A study of the substrate specificity and other properties of the alkaline phosphatase of *Escherichia coli*. *J. Biol. Chem.*, **237**, 841–846.
- Gottesman, M., Simpson, R.T. and Vallee, B.L. (1969) Kinetic Properties of Cobalt Alkaline Phosphatase. *Biochemistry*, **8**, 3776–3783.
- Torriani, A. (1960) Influence of inorganic phosphate in the formation of phosphatases by *Escherichia coli*. *Biochim. Biophys. Acta*, **38**, 460–479.

38. Nigam,V.N., Davidson,H.M. and Fishman,W.H. (1959) Kinetics of hydrolysis of the orthophosphate monoesters of phenol, *p*-nitrophenol, and glycerol by human prostatic acid phosphatase. *J. Biol. Chem.*, **234**, 1550–1554.
39. Neuman,H. (1968) Substrate selectivity in the action of alkaline and acid phosphatases. *J. Biol. Chem.*, **243**, 4671–4676.
40. Crane,R.K. (1955) The substrate specificity of liver glucose-6-phosphatase. *Biochim. Biophys. Acta*, **17**, 443–444.
41. Pilkis,S.J., Pilkis,J., el-Maghrabi,M.R. and Claus,T.H. (1985) The sugar phosphate specificity of rat hepatic 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *J. Biol. Chem.*, **260**, 7551–7556.
42. Hofmann,A., Zdanov,A., Genschik,P., Ruvinov,S., Filipowicz,W. and Wlodawer,A. (2000) Structure and mechanism of activity of the cyclic phosphodiesterase of Appr>p, a product of the tRNA splicing reaction. *EMBO J.*, **19**, 6207–6217.
43. Schwer,B., Sawaya,R., Ho,C.K. and Shuman,S. (2004) Portability and fidelity of RNA-repair systems. *Proc. Natl Acad. Sci. USA*, **101**, 2788–2793.
44. Spinelli,S.L., Consaul,S.A. and Phizicky,E.M. (1997) A conditional lethal yeast phosphotransferase (tpt1) mutant accumulates tRNAs with a 2'-phosphate and an undermodified base at the splice junction. *RNA*, **3**, 1388–1400.