Disruption and overexpression of the *Schizosaccharomyces pombe aps1* gene, and effects on growth rate, morphology and intracellular diadenosine 5′,5‴-*P*^{1},*P*^{5}-pentaphosphate and diphosphoinositol polyphosphate concentrations

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Schizosaccharomyces pombe Aps1 is an enzyme that degrades both diadenosine oligophosphates (Ap_nA, $n = 5$ or 6) and diphosphoinositol polyphosphates {diphosphoinositol pentakisphosphate (*PP*-InsP_5) and bisdiphosphoinositol tetrakisphosphate ([*PP*]$_2$-InsP_4)} *in vitro*. The *in vivo* substrates of Aps1 are unknown. We report here the identification of Ap_5A, *PP*-InsP_5, [*PP*]$_2$-InsP_4 and a novel diphosphoinositol polyphosphate ([*PP*]$_x$-InsP_x) in *S. pombe* using HPLC methods. Ap_5A was present at 0.06 pmol/mg of protein (approx. 4 nM). *PP*-InsP_5, [*PP*]$_x$-InsP_x and [*PP*]$_2$-InsP_4 were present at 15 pmol/mg (approx. 1.1 μM), 15 pmol/mg (approx. 1.1 μM) and 30 pmol/mg (approx. 2.2 μM) respectively, while the intracellular concentration of InsP_6 was 0.5 nmol/mg of protein (approx. 36 μM). Disruption of *aps1* resulted in a 52 % decrease in Ap_6A hydrolase activity *in vitro*, no detectable change in the intracellular Ap_5A concentration, and 3-fold increased intracellular concentrations of *PP*-InsP_5 and [*PP*]$_x$-InsP_x. Disruption of *aps1* resulted in no detectable change in morphology or growth rate in minimal or rich media at 30 °C. Overexpression of *aps1* via two different plasmids that resulted in 60 % and 6-fold increases above wild-type enzymic activity *in vitro* caused no detectable changes in the intracellular concentrations of [*PP*]$_2$-InsP_4, [*PP*]$_x$-InsP_x or *PP*-InsP_5, but paradoxical increases of approx. 2.5- and 55-fold respectively in the intracellular Ap_5A concentration. Overexpression of *aps1* also resulted in a reduced growth rate and in morphological changes, including swollen, rounded and multiseptate cells. No phenotypic changes or changes in intracellular Ap_5A occurred upon overexpression of *aps1*E93Q, which encodes a mutated Aps1 lacking significant enzymic activity. We conclude that Aps1 degrades *PP*-InsP_5 and [*PP*]$_x$-InsP_x *in vivo*.

Key words: adenine nucleotide, diadenosine oligophosphate, fission yeast, inositol phosphate, nudix hydrolase.

INTRODUCTION

Aps1 from *Schizosaccharomyces pombe* has been characterized as a dual-substrate enzyme which is active on diadenosine 5′,5‴-oligophosphates (Ap_nA, $n = 5$ or 6) and diphosphoinositol polyphosphates {diphosphoinositol pentakisphosphate (*PP*-InsP_5) and bisdiphosphoinositol tetrakisphosphate ([*PP*]$_2$-InsP_4)} *in vitro* [1,2]. Ap_5A and Ap_6A have not been identified in any unicellular organism. *PP*-InsP_5 and [*PP*]$_2$-InsP_4 are compounds of unknown function that have not been identified previously in *S. pombe*. *PP*-InsP_5 and [*PP*]$_2$-InsP_4 are known to exist in a number of cell types [3], including the budding yeast *Saccharomyces cerevisiae* [4]. *S. pombe* is a natural inositol auxotroph [5], and InsP_6 is present in *S. pombe* [6]. Ongusaha et al. [6] demonstrated that crude extracts of *S. pombe* can catalyse the synthesis of inositol polyphosphates more polar than InsP_6. It is not known whether diadenosine oligophosphates, diphosphoinositol polyphosphates or both are *in vivo* substrates for Aps1. In the present study Ap_5A, *PP*-InsP_5 and [*PP*]$_2$-InsP_4 were identified in *S. pombe*. To identify the *in vivo* substrate(s) for Aps1, the *aps1* gene was disrupted and overexpressed.

S. pombe Aps1 is one of three enzymes, the others being Ddp1 (Ap_6A hydrolase) from *Sacch. cerevisiae* and diphosphoinositol polyphosphate phosphohydrolase (DIPP) from *Homo sapiens*, that are known to catalyse the hydrolysis of [*PP*]$_2$-InsP_4, *PP*-InsP_5, Ap_5A and Ap_6A *in vitro* [2]. Aps1, Ddp1 and DIPP all contain the nudix (nucleoside diphosphate X) signature sequence motif [also called the Nudt (nudix-type) or mutT motif] **G**x$_5$**E**x$_5$**U**x**REU**x**EE**x**GU**, where U is an aliphatic, hydrophobic amino acid [7]. Most enzymically characterized members of the nudix protein family catalyse a reaction where the substrate is a nucleoside diphosphate attached to some other moiety, X. Exceptions are Aps1, Ddp1 and DIPP, which, as noted above, also degrade diphosphoinositol polyphosphates. Nudix hydrolases may function *in vivo* to degrade potentially deleterious nucleoside derivatives and to regulate the levels of metabolic intermediates that act as cellular signals [7].

Ap_5A and Ap_6A were first reported in biological material in adrenal chromaffin granules [8,9]. Ap_5A and Ap_6A also are found in the dense granules of platelets [10], and more recently Jankowski et al. [11] demonstrated the presence of a higher-phosphate homologue, Ap_7A, in human platelets. Ap_5A is present in rat brain synaptosomes and in the cholinergic synaptic vesicles of the electric ray *Torpedo* [12]. Ap_nA ($n = 3$–7) are proposed to function as extracellular signals involved in vascular tone and Ap_nA ($n = 5$ and 6) to act as neurotransmitters. These proposed

Abbreviations used: Ap_nA, diadenosine 5′,5‴-*P*^{1},*P*^{n}-oligophosphate ($n = 3$–7); DAPI, 4′,6-diamidino-2-phenylindole; DIPP, diphosphoinositol polyphosphate phosphohydrolase; MM, minimal medium; nudix, nucleoside diphosphate X; p_4A, adenosine tetraphosphate; p_5A, adenosine pentaphosphate; *PP*-InsP_5, diphosphoinositol pentakisphosphate; [*PP*]$_2$-InsP_4, bisdiphosphoinositol tetrakisphosphate; [*PP*]$_x$-InsP_x, a novel diphosphoinositol polyphosphate that is probably an isomer of diphosphoinositol pentakisphosphate.

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extracellular functions in higher eukaryotes have been reviewed [12,13].

Diphosphoinositol polyphosphates, originally identified in Dictyostelids [14], are widely distributed in a variety of organisms and cell types [3,15], including *Sacch. cerevisiae* [4]. In *Sacch. cerevisiae*, the intracellular concentrations of *PP*-InsP_5 and [*PP*]$_2$-InsP_4 represent approx. 0.6–0.8 % and 0.7–0.9 % respectively of the concentration of InsP_6 [4,16]. Diphosphoinositol polyphosphates are the most highly phosphorylated inositol compounds found in cells, and 30–50 % of the intracellular content of InsP_6 in mammalian cells ($\sim$ 15–100 μM) cycles through the diphosphoinositol polyphosphates every 1 h [3]. The observation that the cell cycles its InsP_6 content through a more highly phosphorylated form suggests that the cell reaps some benefit from this process. Diphosphoinositol polyphosphates have been proposed to play a role in phosphotransferase reactions, based on the predicted bond energy of 27.6 kJ/mol (6.6 kcal/mol) for the P–O–P linkages [14], and may function indirectly in the phosphorylation of protein targets *in vivo* [17]. Diphosphoinositol polyphosphates may have a role in vesicle trafficking. This potential role is based on their binding to AP3, a neuron-specific protein that localizes to nerve terminals and promotes the formation of clathrin polymers [18], and to coatomer proteins from bovine liver [19] and *Sacch. cerevisiae* [20]. Diphosphoinositol polyphosphates have also been proposed to play a role in signal transduction in mammals through β-adrenergic receptors [21]. More recent work in *Sacch. cerevisiae* points to a possible role for InsP_6 in mRNA export from the nucleus [4,22,23]. InsP_6 is a precursor for *PP*-InsP_5 and [*PP*]$_2$-InsP_4 synthesis in *Sacch. cerevisiae* [24]. Luo et al. [25] reported that *PP*-InsP_5 and [*PP*]$_2$-InsP_4 mediate homologous DNA recombination in a protein kinase C1 mutant of *Sacch. cerevisiae*. Shears [3], Safrany et al. [26] and Irvine and Schell [27] have reviewed the metabolism and possible functions of the diphosphoinositol polyphosphates.

Here we report the identification of Ap_5A, *PP*-InsP_5 and [*PP*]$_2$-InsP_4, and a previously unreported diphosphoinositol polyphosphate ([*PP*]$_x$-InsP_x), in *S. pombe*. We also report the results of the disruption and overexpression of the *aps1* gene.

MATERIALS AND METHODS

S. pombe strains

The *S. pombe* strains used in these investigations were PR1319 h$^-$ *ade6*-210 *leu1*-32 *his7*-366 *ura4*-D18 and PR1319/PR1320 h$^-$/h$^+$ *ade6*-210/*ade6*-216 *leu1*-32/*leu1*-32 *his7*-366/*his7*-366 *ura4*-D18/*ura4*-D18.

Growth

Cells were grown in minimal medium (MM) with appropriate supplements or 2 % YE medium [28] at 30 °C in sterile Erlenmeyer flasks on an orbital shaker running at 275 rev./min. Cultures of 1 or 2 ml of yeast in MM were grown in 14 ml polypropylene round-bottomed tubes for labelling with [^{3}H]-inositol. Cell growth was determined by measuring absorbance at 600 nm using a Beckman DU 7400 spectrophotometer. The linear portion of a plot of log A_{600} against time was used to calculate generation time. In some experiments cell density was determined by counting cells in a haemocytometer at $\times$400 magnification. Cells containing the pREP3X series of vectors [29] were repressed by growth in MM containing 5 μg/ml thiamine. Derepression was accomplished by washing cells in water and subsequently culturing cells in thiamine-free MM for at least 24 h.

Preparation of cellular extracts for Ap_nA measurements

Cells were grown in 500 ml of 2 $\times$ MM with the appropriate supplements [28]. When the cultures had reached the required density, three separate 100 ml samples were harvested by rapid filtration on 1.2 μm-pore-size Millipore filters, suspended in 5 % (w/v) trichloroacetic acid and processed for measurement of Ap_nAs as described previously [30]. Sonicated trichloroacetic acid extracts were stored on ice for at least 45 min before centrifugation to obtain trichloroacetic acid supernatants and crude pellets. Strains grown under the same conditions were harvested by centrifugation and used for determining the Ap_6A hydrolase activity, as described below.

Measurement of intracellular Ap_nA concentrations

Trichloroacetic acid supernatants were purified by column chromatography on boronate-derivatized resin [30]. Fractions containing Ap_nAs were freeze dried, dissolved in 1 ml of 20 mM Tris/HCl, pH 8.5, treated with alkaline phosphatase, and analysed quantitatively for Ap_nAs by HPLC [30]. Ap_nAs were eluted with 0.475 M ammonium bicarbonate, pH 8.5. Under these elution conditions, the majority of intracellular Ap_4A is washed off the precolumn prior to switching effluent to the Mono Q column for analysis. Identification of Ap_5A in samples was based on retention time in comparison with standard Ap_5A, co-elution with standard Ap_5A added to samples and degradation of the sample HPLC peak by Aps1 and snake venom phosphodiesterase.

Measurement of Ap_6A hydrolase activity

Cells were harvested by centrifugation from mid- to late-exponential-phase cultures, and crude supernatants were prepared as described previously [30]. Ap_6A hydrolase activity was assayed using 200 μM [^{3}H]Ap_6A as substrate, with subsequent separation of products and residual substrate using chromatography on boronate-derivatized resin [30]. [^{3}H]Ap_6A was synthesized from [2,8-^{3}H]ATP (Amersham/Pharmacia) by reaction with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide in Hepes buffer, pH 6.5, containing $MgCl_2$ [31]. [^{3}H]Ap_6A was purified by chromatography on DEAE-Sephacel eluted with a gradient of ammonium acetate. The chemical purity was estimated to be 94 % based on HPLC analytical chromatography on a Mono Q column (Amersham/Pharmacia). Assays were performed using dialysed crude supernatant in the presence of 50 mM Hepes, pH 7.5, 1 mM $MnCl_2$ and 0.2 mg/ml BSA. Activity was expressed as nmol of AXP formed/min per mg of protein, where AXP equals the sum of adenosine pentaphosphate (p_5A), adenosine tetraphosphate (p_4A), ATP, ADP and AMP. Although Aps1 will degrade Ap_nA (n = 4–6), we assayed Ap_6A hydrolase activity because Ap_6A is the optimal substrate *in vitro* among the Ap_nAs [1].

Measurement of inositol polyphosphates

Strains were grown in 1 or 2 ml of appropriately supplemented MM containing 50 μCi of [^{3}H]inositol/ml (Amersham) at 30 °C and 220 rev./min. Cells were labelled for 72–96 h. Exponential-phase cultures were obtained by inoculating 1 ml of fresh medium with 20–25 μl of the labelled culture and allowing growth as above for 24–48 h. Growth was terminated by centrifuging for 1 min at 10000 ***g***, washing the pellet with 1 ml of MM, then resuspending in 50 μl of 2 M perchloric acid. Following a 15 min incubation on ice, the sample was subjected to centrifugation for 1 min at 10000 ***g*** to pellet protein. The supernatant was neutralized with an appropriate volume (usually 55 μl) of 1 M

K_2CO_3 containing 5 mM EDTA. Samples were incubated at 4 °C for 15 min with occasional vortexing to allow the precipitation of potassium perchlorate and release of CO_2, then spun again (10000 ***g***, 5 min, 4 °C). The supernatant was diluted 5-fold with 1 mM Na_2EDTA and stored at −20 °C until analysed. Samples were loaded on to a 4.6 mm × 125 mm Partisphere 5 μm SAX HPLC column. Inositol phosphates were eluted at 1 ml/min by the following gradient generated by mixing buffer A (1 mM Na_2EDTA) and buffer B [buffer A plus 1.3 M $(NH_4)_2HPO_4$, pH 3.85, with H_3PO_4; total P_i = 2.6 M]: 0–10 min, 0 % B; 10–25 min, 0–35 % B; 25–105 min, 35–100 % B; 105–115 min, 100 % B. Fractions of 1 ml were collected and radioactivity was determined using liquid-scintillation counting [21]. Standards of [^{3}H]inositol 1,3,4,5,6-pentakisphosphate and a mixture of three inositol pentakisphosphates which co-elute on HPLC (D/L-inositol 1,2,4,5,6-pentakisphosphate and inositol 1,2,3,4,6-pentakisphosphate) were prepared from [^{3}H]InsP_6 (NEN) by alkaline hydrolysis, as described by Stephens [32]. Standards of *PP*-[^{3}H]InsP_5 and [*PP*]$_2$-[^{3}H]InsP_4 were prepared by phosphorylation of [^{3}H]InsP_6 using ATP and partially purified rat brain InsP_6 kinase and *PP*-InsP_5 kinase respectively [2]. Identification of inositol polyphosphates in samples was based on retention time in comparison with standards.

Construction of a vector for disruption of *aps1*

pUR18*aps1*, which we constructed previously [1], was digested with *Eco*RI and *Hin*dIII. The resulting fragment, which contained the *aps1* open reading frame flanked by 310 bp of *S. pombe* 5′ sequence and 291 bp of *S. pombe* 3′ sequence, was ligated into *Eco*RI/*Hin*dIII-digested pk184 plasmid [33]. The resulting plasmid, named pk184*aps1*, was digested with *Sca*I and *Nsi*I. This digestion removed the entire open reading frame of *aps1* and left 296 bp of the *S. pombe* upstream sequence and 279 bp of the *S. pombe* downstream sequence in place. The deleted fragment was replaced by ligating in a *Sca*I/*Nsi*I-digested 1994 bp PCR product containing the *his7* gene. The PCR product was generated using the primers 5′-AGACAGTACTTTGTACGTAA-AGGCG-3′ and 5′-ATATATGCATCCGGGGATCTCTT-3′ (*Sca*I and *Nsi*I sites are underlined) and plasmid pEA2 [34] as template. The resulting plasmid was named pUR18*aps1*Δ*his7*.

Disruption of *aps1*

An *Eco*RI/*Bgl*I digest of pUR18*aps1*Δ*his7* (1.5 μg) was used to transform [35] *S. pombe* PR1319/1320 and disrupt *aps1*. Transformants were picked and restreaked on to YE plates. These plates were replica-plated on to MM lacking histidine to ensure *his7* retention. A total of 30 transformants were screened for *aps1* disruption by Southern blotting.

Southern blots

Genomic DNA was isolated as described in [36]. Southern blotting was performed as described in [37]. Probes were labelled using a Sigma random primer labelling kit (Prime-2) and Easy-Tides [^{32}P]dCTP (NEN).

Sporulation and dissection of tetrads

PR1319/1320 *aps1*$^+$/*aps1*Δ::*his7* was sporulated and tetrads were dissected as described in [28]. Viable spores on 2 % YE agar were replicate-plated on to MM agar lacking histidine.

Site-directed mutagenesis of *aps1*

Site-directed mutagenesis was accomplished using the unique site elimination method [38]. A mutagenic primer, 5′-CTGCCCTTCGCGAAGGTTGGGAACAAGGCGGAC-3′ (mutations are underlined), was annealed to denatured pSGA02*aps1* DNA [1] and the resulting complex was used as template for T4 DNA polymerase. The resulting plasmid was named pSGA02*aps1*E93Q. The first base change in the primer generated a unique restriction nuclease (*Nru*I) site that permitted screening of transformant colonies for plasmid containing the mutated sequence. The second base change in the primer resulted in glutamate-93 of Aps1 being changed to a glutamine residue. The base changes in *aps1* were verified by DNA sequencing.

Overexpression of *aps1* and *aps1*E93Q

Wild-type Aps1 and the Aps1E93Q mutant were overexpressed using five plasmids: pUR18*aps1*, pPOX*aps1*, pPOX*aps1*E93Q, pREP3X*aps1* and pREP3X*aps1*E93Q. pUR18*aps1* has been described previously [1]. pPOX*aps1* and pPOX*aps1*E93Q were made as follows. PCR primers 5′-GGCAAAGCTTATGCTT-GAAAATAACGGAAG-3′ and 5′-GGACTCTAGATCAGT-TTTCCTCTTTAATG-3′ (*Hin*dIII and *Xba*I sites are underlined) were used to amplify the *aps1* and *aps1*E93Q open reading frames from plasmids pSGA02*aps1* [1] and pSGA02*aps1*E93Q respectively. The PCR products were digested with *Hin*dIII and *Xba*I and ligated into *Hin*dIII/*Xba*I-digested pPOX [30], yielding pPOX*aps1* and pPOX*aps1*E93Q. The open reading frames of *aps1* and *aps1*E93Q in pPOX were verified by DNA sequencing. pREP3X*aps1* and pREP3X*aps1*E93Q were made as follows. The PCR primers 5′-TACACTCGAGATGCTTG-AAAATAACGGAAG-3′ and 5′-GGACGGATCCTCAGTT-TTCCTCTTTAATG-3′ (*Xho*I and *Bam*HI sites are underlined) were used to amplify both wild-type and mutant *aps1* open reading frames from pSGA02*aps1* and pSGA02*aps1*E93Q respectively. These PCR products were digested with *Bam*HI and *Xho*I and ligated into *Bam*HI/*Xho*I-digested pREP3X [29]. The sequences of both open reading frames in pREP3X were verified by DNA sequencing.

Microscopy

Images of fluorescent samples were acquired using an Olympus BX60 microscope and a digital Spot Camera (Diagnostics, Inc.). DAPI (4′,6-diamidino-2-phenylindole) and calcofluor staining of ethanol-fixed *S. pombe* were done as described in [28]. Differential interference contrast images were acquired using an Olympus IX70 microscope and a Hamamatsu digital camera.

Determination of protein mass

Crude pellets obtained by centrifugation of trichloroacetic acid extracts were washed in cold 95 % (v/v) ethanol and dissolved in 2 ml of 2 M NaOH for determination of protein [39]. Protein masses in crude supernatants used to assay enzymic activity were also measured as in [39].

Data analysis

For measurement of Ap_5A and the diphosphoinositol polyphosphates, three to six independent samples of each strain were analysed. For Ap_5A, values are expressed as pmol of Ap_5A per mg of protein that was measured quantitatively in trichloroacetic acid pellets as described above. We estimated the *in vivo* concentration of Ap_5A in *S. pombe* from the value of pmol of

Ap_5A/mg of protein using parameters described previously [30]. Values for PP-InsP_5, $[PP]_2$-InsP_4 and $[PP]_x$-InsP_x are expressed as a percentage of InsP_6, which is quantitatively the most abundant higher inositol polyphosphate derived from the [^{3}H]-inositol precursor [6]. We estimated the *in vivo* concentration of InsP_6 in *S. pombe* using the same parameters as referenced above for Ap_5A. We assumed 100 % recovery of Ap_5A, InsP_6 and the

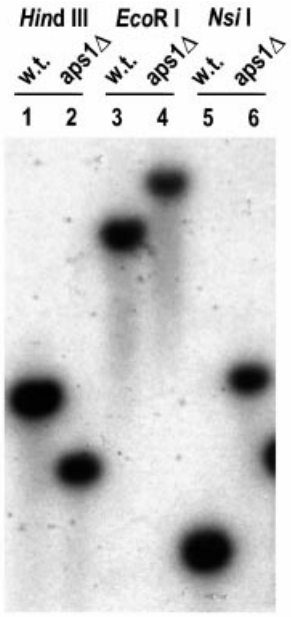

Figure 1 Southern blot analysis of genomic DNA from *S. pombe* PR1319 and *S. pombe* PR1319*aps1*Δ::*his7* probed with 300 bp of [^{32}P]dCTP-labelled DNA from the 5′ flanking region of *aps1*

Lanes 1 and 2 contain *Hin*dIII-digested genomic DNA from wild-type (w.t.) *S. pombe* PR1319 and *S. pombe* PR1319*aps1*Δ::*his7* respectively. Lanes 3 and 4 contain *Eco*RI-digested genomic DNA from *S. pombe* PR1319 and *S. pombe* PR1319*aps1*Δ::*his7* respectively. Lanes 5 and 6 contain *Nsi*I-digested genomic DNA from *S. pombe* PR1319 and *S. pombe* PR1319*aps1*Δ::*his7* respectively. The size of each band in each lane (calculated using *Hin*dIII-digested λgt11 DNA to generate a standard curve) is as follows, along with predicted sizes for each band (calculated using DNA sequence information from the *S. pombe* genome sequencing project) in parentheses: lane 1, 2500 bp (2645 bp); lane 2, 1800 bp (1946 bp); lane 3, 5500 bp (5101 bp); lane 4, 6900 bp (6885 bp); lane 5, 1300 bp (1319 bp); lane 6, 2900 bp (2653 bp).

Table 1 Ap_6A hydrolase activity in *S. pombe* lacking or overexpressing *aps1*

Data were generated by assaying dialysed crude supernatant fractions from exponential-phase cells grown in MM (lacking thiamine) with supplements at 30 °C, as described in the Materials and methods section. AXP represents AMP + ADP + ATP + p_4A + p_5A. Values are means ± S.D. (n = 3–4).

Strain	Activity (nmol of AXP/min per mg of protein)
PR1319	1.86 ± 0.08
PR1319*aps1*Δ::*his7*	0.89 ± 0.28
PR1319 + pUR18	1.60 ± 0.08
PR1319 + pUR18*aps1*	2.61 ± 0.21
PR1319 + pREP3X	2.26 ± 0.28
PR1319 + pREP3X*aps1*	14.4 ± 1.87
PR1319 + pREP3X*aps1*E93Q	2.59 ± 0.23

Table 2 Growth rates of *S. pombe* lacking or overexpressing *aps1*

Growth rates were determined for cultures at 30 °C in MM (lacking thiamine) with supplements, as described in the Materials and methods section. Values are means ± S.D. (n = 3–4).

Strain	Generation time (h)
PR1319	3.6 ± 0.3
PR1319*aps1*Δ::*his7*	4.1 ± 0.3
PR1319 + pUR18	3.8 ± 0.1
PR1319 + pUR18*aps1*	4.6 ± 0.2
PR1319 + pREP3X	4.6 ± 0.1
PR1319 + pREP3X*aps1*	11.1 ± 3.7
PR1319 + pPREP3X*aps1*E93Q	5.5 ± 0.1

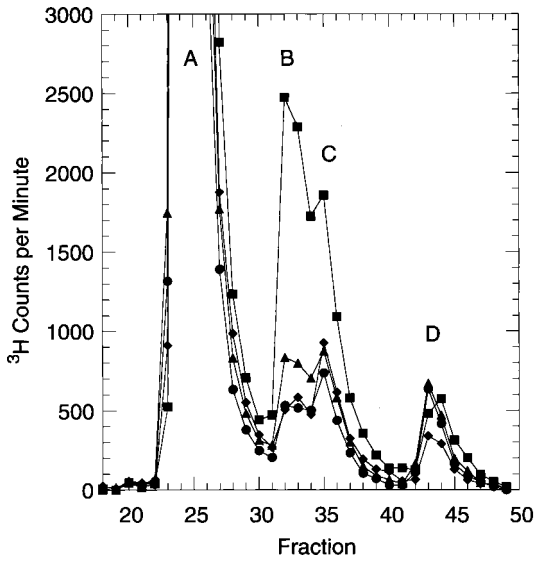

Figure 2 HPLC analysis of intracellular soluble inositol phosphates from *S. pombe* lacking or overexpressing *aps1* after steady-state labelling of cells with [^{3}H]inositol

◆, *S. pombe* PR1319; ■, *S. pombe* PR1319*aps1*Δ::*his7*; ▲, *S. pombe* PR1319 transformed with pUR18; ●, *S. pombe* PR1319 transformed with pUR18*aps1*. Peaks are labelled as follows: A, InsP_6; B, $[PP]_x$-InsP_x; C, PP-InsP_5; D, $[PP]_2$-InsP_4. Peaks were identified by comparison with the elution times of known standards.

Table 3 Intracellular concentrations of diphosphoinositol polyphosphates in *S. pombe* lacking or overexpressing *aps1*

Data were generated from exponential-phase cultures grown in MM (lacking thiamine) at 30 °C with supplements, as described in the Materials and methods section. Values are means ± S.D. (n = 3–4), and are presented as a percentage of InsP_6 levels. InsP_5(1), inositol 1,3,4,5,6-pentakisphosphate; InsP_5(2) designates three inositol pentakisphosphates which co-elute on HPLC: D/L-inositol 1,2,4,5,6-pentakisphosphate and inositol 1,2,3,4,6-pentakisphosphate.

	Level (% of InsP_6)				
Strain	InsP_5(1)	InsP_5(2)	$[PP]_x$-InsP_x	PP-InsP_5	$[PP]_2$-InsP_4
PR1319	1.8 ± 0.6	1.9 ± 0.6	3.0 ± 1.0	3.0 ± 2.0	6.0 ± 2.0
PR1319*aps1*Δ::*his7*	2.9 ± 0.8	2.0 ± 1.0	10.0 ± 0.8	9.0 ± 3.0	8.0 ± 0.4
PR1319 + pUR18	3.0 ± 2.0	1.7 ± 0.4	3.0 ± 1.0	4.0 ± 1.0	6.0 ± 1.0
PR1319 + pUR18*aps1*	2.4 ± 0.8	2.0 ± 0.6	1.3 ± 0.4	4.7 ± 0.7	11.0 ± 2.0
PR1319 + pREP3X	4.0 ± 1.0	4.0 ± 0.7	2.6 ± 0.8	3.0 ± 2.0	2.9 ± 0.5
PR1319 + pREP3X*aps1*	3.4 ± 0.6	3.1 ± 0.3	1.8 ± 0.7	3.0 ± 2.0	4.0 ± 2.0
PR1319 + PREP3X*aps1*E93Q	2.2 ± 0.8	3.0 ± 1.0	3.0 ± 1.0	3.0 ± 1.0	4.0 ± 1.0

Table 4 Intracellular Ap_5A concentration in *S. pombe* lacking or overexpressing *aps1*

Cells were harvested at mid- to late-exponential phase, and Ap_5A was measured as described in the Materials and methods section. Values are means ± S.D. ($n = 3$, except $n = 6$ for PR1319).

Strain	Ap_5A concentration (pmol/mg of protein)
PR1319	0.06 ± 0.03
PR1319*aps1*Δ::*his7*	0.03 ± 0.01
PR1319 + pUR18	0.06 ± 0.01
PR1319 + pUR18*aps1*	0.16 ± 0.01
PR1319 + pREP3X	0.06 ± 0.01
PR1319 + pREP3X*aps1*	3.31 ± 0.05
PR1319 + pREP3X*aps1*E93Q	0.07 ± 0.02

diphosphoinositol polyphosphates in analyses of cell extracts, so the values reported are minimum values.

RESULTS

Disruption of *aps1*

A total of 30 potential *aps1* disruptant diploid transformants were screened for *aps1* disruption by Southern blotting. The one positive transformant was sporulated and 17 tetrads were dissected. Of these, 15 tetrads contained four viable spores, indicating that *aps1* is not essential, and in each case the *his7* marker segregated 2 × 2. A haploid h^- *his7*$^+$ colony was selected for further analysis. Southern blots performed on genomic DNA from this haploid verified *aps1* disruption (Figure 1) and a single site of *his7* integration (results not shown). A PR1319*aps1*Δ::*his7* strain was isolated and used in subsequent experiments. Disruption of *aps1* had no apparent effect on sporulation in heterozygous diploids, and reduced Ap_6A hydrolase activity in a haploid crude supernatant fraction by approx. 52% (Table 1). PR1319*aps1*Δ::*his7* exhibited normal morphology, mated with wild-type cells of the opposite mating type, and exhibited the same growth rate as wild-type cells in both rich medium (generation time of 2.3 ± 0.1 h for both wild-type and *aps1* disruptant strains; $n = 3$) and MM (Table 2).

Measurement of intracellular inositol polyphosphate concentrations in wild-type and *aps1* disruptant strains of *S. pombe*

The only phenotypic change observed in the disruptant strain other than the decrease in Ap_6A hydrolase activity was an increase in the intracellular concentrations of two diphosphoinositol polyphosphates. Figure 2 shows a representative HPLC trace of inositol polyphosphate extracts from *S. pombe* prepared after steady-state labelling with [^{3}H]inositol. This analysis identified the diphosphoinositol polyphosphates *PP*-InsP_5 (peak C), $[PP]_2$-InsP_4 (peak D) and a previously unreported diphosphoinositol polyphosphate, $[PP]_x$-InsP_x (peak B). The *aps1* disruptant strain showed a clear increase in peak B, which eluted near a *PP*-InsP_5 standard. Based on its relative elution position, this peak is likely to represent $[PP]_2$-InsP_3. In addition, the shoulder on the trailing edge of the $[PP]_x$-InsP_x peak in the disruptant strain co-eluted with a *PP*-InsP_5 standard (peak C). The masses of $[PP]_x$-InsP_x, *PP*-InsP_5 and $[PP]_2$-InsP_4 are expressed as a percentage of that of InsP_6 in Table 3. In wild-type *S. pombe* the intracellular concentration of InsP_6 was calculated to be 0.5 ± 0.2 nmol/mg of protein or 36 ± 17 μM ($n = 4$), and the intracellular concentrations of *PP*-InsP_5, and $[PP]_2$-InsP_4 were calculated to be 1.1 μM and 2.2 μM respectively. The intracellular concentrations of *PP*-InsP_5 and $[PP]_x$-InsP_x were

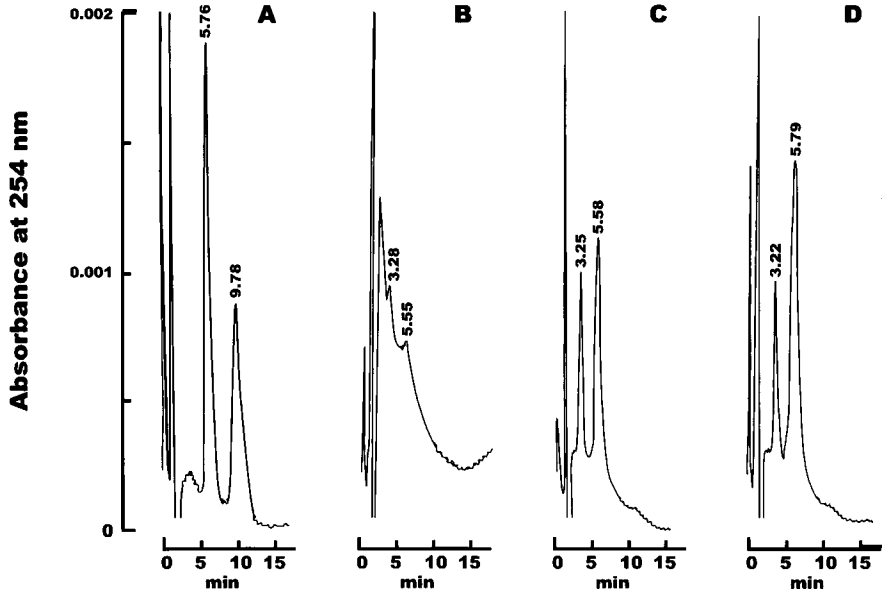

Figure 3 HPLC analysis of intracellular Ap_nA from *S. pombe* extracts

(**A**) A 1 ml aliquot of 20 mM Tris/HCl, pH 8.5, containing 51 pmol each of standard Ap_5A and Ap_6A was loaded on to a Mono Q column and eluted as described in the Materials and methods section. Ap_5A was eluted at 5.76 min, and Ap_6A at 9.78 min. (**B**) Sample from *S. pombe* 1319pREP3X corresponding to 30 mg of protein; Ap_5A was eluted at 5.55 min. The peak at 3.28 min is residual Ap_4A that was not eluted completely from the pre-column. (**C**) Sample from *S. pombe* 1319pREP3X*aps1* corresponding to 8.5 mg of protein; Ap_5A was eluted at 5.58 min and residual Ap_4A at 3.25 min. (**D**) Same sample as in (**C**) with 20 pmol of standard Ap_5A added.

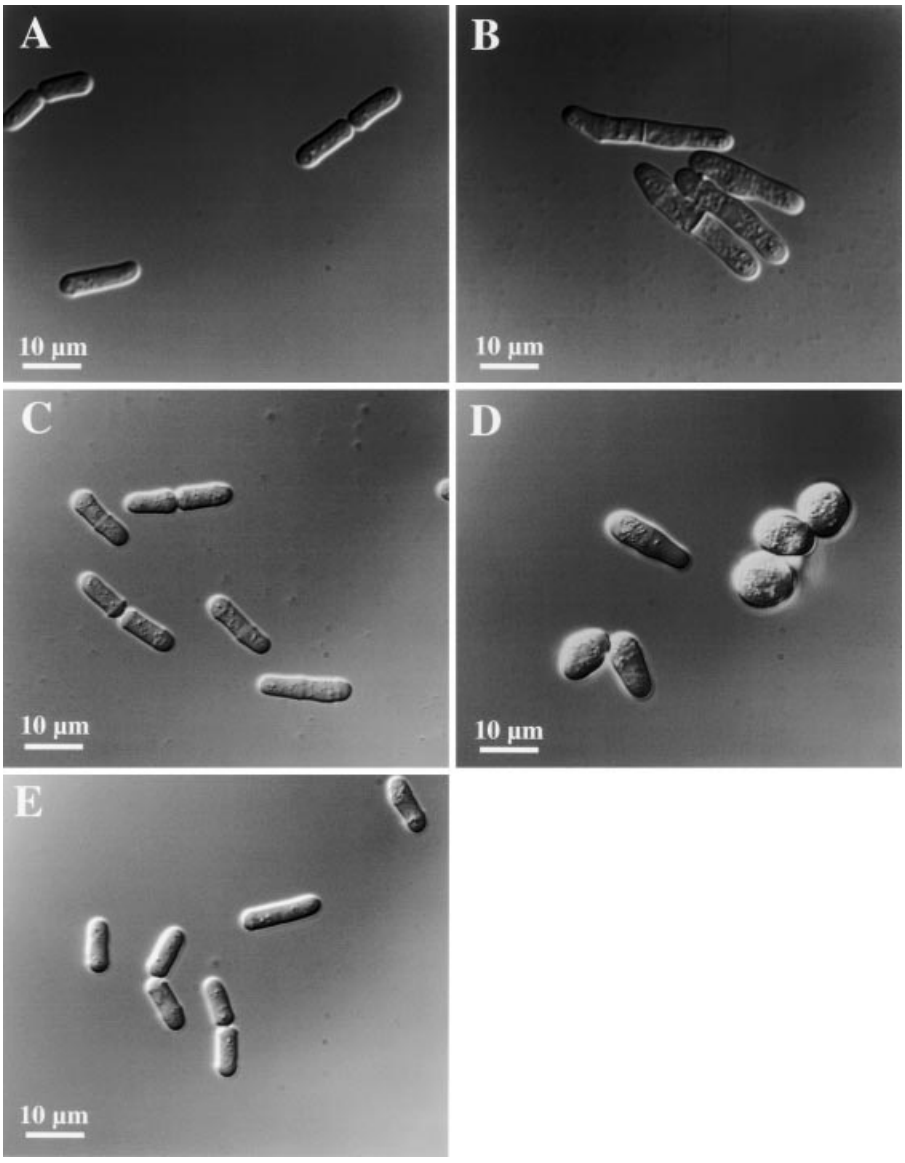

Figure 4 Differential interference contrast microscopy of exponential-phase *S. pombe* transformed with control or *aps1* overexpression plasmids

S. pombe PR1319 was transformed with: (**A**) pUR18, (**B**) pUR18*aps1*, (**C**) pREP3X, (**D**) pREP3X*aps1*, and (**E**) pREP3X*aps1*E93Q.

increased by approx. 3-fold in the *aps1* disruptant. There were no significant changes in the intracellular levels of $[PP]_2$-InsP_4, InsP_6, inositol 1,3,4,5,6-pentakisphosphate or D/L-inositol 1,2,4,5,6-pentakisphosphate + inositol 1,2,3,4,6-pentakisphosphate in the *aps1* disruptant.

Measurement of intracellular Ap_5A concentrations in wild-type and *aps1* disruptant strains of *S. pombe*

The intracellular concentration of Ap_5A in wild-type *S. pombe* was 0.06 ± 0.03 pmol of Ap_5A/mg of protein (approx. 4 ± 2 nM), and this concentration was unchanged by transformation of the wild-type strain with the plasmids pUR18 and pREP3X (Table 4). Figure 3(B) shows a representative HPLC profile of an Ap_nA extract from *S. pombe* 1319pREP3X. Figure 3(B) also represents a typical profile for wild-type and pUR18 transformant strains, since the Ap_5A concentration is the same in these three strains (Table 4). Disruption of *aps1* had no significant effect on the intracellular concentration of Ap_5A (Table 4). We were unable to detect Ap_6A in any of the *S. pombe* strains in which we measured Ap_5A. If Ap_6A is present in *S. pombe* during exponential growth, we estimated that its intracellular concentration must be less than 1 nM, based on the limit of instrument detectability.

Overexpression of *aps1*

aps1 was overexpressed via three different plasmids that resulted in two different levels of measurable Ap_6A hydrolase activity. PR1319 transformed with pUR18*aps1* exhibited an approx. 60% increase in Ap_6A hydrolase activity in comparison with the control plasmid transformant (Table 1). This strain also showed a 20% increase in generation time (Table 2), and the cells were slightly swollen, showing increases in both length and width (Figures 4A and 4B). PR1319 cells transformed with pUR18

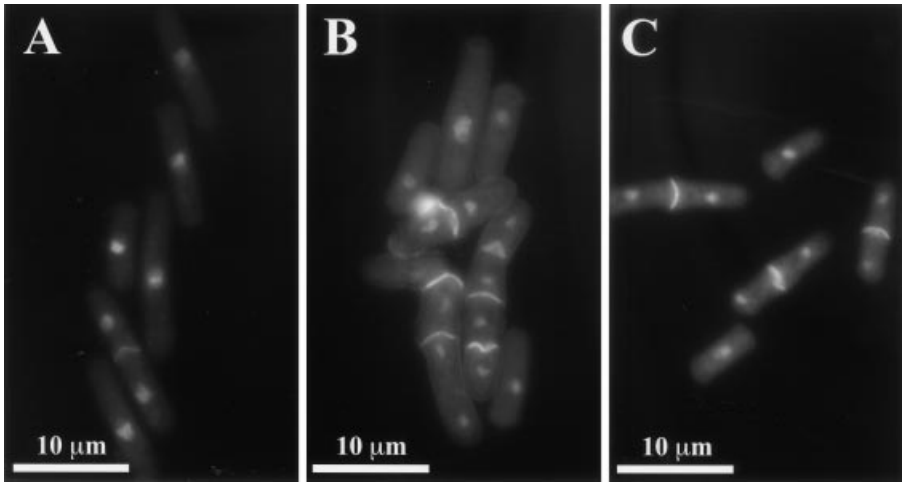

Figure 5 Fluorescence microscopy of DAPI/calcofluor-stained *S. pombe* transformed with pPOX, pPOX*aps1* or pPOX*aps1*E93Q

(**A**) Exponential-phase *S. pombe* PR1319 transformed with pPOX; (**B**) resuspended microcolony of *S. pombe* PR1319 transformed with pPOX*aps1*; (**C**) exponential-phase *S. pombe* PR1319 transformed with pPOX*aps1*E93Q.

were 12.8 ± 1.1 μm long and 2.4 ± 0.1 μm wide at division, while PR1319 cells transformed with pUR18*aps1* were 15.2 ± 3.0 μm long and 3.7 ± 0.6 μm wide at division ($n = 11$). PR1319 transformed with pREP3X*aps1* exhibited an approx. 6-fold increase in Ap_6A hydrolase activity in comparison with control plasmid transformant (Table 1), and an approx. 2.4-fold increase in generation time (Table 2). The morphological changes that occurred in PR1319 transformed with pREP3X*aps1* appeared to increase in severity along with the increased enzymic activity. These cells were also swollen and cultures contained round cells, 'round bottom flask'-shaped cells, and some dividing cells in which it appeared that one half of the cell died before completing cell separation (Figures 4C and 4D). Transformation of PR1319 with pPOX*aps1* resulted in transformants that grew as microcolonies on agar plates. We could not culture these transformants reproducibly in liquid medium. Hence growth of a sufficient mass of yeast for measuring Ap_6A hydrolase activity was not possible. We believe the the Aps1 expression level in PR1319 pPOX*aps1* is higher than in PR1319 pUR18*aps1* or PR1319 pREP3X*aps1*, based on the lack of growth of PR1319 pPOX*aps1* in liquid medium and the altered morphology of the transformants (Figure 5). In addition, previous overexpression of *S. pombe aph1* using pPOX has been shown to give high expression levels of Aph1, an Ap_4A hydrolase [30]. Microscopic examination of microcolonies of PR1319 transformed with pPOX*aps1* revealed enlarged cells with grossly altered cell morphology, including rounded, elongated and multiseptate cells (Figure 5).

Overexpression of *aps1*E93Q

To ascertain that the enzymic activity of Aps1 is required for manifestation of the observed phenotypic changes upon overexpression of *aps1*, a site-directed mutant, Aps1E93Q, was overexpressed. This mutant of Aps1 was predicted to have negligible catalytic activity, based on knowledge of residues critical for enzymic activity in other nudix hydrolases [40,41]. An E93Q mutation in Aps1 corresponds to an E70Q mutation in human DIPP and an E57Q mutation in *E. coli* MutT, and both of the latter mutated enzymes are catalytically inactive [40,41]. Assay of wild-type Aps1 and Aps1E93Q enzymes (both purified to apparent homogeneity using previously described methods [1]) with 200 μM [^{3}H]Ap_6A showed that Aps1E93Q had 3×10^{-4} times the Ap_6A hydrolase activity of Aps1. PR1319 transformed with pREP3X*aps1*E93Q showed no increase in Ap_6A hydrolase activity (Table 1), no detectable change in morphology (Figure 4E), and an insignificant increase in generation time (Table 2) in comparison with the control plasmid transformant. Similarly, PR1319 transformed with pPOX*aps1*E93Q showed no detectable change in morphology (Figure 5) and exhibited the same growth rate as PR1319pPOX (results not shown).

Measurement of intracellular diphosphoinositol polyphosphate and Ap_5A concentrations in *S. pombe* overexpressing *aps1*

Measurement of diphosphoinositol polyphosphates in PR1319 transformed with pUR18*aps1* or pREP3X*aps1* revealed no significant changes in the intracellular concentrations of $[PP]_x$-InsP_x, PP-InsP_5 or $[PP]_2$-InsP_4 in comparison with corresponding control strains transformed with plasmid alone or pREP3X*aps1*E93Q (Table 3). PR1319 transformed with pUR18*aps1* or pREP3X*aps1* displayed a 2.5-fold or 55-fold increase respectively in the intracellular Ap_5A concentration when compared with the corresponding control strain transformed with plasmid alone or pREP3X*aps1*E93Q (Table 4 and Figures 3C and 3D).

DISCUSSION

To our knowledge, this is the first report of Ap_5A in any unicellular organism. The estimated intracellular concentration of 4 nM Ap_5A is approx. 14-fold lower than the intracellular concentration of Ap_4A in *S. pombe* [30]. The intracellular source of Ap_5A is unknown. *In vivo* synthesis of Ap_5A by aminoacyl-tRNA synthetases analogous to the synthesis of Ap_4A and Ap_3A would require p_4A as substrate. Neither p_5A nor p_4A have been reported in *S. pombe*, but both are present in *Sacch. cerevisiae* during sporulation [42].

The present work also shows that *S. pombe* contains *PP*-InsP_5 and $[PP]_2$-InsP_4, as well as $[PP]_x$-InsP_x, a diphosphorylated inositol polyphosphate not described previously in intact cells. The intracellular concentrations of *PP*-InsP_5 and $[PP]_2$-InsP_4 in *S. pombe* of 1.1 μM and 2.2 μM respectively are approx. 5- and 7-fold higher than the corresponding values in *Sacch. cerevisiae* [4]. *S. pombe* showed decreased levels of $[PP]_2$-InsP_4 [3 ± 2 % of InsP_6 level; mean $\pm$ S.D., $n = 5$)] in stationary phase (S. T. Safrany, unpublished work). The intracellular concentrations of diphosphoinositol polyphosphates in mammalian cells are approx. 0.5–3 % of the InsP_6 concentration [3], revealing that the relative levels of these compounds in *S. pombe* (Table 3) are similar to the relative levels in mammalian cells.

Based on the elution of $[PP]_x$-InsP_x relative to *PP*-InsP_5, the identity of $[PP]_x$-InsP_x is likely to be $[PP]_2$-InsP_3, described recently as a twice-phosphorylated product of InsP_5 [16]. The accumulation of such levels of $[PP]_x$-InsP_x would suggest that, while levels of InsP_5s are low in *S. pombe*, their turnover is rapid. Indeed, the accumulations of $[PP]_x$-InsP_x and *PP*-InsP_5 are comparable, despite the steady-state level of the InsP_5 precursor for $[PP]_x$-InsP_x being 50-fold lower than that of the InsP_6 precursor for *PP*-InsP_5. This suggests that $[PP]_x$-InsP_x is the most metabolically active diphosphoinositol polyphosphate.

Disruption of *aps1* led to significant increases in the intracellular concentrations of $[PP]_x$-InsP_x and *PP*-InsP_5, but no detectable changes in the intracellular concentrations of Ap_5A and $[PP]_2$-InsP_4. Based on these results, we conclude that Aps1 functions *in vivo* to degrade the diphosphoinositol polyphosphates $[PP]_x$-InsP_x and *PP*-InsP_5, rather than Ap_5A. This conclusion is supported by K_m values of Aps1 of 31 nM for *PP*-InsP_5 [2] and 22 μM for Ap_5A [1], and the *in vivo* concentrations of these two compounds. It is unclear why levels of $[PP]_2$-InsP_4 were unchanged, given the *in vitro* activity on this compound [2], but this may reflect tight regulation or compartmentation.

Based on results obtained with the *aps1* diruptant strain, we expected the intracellular concentrations of $[PP]_x$-InsP_x and *PP*-InsP_5 to decrease and those of Ap_5A and $[PP]_2$-InsP_4 to remain unchanged upon overexpression of *aps1*. However, overexpression of *aps1* via pUR18 and pREP3X, as indicated by increased enzymic activity, did not correlate with changes in the intracellular concentration of any of the measured inositol polyphosphates. These measurements only reflect whole-cell changes in diphosphoinositol polyphosphate concentrations, and would not necessarily detect any changes in localized pools. Overexpression of *aps1* led to a paradoxical increase in the intracellular Ap_5A concentration. While the reason for this increase is not clear, one possibility is that the slow growth and swollen state of the cells caused by *aps1* overexpression somehow affected the rate of Ap_5A synthesis or its rate of degradation by another enzyme. The failure of Ap_5A levels to increase when the inactive point mutant was overexpressed suggests that Aps1 may be directly responsible for the increased Ap_5A, i.e. that under the conditions in some microenvironment in the cell, the enzyme can synthesize Ap_5A. However, in *in vitro* tests with Aps1, 1 mM ATP and various combinations of metals and pH, we did not detect Ap_5A synthesis (results not shown).

Overexpression of *aps1* resulted in phenotypic changes in growth rate and cell morphology. The extent of these changes clearly depended upon the level of overexpression and the enzymic activity of Aps1. The pUR18*aps1* transformant strain exhibited a phenotypic change in which the cells appeared swollen. We could find no reports of an identical phenotypic change in *S. pombe*. The pREP3X*aps1* transformant strain showed more drastic phenotypic changes. These cells appeared to be more rounded, and they were morphologically similar to *S. pombe* with defects in cell wall synthesis [43]. Overexpression of *aps1* via pPOX*aps1* resulted in almost no cell growth and the presence of a low percentage of multiseptate cells. Multiseptate cells are characteristic of many different mutant strains of *S. pombe*, including some with defective cell wall synthesis [43].

The molecular basis for the phenotypic changes occurring upon overexpression of *aps1* is unknown. One possibility is perturbation of adenine mononucleotide metabolism by Ap_5A-mediated inhibition of adenylate kinase. The intracellular concentrations of Ap_5A obtained upon overexpression of *aps1* are 3–60 times greater than the K_i value of 2.0–3.6 nM for the inhibition of adenylate kinase activity by Ap_5A *in vitro* [44]. When the intracellular Ap_4A concentration in *S. pombe* [30] was approx. 1.5 times the K_i value of 11 μM for *in vitro* inhibition of adenylate kinase activity by Ap_4A [45], no phenotypic changes were observed [30].

A second possibility is perturbation of $[PP]_x$-InsP_x and *PP*-InsP_5 function. Work in *Sacch. cerevisiae* demonstrated that InsP_6 is required for mRNA export from the nucleus [4,22,23]. InsP_6 is a precursor for *PP*-InsP_5 synthesis in *Sacch. cerevisiae* through the action of an InsP_6 kinase encoded by *KCS1* [24]. Saiardi et al. [24] suggested that inositol polyphosphates, including *PP*-InsP_5 and $[PP]_2$-InsP_4, rather than InsP_6 itself, play a direct role in mRNA export. However, wild-type levels of mRNA export in a *kcs1*Δ yeast strain [16,46] and lack of identification of *KCS*1 in a synthetic lethal screen of a *gle1* mRNA export mutant strain [22,46] weigh against this proposal.

The *kcs1*Δ yeast strain exhibits abnormally small and fragmented vesicles rather than wild-type vacuoles, which suggests that *PP*-InsP_5 or $[PP]_2$-InsP_4 functions in vacuolar protein trafficking or vesicle fusion [16]. Recent results obtained with Kcs1 mutated in specific sequence motifs revealed different functional roles of the protein. Mutagenesis of the 'kinase' motif demonstrates that *PP*-InsP_5 and/or $[PP]_2$-InsP_4 are required for biogenesis of the yeast vacuole, integrity of the cell wall, and resistance to high NaCl concentrations [47]. Mutagenesis of a leucine heptad motif, which did not affect the synthesis of *PP*-InsP_5 and $[PP]_2$-InsP_4, demonstrates that this element of Kcs1 was also necessary for vacuolar biogenesis and cell wall integrity, but not resistance to NaCl stress [47]. The results of these groups [16,47] extend previous results cited in the Introduction indicating that diphosphoinositol polyphosphates may function in vesicle trafficking. If $[PP]_x$-InsP_x and/or *PP*-InsP_5 function in vesicle trafficking and cell wall maintenance, then overexpression of *aps1* could be inhibitory to these processes. Conceivably, the changes in morphology observed upon overexpression of *aps1* are a result of altered protein trafficking necessary for cell wall synthesis or establishment of cell shape and size.

Yamashita et al. [48] reported that *cut20* (cell untimely torn) encodes a protein that is a component of the 20 S cyclosome complex that promotes the metaphase–anaphase transition in *S. pombe*. *cut20* is identical with *lid1* [48], which Berry et al. [49] demonstrated to be an essential gene. A *cut20* temperature-sensitive mutant exhibits mitotic arrest with aberrant morphology, including condensed chromosomes, undivided nucleus cut by a septum, and displaced nucleus. This temperature-sensitive mutant is complemented by high-dosage expression of a gene that Yamashita et al. [48] designated *stw1*. Stw1 [48] is identical with Aps1 [1]. In light of the phenotypic changes that we observed upon overexpression of *aps1* (Figures 4 and 5), the suppression of *cut20* by overexpression of *stw1*, i.e. *aps1*, is somewhat surprising. The net cellular effect of *aps1* (*stw1*) is undoubtedly dependent upon its level of expression. Yamashita et al. [48] did not report any results on overexpression of *stw1* alone in a wild-type strain. They did show that Stw1 (Aps1) is not

a component of the cyclosome, and they suggested that Stw1 (Aps1) might function as a positive regulator of cyclosome formation [48]. The functional relationships among Aps1 (Stw1), Cut20 (Lid1) and other proteins related to the cyclosome remain to be determined.

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