Disruption and overexpression of the *Schizosaccharomyces pombe* aph1 gene and the effects on intracellular diadenosine 5’‚5''’-P1,P4-tetraphosphate (Ap4A), ATP and ADP concentrations

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Diadenosine oligophosphates are ubiquitous compounds that were discovered over 30 years ago. Diadenosine 5’‚5''’-P1,P4-tetraphosphate (Ap4A) is the most studied member of this family, and its function in yeast is unknown. To investigate possible functions, we changed the intracellular Ap4A concentration in *Schizosaccharomyces pombe* via disruption and overexpression of the aph1 gene, which encodes an Ap4A hydrolase (Aph1). *S. pombe* Aph1 is 52% identical with a human tumour suppressor protein, Fhit, in a core region of 109 amino acids. Disruption of aph1 resulted in an 85% decrease in intracellular Ap4A hydrolyase activity and a 290-fold increase in the intracellular Ap4A concentration. The disruption and subsequent increase in intracellular Ap4A concentration had no significant effect on the growth of *S. pombe*. Overexpression of the *S. pombe* aph1 gene, resulting in 17- and 84-fold increases in Ap4A hydrolyase activity above wild-type levels, resulted in 60 and 80% decreases respectively in the intracellular Ap4A concentration. This represents the first report of a decrease in the intracellular Ap4A concentration in response to overexpression of a degradative enzyme in any eukaryotic organism. We describe a new *S. pombe* expression plasmid, pPOX, which was used to achieve the largest increase in expression of aph1. Overexpression of aph1 at the highest level resulted in a 46% increase in generation time in comparison with the control strain. Neither overexpression nor disruption had any effect on the intracellular ATP or ADP concentrations. This is the first report of ADP and ATP concentrations in *S. pombe*. These data also indicate that Aph1 functions in *vivo* to degrade Ap4A, and that high-level overexpression of this enzyme reduces the growth rate.

Key words: adenine nucleotides, diadenosine oligophosphates, fission yeast, gene manipulation.

INTRODUCTION

The diadenosine oligophosphates are a family of compounds first discovered over 30 years ago [1]. These compounds consist of two adenosine moieties joined together through their 5’ carbons by a chain of phosphate molecules to give a diadenosine 5’‚5''’-P1,P4-oligophosphate (ApnA; n = 2–7) [2]. ApnA, a commonly studied family member, has been found in all tissues and organs examined for its presence, usually at levels of between 0.01 and 1 µM [3]. The discovery that the Fhit tumour suppressor protein behaves as an ApnA hydrolase in *vivo* [4] has increased interest in the *vivo* function(s) of diadenosine oligophosphates. The fission yeast *Schizosaccharomyces pombe* contains an Ap4A hydrolase (Aph1) [5] which is 52%, identical with Fhit in a core region of 109 amino acids [6]. The present study was aimed at gaining insight into the functions of Aph1 and Ap4A in the fission yeast *S. pombe* using genetic manipulation of the aph1 gene. Clues as to the *vivo* function of Aph1 and Ap4A in *S. pombe* might also be useful in determining the *vivo* function of the Fhit tumour suppressor.

The only known method of *vivo* synthesis of Ap4A occurs as a side reaction during the charging of a tRNA molecule [7]. This reaction was verified as an *vivo* source of Ap4A in *Escherichia coli* by overexpressing the genes encoding four different tRNA synthetases that synthesize Ap4A *vivo* and measuring the concomitant increases in the intracellular Ap4A concentration [7]. Three enzymes have been identified in yeasts, two in *Saccharomyces cerevisiae* and one in *S. pombe*, that degrade Ap4A. The *S. pombe* aph1 gene product (the subject of the present study) is an orthologue of Fhit [6], but prefers Ap4A as substrate [5]. Aph1 contains a histidine triad (HIT) sequence motif and is a member of the GAFH (GalT, Ap4A phosphorylase, Fhit, Hnt) protein family [8]. Ap4A phosphorylases I and II, encoded by the APA1 and APA42 genes from *Sacch. cerevisiae* respectively [9–11], are both members of the GAFH protein family [8] and prefer Ap4A as substrate.

Here, we report the use of molecular genetic techniques to both raise and lower the intracellular concentration of Ap4A. Specifically, we report the disruption and overexpression of the *S. pombe* aph1 gene. Disruption of aph1 led to a 290-fold increase in the intracellular Ap4A concentration, but no change in growth rate. Overexpression of aph1 via a new *S. pombe* expression plasmid, leading to an 84-fold increase in Ap4A hydrolyase activity, resulted in an 80% decrease in the intracellular Ap4A concentration. This strain exhibited a 46% increase in generation time. There was no significant change in the intracellular ATP and ADP concentrations in either the disruption or the overexpression strain. These experiments show for the first time that genetic manipulation of an Ap4A degradative enzyme can be

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**Abbreviations used:** ApnA, diadenosine 5’‚5''’-P1,P4-oligophosphate (n = 2–7); ApnN, adenosine (5’)tetraphospho(5’)nucleoside

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used to lower the intracellular Ap4A concentration in a eukaryotic organism. These results also indicate that the high-level overexpression of the aph1 gene may affect the rate of cell growth. This is also the first report on the intracellular concentrations of ATP and ADP in *S. pombe*.

A preliminary report of this work has been published [12].

**MATERIALS AND METHODS**

**S. pombe strains**

The *S. pombe* strain used as wild type was PR1319 h+ ade6-210 leu1-32 his7-366 ura4-D18. The aph1 disruption and overexpression strains were constructed as described below.

**Growth**

Cells were grown using standard procedures [13]. Generation times were calculated from the linear portion of a plot of log *D* vs against time. In some experiments, cell density was determined by counting cells in a haemocytometer.

**Construction of the aph1 disruption vector**

pBluescript II KS(+) (Stratagene) containing the aph1 gene on a 1.2 kb EcoRI fragment was used as the source of aph1 [5]. A 441 bp fragment of the open reading frame of aph1 was removed by treatment of this plasmid with *Bsa*I and *Bsa*36I. This fragment was replaced by a 2.1 kb fragment containing the ura4 gene from *S. pombe* that was cut out of the pUD18 plasmid [14] with *Nar*I and *Asel*. The replacement was accomplished by filling the AT overlaps of the two fragments to be ligated together with the Klenow fragment of *E. coli* DNA polymerase I, resulting in both fragments having one GC overhang and one blunt end. The two fragments were ligated together with T4 DNA ligase, and the resulting plasmid was named pDis22. This plasmid contains the ura4 gene flanked by 359 bp of the 5′ region of aph1 and 386 bp of the 3′ region of aph1.

**Disruption of the aph1 gene**

Approx. 5 μg of EcoRI-digested pDis22 was used to transform *S. pombe* PR1319 using a lithium acetate-based procedure [15]. A total of 18 transformants were screened for a decrease in Ap4A hydrolase activity. Transformants were grown overnight in 50 ml of rich medium. The cells were then harvested, and the crude supernatants were prepared and assayed as described below.

**Southern blots**

Genomic DNA was isolated as described in [16]. Southern blotting was done as described in [17]. The aph1 CDNA probe was labelled using a Sigma random primer labelling kit and [32P]dCTP (New England Nuclear).

**Construction of pPOX**

pPOX was constructed from pDblet [18] and pCDNA 3.0 (Invitrogen). Initially, an *Xho*I site in the multiple cloning site of pDblet was eliminated by cleaving with *Xho*I, blunting both ends with the Klenow fragment of *E. coli* DNA polymerase I and religating the ends. This plasmid was named pDblet2. The cytomegalovirus-promoter–MCS-bovine-growth-hormone (where MCS is multiple cloning site) poly(A) tail cassette from pCDNA 3.0 was amplified with PCR primers 5′-ataaacggcaggttaggtggtgcgttt-3′ and 5′-agtgcattactttgctgcgt-3′ (*SacII* and * ClaI* sites are underlined). The resulting PCR product and pDblet2 were digested with *SacII* and *ClaI* and ligated together with T4 ligase to yield pPOX (Pombe Overexpression).

**Construction of pPOXluc**

pPOXluc was constructed by ligating together *HindIII/XhoI*-digested pPOX and the *HindIII/XhoI* fragment from pGEM-luc (Promega) that contains luc.

**Assay of luciferase activity**

Crude supernatant fraction from *S. pombe* PR1319 transformed with pPOXluc was assayed in 100 μl of 25 mM Hepes, pH 7.75, containing 4 mM MgCl2, 25 nM ATP and 2 μM luciferin (Sigma). Relative light output was measured in a Tropix luminometer.

**Overexpression of aph1**

Two plasmids were used for overexpression of aph1. First, aph1 was overexpressed using pYH1 [5]. This plasmid is pFL20 containing a 3.0 kb fragment of *S. pombe* genomic DNA that contains the aph1 gene. The second plasmid used for overexpression of aph1 was pPOXaph1. pPOXaph1 was made by removing the open reading frame of aph1 from pYH6 [5] by digestion with EcoRI and *XhoI* and ligating this fragment into EcoRI/XhoI-digested pPOX. Both plasmids were verified by DNA sequencing. *S. pombe* PR1319 was transformed with plasmids using a lithium acetate-based procedure [15].

**SDS/PAGE analysis**

Crude supernatant fractions were obtained from exponential-phase cultures of *S. pombe* PR1319 transformed with pPOXaph1 or pPOXluc. Crude supernatant samples were subjected to electrophoresis on 15% (w/v) polyacrylamide gels under denaturing protein conditions using a discontinuous buffer system [19]. Gels were stained in Coomassie Blue and destained in 10% trichloroacetic acid. Molecular mass markers were from Sigma (Sigma Mark VII-L, SDS 7).

**Preparation of cellular extracts for nucleotide measurements and measurement of Ap4A hydrolase activity**

Cells were grown in 250 or 500 ml of 2 × minimal medium with the appropriate supplements [13]. When the cells reached the appropriate density, three independent samples (50 or 100 ml each) from each culture were removed and harvested by rapid filtration on 1.2 μm-pore-size Millipore filters, suspended in 5% (w/v) trichloroacetic acid containing 2 pmol of [3H]Ap4A as an internal standard, and processed for measurement of Ap4A as described previously [20]. One 1-ml rinse of the filter with 5% (w/v) trichloroacetic acid was added to the resuspended sample before sonication, and the sonicated trichloroacetic acid extracts sat on ice for at least 45 min before centrifugation (43,000 g, 30 min) to obtain trichloroacetic acid supernatants and crude pellets. The remaining 100 or 200 ml of cell culture was harvested and used for determining the Ap4A hydrolase activity (see below).

**Measurement of intracellular Ap4A concentration**

Trichloroacetic acid supernatants were purified using chromatography on boronate-derivatized resin, treated with alkaline phosphatase and analysed quantitatively for Ap4A by HPLC [21] with the following modifications: a Mono-Q HR 5/5 column
(Amersham Pharmacia Biotech) was used instead of a Partisil SAX column, and the resin used in the precolumn was Source 30Q resin (Amersham Pharmacia Biotech). Ap₄A was eluted with 0.2 M ammonium bicarbonate, pH 8.5. The [³H]Ap₄A radioactivity in a portion of the eluted Ap₄A was measured by liquid scintillation counting to determine recovery for each sample. Measured Ap₄A values were corrected for recoveries, which averaged 50 ± 6 % (n = 41).

Measurement of intracellular ATP and ADP concentrations

An aliquot of each trichloroacetic acid supernatant was diluted into 20 mM NH₄HCO₃, pH 8.5, and then subjected to gradient elution from 50 mM to 383 mM NH₄HCO₃, pH 8.5, over a 30 min period on a MonoQ column, to analyse ATP and ADP levels. Mononucleotides were measured quantitatively by area integration of peaks in comparison with standards. Recoveries were assumed to be 100 %. We were unable to measure AMP under these conditions due to contaminants eluting with or near the AMP peak.

Measurement of Ap₄A hydrolase activity

Cells from the same cultures used for measurement of the nucleotides were harvested by centrifugation as above, and crude supernatants were prepared as described previously [5]. Ap₄A hydrolase activity was assayed using 100 μM [³H]Ap₄A as substrate, with subsequent separation of products and residual substrate using chromatography on boronate-derivatized resin [22]. Assays were carried out using both dialysed and undialysed crude supernatant in the presence or absence of 0.2 mg/ml BSA. Neither dialysis nor BSA affected the measured activities, but the BSA was necessary to stabilize the enzyme at high dilution. Activity was expressed as nmol of AXP formed/min per mg of protein, where AXP = AMP + ATP.

Determination of protein mass

Crude pellets obtained by centrifugation of trichloroacetic acid extracts were washed with 95 % (v/v) ethanol and dissolved in 2–3 ml of 2 M NaOH for determination of protein [23]. Protein masses in crude supernatant fractions used in assay of Ap₄A hydrolase activity also were measured as in [23].

Data analysis

For measurement of nucleotides, 3–12 independent samples of each strain were analysed. Values (mean ± S.D.) are expressed in pmol or nmol of nucleotide per mg of protein, which was measured quantitatively in trichloroacetic acid pellets as described above. Statistical analyses were performed with Excel software using a two-tailed group t-test assuming unequal variance. Statistical significance was assumed at P < 0.05.

To estimate the in vivo concentration of nucleotides in S. pombe, we used the following parameters. Based on the cell density of cultures at the time of harvest and the protein mass in trichloroacetic acid pellets, we calculated a value of 9.5 ± 1.9 pg of protein per cell (n = 12). This value is in the range of values of 7–14 pg of protein per cell reported for cells grown under different conditions [24,25]. The volume of a single cell was taken as 0.14 pl, based on reported values of 0.140–0.148 pl/cell [26–28].

![Figure 1 Southern blot analysis of genomic DNA from PR1319 and PR1319 aph1Δ::ura4 probed with [³2P]dCTP-labelled aph1 cDNA](https://example.com/image)

**Table 1** Ap₄A hydrolase activity in Schizosaccharomyces pombe

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ap₄A hydrolase activity (nmol of AXP/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR1319</td>
<td>1.77 ± 0.15</td>
</tr>
<tr>
<td>PR1319 aph1Δ::ura4</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td>PR1319 + pFL20</td>
<td>1.37 ± 0.25</td>
</tr>
<tr>
<td>PR1319 + pFL20 aph1</td>
<td>23.6 ± 3.76</td>
</tr>
<tr>
<td>PR1319 + pDX</td>
<td>1.70 ± 0.20</td>
</tr>
<tr>
<td>PR1319 + pDX aph1</td>
<td>143 ± 22.7</td>
</tr>
</tbody>
</table>
Table 2  Intracellular Ap4A, ATP and ADP concentrations in Schizosaccharomyces pombe

Data were generated from exponential-phase cultures grown in minimal medium with the appropriate supplements at 30 °C, as described in the Materials and methods section. Values are means ± S.D. for the numbers of measurements shown in parentheses. Intracellular concentrations of Ap4A, ATP and ADP were estimated using the cell parameters, as described in the Materials and methods section. Significance of differences: *P < 0.05 compared with wild-type strain PR1319; †P < 0.05 compared with strain transformed with the corresponding control plasmid. The Ap4A concentration (pmol/mg) with PR1319 + pPOXaph1 is not statistically significantly different from that with PR1319 + pFL20aph1 at the P = 0.05 level.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ap4A (pmol/mg of protein)</th>
<th>ATP (nmol/mg)</th>
<th>ADP (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR1319 (n = 12)</td>
<td>0.84 ± 0.39</td>
<td>13.9 ± 1.6</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>PR1319+aph1Δ::ura4 (n = 6)</td>
<td>245 ± 98†</td>
<td>14.3 ± 2.0</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>PR1319+pFL20 (n = 8)</td>
<td>0.69 ± 0.08</td>
<td>15.0 ± 1.3</td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td>PR1319+pFL20aph1 (n = 5)</td>
<td>0.28 ± 0.18†</td>
<td>15.7 ± 3.0</td>
<td>3.4 ± 0.6</td>
</tr>
<tr>
<td>PR1319+pPOX (n = 3)</td>
<td>0.70 ± 0.10</td>
<td>15.0 ± 1.2</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>PR1319+pPOXaph1 (n = 3)</td>
<td>0.14 ± 0.06†</td>
<td>13.0 ± 0.8</td>
<td>3.8 ± 0.4</td>
</tr>
</tbody>
</table>

Table 3  Growth rates of Schizosaccharomyces pombe lacking or overexpressing aph1

Generation times were determined from cultures at 30 °C in minimal medium with appropriate supplements, as described in the Materials and methods section. Values are means ± S.D. (n = 3), except where indicated († measured values in two experiments). Significance of difference compared with strain transformed with the corresponding control plasmid: *P < 0.05.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Generation time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR1319</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>PR1319+aph1Δ::ura4</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>PR1319+pFL20</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>PR1319+pFL20aph1</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td>PR1319+pPOX</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td>PR1319+pPOXaph1</td>
<td>5.4 ± 0.4*</td>
</tr>
<tr>
<td>PR1319+pPOXlac</td>
<td>3.8, 4.2†</td>
</tr>
</tbody>
</table>

the presence of another enzyme relatively specific for Ap4A. Disruption of the aph1 gene had no detectable effect on the growth rate in minimal medium (Table 3) or in rich medium. The generation times in rich medium were 2.3 ± 0.1 h (n = 3) and 2.0 ± 0.1 h (n = 3) for wild-type and aph1 disruptant strains respectively. The aph1Δ::ura4 strain mated with wild-type cells of the opposite mating type. An aph1+/aph1Δ::ura4 diploid sporulated, and its morphology and size at cell division were the same as that of the diploid wild-type strain. Although the intracellular concentration of Ap4A was increased by a factor of 290 in the aph1 disruptant strain, the intracellular ATP and ADP concentrations were essentially the same as in the wild-type strain (Table 2).

Overexpression of aph1

The aph1 gene was overexpressed under the control of its own promoter on the plasmid pFL20 (pFL20aph1). S. pombe containing pFL20aph1 exhibited a 17-fold increase in Ap4A hydrolase activity (Table 1) and a 60% decrease in intracellular Ap4A concentration (Table 2) relative to the pFL20 control strain. Overexpression of aph1 via pFL20 had no significant effect on the intracellular concentrations of ADP or ATP (Table 2). To express aph1 at a higher level, a new S. pombe expression plasmid was constructed as described in the Materials and methods section. This vector was named pPOX (Pombe Overexpression) (Figure 2). The aph1 gene was overexpressed under the control of the cytomegalovirus promoter on pPOX. S. pombe transformed with pPOXaph1 exhibited an 84-fold increase in Ap4A hydrolase activity (Table 1) and an 80% decrease in intracellular Ap4A concentration (Table 2) relative to the pPOX control strain. Overexpression of aph1 via pPOX did not significantly affect the intracellular concentrations of ADP or ATP (Table 2), nor did it affect cell morphology. Overexpression of aph1 via pPOX increased the generation time by about 46% relative to the pPOX control strain (Table 3).

To address the possibility that the decreased growth rate in S. pombe PR1319 transformed with pPOXaph1 was simply related
DISCUSSION

This is the first report of the measurement of intracellular Ap$_A$, ATP and ADP concentrations in *S. pombe*. The measured value of 0.84 pmol of Ap$_A$/mg of protein and the estimated concentration of 57 nM Ap$_A$ are generally lower than the corresponding values from *Sacch. cerevisiae*. Previously reported values in *Sacch. cerevisiae* are 3.6 pmol of Ap$_A$/mg of protein [11,21] and 0.58 pmol of Ap$_A$/mg of protein [20]. These values can be estimated as concentrations of 310 nM and 50 nM Ap$_A$ respectively, using cell parameters for *Sacch. cerevisiae* [30]. Concentrations of 550 nM Ap$_A$ [31], 400 nM adenosine(5')-triphospho(5')-nucleoside (Ap$_N$) [32] and 80 nM Ap$_N$ [33] have also been reported. The basal concentration of Ap$_A$ in *S. pombe* is similar to the basal concentration determined in other organisms and cells, including *Physarum, Tetrahymena* and CHO cells [3].

The concentrations of 0.94 mM ATP and 0.22 mM ADP in *S. pombe* are also lower than corresponding values in *Sacch. cerevisiae*, which range from 1.3 to 5.2 mM ATP [31,34-40] and from 0.30 to 0.66 mM ADP [35,37-40]. (Some values reported in the cited references were converted to mM concentrations using cell parameters for *Sacch. cerevisiae* [30].) In both fission and budding yeasts, the concentration of Ap$_A$ is about 0.0001 times that of ATP.

Disruption of *aph1* in *S. pombe* resulted in a much larger increase in intracellular Ap$_A$ concentration than occurred upon disruption of two genes encoding Ap$_A$ degradative enzymes in *Sacch. cerevisiae*. Upon disruption of *APA1*, which encodes Ap$_A$ phosphorylase I [9], the concentration of intracellular Ap$_A$ in *Sacch. cerevisiae* increases by a factor of 3-5 [10,20], while disruption of *APA2* causes no detectable change in the intracellular Ap$_A$ concentration [10]. However, disruption of both phosphorylase genes leads to a 38-fold increase in intracellular Ap$_A$ concentration [10]. The 290-fold increase in intracellular Ap$_A$ concentration upon disruption of *aph1* occurred in spite of the residual Ap$_A$ degradative activity. This result suggests that either the degradative enzymes or Ap$_A$, or both, may be compartmentalized in *S. pombe*. Although disruption of the genes encoding Ap$_A$ degradative enzymes was successful in increasing intracellular Ap$_A$ in both yeasts, neither exhibited a detectable change in phenotype. *S. pombe* with *aph1* disrupted showed no change in growth rate in minimal and rich media, and appeared to mate and sporulate normally. *Sacch. cerevisiae* strains with either *APA1* or *APA2*, or both *APA1* and *APA2*, disrupted show no change in growth rate in minimal medium and are capable of sporulating and mating normally [10].

In addition to the manipulations performed on *APA1* and *APA2*, the *APH1* gene from *Sacch. cerevisiae*, which encodes an Ap$_A$ hydrolase, has been disrupted alone and in an *apa1*::HIS3 *apa2*::URA3 background [32]. Disruption of *APH1* alone leads to a 31-fold increase in the intracellular Ap$_N$ concentration and no change in growth rate. The disruption of all three Ap$_A$-degrading enzymes leads to a 750-fold increase in intracellular Ap$_N$, to a concentration of approx. 300 µM [32]. The *apa1*::HIS3 *apa2*::URA3 *aph1*::TRPI strain exhibits a 50% increase in generation time relative to wild type. The authors attributed this increase to the greatly increased intracellular concentration of Ap$_N$ [32]. In contrast, no effect on growth rate was observed when the Ap$_A$ concentration was increased to approx. 16 µM in the *S. pombe* *aph1*::ura4 strain (Tables 2 and 3).

The absence of detectable phenotypic changes accompanying the large increase in intracellular Ap$_A$ concentration observed in the *S. pombe* *aph1*::ura4 strain has several implications. First, comparison of loss-of-function data between Fhit and Aph1 suggests that the two enzymes do not have the same function in *vivo*. Murphy et al. [41] measured the concentrations of Ap$_A$ and Ap$_E$ in several tumour cell lines expressing Fhit at various levels. Ap$_A$ was undetectable in two Fhit-positive cell lines, while the levels of Ap$_A$ were higher and variable in lines with low or undetectable levels of Fhit. The level of Ap$_A$ was about the same in the two sets of cells. These workers concluded that Fhit regulates the level of Ap$_A$, and not Ap$_E$, in *vivo*. The decrease in the intracellular concentration of Ap$_A$ that occurs upon overexpression of *aph1*, coupled to the increase in intracellular Ap$_A$ concentration upon disruption of *aph1*, clearly indicate that Aph1 regulates the level of Ap$_A$ in *vivo*. The conclusion that Aph1 degrades Ap$_A$ in *vivo* while Fhit does not was expected, based on data on their in *vivo* substrate specificities [4,29]. In addition, loss of *aph1* appears to have no major affect on *S. pombe* cell growth, while loss of *FHI1* in mammalian cells is correlated with abnormal cell growth resulting in tumorigenesis [8]. Secondly, the lack of phenotypic change seen in the *aph1* disruptant strain also indicates that fission yeast can tolerate large increases in intracellular Ap$_A$ concentration with no detectable effect on growth. The increase in intracellular Ap$_A$ also does not appear sufficient to cause alterations in the size of cells at division in fission yeast, as it has been suggested to do in *E. coli* [42,43]. Nishimura [43] demonstrated that two mutants of *E. coli* with 15-100-fold increases in Ap$_A$ exhibited reduced cell size at division without a change in growth rate. *S. pombe* PR1319*aph1*::ura4 showed no such change in cell size at division (results not shown).

The present work is the first report of the use of genetic manipulation to successfully lower the intracellular Ap$_A$ concentration in any eukaryotic organism. Previous work attempting to achieve a similar goal in *Sacch. cerevisiae* resulted in a paradoxical increase in intracellular Ap$_A$ concentration upon overexpression of Ap$_A$ phosphorylase I, in spite of a maximal 90-fold increase in phosphorylase activity in *vivo* [11,20]. Mechulam et al. [44] demonstrated that overexpression of *E. coli* *apaH*, which encodes a symmetrical Ap$_A$ hydrolase, decreased the intracellular Ap$_A$ concentration in *E. coli* by a factor of 10.

The finding that increased expression of Aph1 in a wild-type strain of *S. pombe* decreased the rate of growth (Table 3) also has several implications. First, the decreased growth rate of *S. pombe* PR1319 transformed with pPOXaph1 (Table 3) could be related to the decreased intracellular Ap$_A$ concentration. However, since the intracellular Ap$_A$ concentration in this strain is not statistically significantly different from that found in *S. pombe* PR1319 transformed with pFL20aph1, a strain that showed no detectable change in growth rate, a decrease in the intracellular Ap$_A$ concentration may not be the cause of the decreased growth rate in *S. pombe* PR1319 transformed with pPOXaph1. Secondly, while not strictly correlated with Ap$_A$ levels, the decreased growth rate in *S. pombe* PR1319 transformed with pPOXaph1 does seem to be specific for overexpression of *aph1*.

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rather than being an effect of general high-level protein over-expression. Thirdly, comparison of overexpression data for Aph1 and Fhit reveals the following. Increased expression of Fhit in mammalian cells containing endogenous Fhit has no detectable effect on growth [45]. However, re-introduction of Fhit into FHIT-minus cells results in suppression of tumorigenicity of cancer cells in nude mice [45,46] and growth inhibition of cancer cells due to cell cycle arrest and increased apoptosis [45,47]. Interestingly, the decreased growth rate displayed by MKN74 gastric cancer cells overexpressing Fhit upon injection into nude mice does not appear to require Ap$_4$A hydrolysis. Overexpression of a mutant form of Fhit (Fhit H96N) that displays Ap$_4$A hydrolase activity reduced to 1/10$^4$ that of wild-type Fhit also suppresses tumorigenicity [46]. This suggests that binding of substrate and/or interaction of Fhit with another protein is more important in tumour suppression than is hydrolytic activity [46]. It has been shown that Fhit H96N binds Ap$_4$A with 4-fold less affinity than wild-type Fhit [48]. Overexpression of Aph1 at a high level also results in growth inhibition in S. pombe, but, as stated above, we have not been able to strictly correlate this growth inhibition with changes in the intracellular Ap$_4$A concentration. So, as is the case with Fhit, the observed growth inhibition upon overexpression of Aph1 may not involve hydrolysis of an Ap$_4$A. This is consistent with Aph1 and Fhit sharing a similar function in vivo related to cell growth that does not require Ap$_4$A degradation.

In conclusion, these data indicate that disruption of aph1 and the resulting 290-fold increase in the intracellular Ap$_4$A concentration have no detectable effect on S. pombe growth under the conditions tested, and do not appear to affect mating and sporulation. On the other hand, high-level expression of Aph1 appears to slow growth rate, but the inhibition of growth is not clearly linked to changes in intracellular Ap$_4$A concentration. Comparison of in vitro data and in vivo overexpression and loss-of-function data for Aph1 and Fhit suggests that these two enzymes degrade different Ap$_4$As in the cell, but that their mechanism of growth inhibition involving a reaction unrelated to Ap$_4$A degradation may yet prove similar.

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