Negative regulation of mitosis by the fission yeast protein phosphatase ppa2

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To understand the role of the type 2A-like protein phosphatase in the cell division cycle, we investigated the mutant phenotypes obtained when the fission yeast ppa1+ and ppa2+ phosphatase genes (which encode polypeptides with ~80% identity to mammalian type 2A phosphatases) were either deleted or overexpressed. We also investigated the in vivo effect of okadaic acid, an inhibitor of protein serine/threonine phosphatases, on cell division. We show that ppa2+ interacts genetically with the cell cycle regulators cdc25+ and wee1+, as a ppa2 deletion is lethal when combined with wee1-50 but partially suppresses the conditional lethality of cdc25-22 mutation. Evidence that ppa2+ negatively controls the entry into mitosis, possibly through the regulation of cdc25 tyrosine phosphorylation, is presented. ppa2 phosphatase is abundant in the cytoplasm, in contrast to the type 1-like phosphatase dis2, which is enriched in the nucleus. Overproduced ppa1 or ppa2 proteins accumulate in the cytoplasm near the nuclear periphery, and cells arrest in interphase. Okadaic acid-treated cells, like a dis2 mutant strain or overexpressing the ppa2 deletion, are short in length and display protein hyperphosphorylation.

Cytokinesis is also inhibited, producing binucleated cells. We show that ppa2 is the genetic locus controlling okadaic acid sensitivity. The ppa2 deletion reveals the same hyperphosphorylated proteins as okadaic acid. When a strain deleted for ppa2 is treated with okadaic acid, cell size is reduced further to that of wee1-50 mutant strain or overexpressing the cdc25+ gene product, suggesting functional relationship of ppa2 with the cdc25 tyrosine phosphatase and/or the wee1 kinase in cell cycle control.

[Key Words: Mitosis; okadaic acid; cytokinesis; wee1 kinase; cdc25 tyrosine phosphatase; cdc2 kinase]

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Protein serine/threonine phosphatases are essential in the cell division cycle. This was demonstrated by employing mitosis-blocked mutants in the filamentous fungi Aspergillus nidulans BimG (Doonan and Morris 1989) and the fission yeast Schizosaccharomyces pombe dis2+ (Ohkura et al. 1989) genes, which encode polypeptides highly similar (~80% identical) to PP1. A Drosophila mutant in one of the four PP1-related genes was found to produce a similar mitotic defect (Axton et al. 1990). Definitive evidence for the essentiality of PP1-related phosphatases in cell division was provided in fission yeast by the lethality of the double gene disruption of dis2+ and sds21+ (designated Δdis2-Δsds21), the latter gene codes for a polypeptide highly similar (~79%) to dis2+ (Ohkura et al. 1989). Single gene deletion mutants (Δdis2 or Δsds21) were viable.

Other phosphatase genes encoding polypeptides highly similar (~80% identical) to PP2A are present in fission and budding yeasts (Kinoshita et al. 1990; Sneddon et al. 1990, Ronne et al. 1991; Sutton et al. 1991). From multiple gene disruption and point analyses, the fission yeast PP2A-related phosphatase genes ppa1+ and ppa2+ were shown to share an essential function for cell division; a double deletion, Δppa1-Δppa2 is lethal (Kinoshita et al. 1990). On the other hand, the combination of the PP1-related Adis2 and the PP2A-related Δppa2 gene disruptants is viable, suggesting that they do not share an essential function (Kinoshita et al. 1990). Consistently, cold-sensitive phosphatase mutants of dis2 and ppa2 exhibit strikingly different cell cycle phenotypes (Ohkura et al. 1989; Kinoshita et al. 1990); dis2-11 exhibits aberrant mitosis, whereas cold-sensitive ppa2 cells are short in cell size and display normal mitotic figures. Because the in vivo substrates for ppa1 and ppa2 phosphatases in fission yeast are not known, the molecular basis for these distinct phenotypes, namely, chromosome nondisjunction and short cell size phenotypes, are little understood. However, these phosphatases may well be the candidates controlling the main cell cycle regulators, cdc2, cyclin, cdc25, and wee1, as they are all phosphorylated.

Okadaic acid isolated from the black sponge Halichondria okadai is a polyether compound of a C32 fatty acid and a potent tumor promoter (Suganuma et al. 1988). It is a highly effective inhibitor (Biolajan and Takai 1988) for mammalian type 1 and 2A phosphatases (designated PP1 and PP2A, respectively; for review, see Cohen 1989a). Approximately 10 and 100 nM of the drug inhibits most
of the PP2A and PP1 activities, respectively. As okadaic acid activates the cdc2/cyclin kinase in Xenopus egg extracts (Felix et al. 1990) and because the purified PP2A catalytic subunit is capable of inhibiting the conversion from the inactive maturation promoting factor [MPF (Masui and Markert 1971)] to the active one in Xenopus (Lee et al. 1990), PP2A-related phosphatases may have a universal role in the cell division cycle from yeast to higher eukaryotic cells. However, the way in which type 2A phosphatases are involved in cell division is not known. The cdc25+ gene product is known to be a mitotic inducer (Russell and Nurse 1986; Nurse 1990) and was shown recently to be a protein tyrosine phosphatase (Dunphy and Kumagai 1991; Gautier et al. 1991; Kumagai and Dunphy 1991; Millar et al. 1991; Strausfeld et al. 1991; Lee et al. 1992) specific for the Tyr-15 residue of cdc2 kinase (Gould and Nurse 1989). The cdc25+ gene product was shown to positively regulate the G2/M transition by directly dephosphorylating the cdc2 kinase. A protein phosphatase activity sensitive to okadaic acid appears to be involved in inhibiting the cdc25 tyrosine phosphatase (Izumi et al. 1992; Kumagai and Dunphy 1992). The wee1+ gene product is a mitotic inhibitor, encoding a kinase responsible for tyrosine phosphorylation of the cdc2 kinase (Russell and Nurse 1987; Nurse 1990; Honda et al. 1992; Parker et al. 1992; Parker and Piwnica-Worms 1992).

In this paper we report the roles of ppa1 and ppa2 phosphatases in mitosis and address the question of how okadaic acid affects the cell division of S. pombe. Although the in vitro effect of okadaic acid on mammalian and yeast PP2A and PP1 activities is well defined (Cohen 1989a,b; Cohen et al. 1989; Kinoshita et al. 1990), the effect of the drug on in vivo cell growth in yeast has not been investigated. We will show that okadaic acid affects mitosis and cytokinesis and that one target of the drug is the product of the ppa2+ gene.

The second question we address is how ppa1+ and ppa2+ genes are involved in cell cycle control and how they might interact with cell cycle regulator genes. We investigate the mutant phenotypes of deletion or overexpression of these phosphatase genes and provide evidence from experiments employing various multiple mutants that the ppa2+ gene product is implicated in the regulation of cdc2 kinase tyrosine phosphorylation by interaction with cdc25 tyrosine phosphatase or the wee1 tyrosine kinase.

The third question we ask is how ppa1 and ppa2 phosphatase proteins exist in cells and what kind of substrate proteins are regulated by dephosphorylation. We identify several polypeptides as potential substrates that were hyperphosphorylated in the phosphatase mutants or okadaic acid-treated cells. We raised antibodies against ppa2 that also recognize ppa1. Immunoblotting suggested that ppa2 and ppa1 are major and minor type 2A-like phosphatases, respectively. These phosphatases were enriched in the cytoplasm in striking contrast to the nuclear-enriched dis2/sds21 phosphatases. Sola et al. (1991) reported that the type 2A, but not the type 1, phosphatase dephosphorylates the cdc2 phosphorylation sites in histone H1. Hence, we examined the phosphatase activity of histone H1 phosphorylated by S. pombe cdc2 kinase and found that ppa2 can dephosphorylate histone H1, but the bulk of histone H1 phosphatase in S. pombe extracts consists of the type 1-like phosphatases dis2 and sds21.

Results

Okadaic acid inhibits S. pombe cell division

To identify the in vivo target of okadaic acid, we examined whether the drug inhibits cell growth of S. pombe. The division rates of wild type and phosphatase mutants at 33°C were obtained by counting the cell number in the rich YPD liquid medium containing 0, 10, and 20 μM okadaic acid (Fig. 1). In the presence of 10 μM okadaic acid, the growth rates of both wild-type and phosphatase mutants were reduced by 20% by 10 μM and -90% by 20 μM okadaic acid (Fig. 1). In the presence of 10 μM okadaic acid, the growth rates of both wild-type and Δppa2 were affected. However, Δppa2 was affected much more strongly—the cells grew exceedingly slowly or division was blocked. The division rate of Δppa2 was ~50% reduced by 10 μM and ~90% by 20 μM okadaic acid, whereas that of wild type was reduced only 20% by 20 μM drug. In the absence of the drug (broken lines), the division rates of wild type and Δppa2 were similar.

![Figure 1. Okadaic acid inhibits fission yeast cell division. Exponentially growing wild-type and Δppa2 cells in YPD liquid medium were transferred to fresh YPD containing 0, 10, or 20 μM okadaic acid (OA) and incubated at 33°C. An aliquot of each culture was taken at 2-hr intervals, and the cell number was measured.](image-url)
A type 2A-like phosphatase deletion mutant Appa2 is hypersensitive to okadaic acid

Various S. pombe strains were also examined in YPD containing 0, 10, and 20 μM okadaic acid (Table 1). Δppa2 carrying a multicopy plasmid containing the wild-type ppa2+ gene grew as wild type in the presence of okadaic acid, indicating that the loss of ppa2+ is responsible for hypersensitivity to okadaic acid. Δppa2 carrying a plasmid with the cold-sensitive ppa2 gene (Kinoshita et al. 1990) is slightly more sensitive to okadaic acid than Δppa2; multicopy ppa2cs appears to have a weak dominant-negative effect on the drug sensitivity. Other phosphatase mutants, Δppa1 (Kinoshita et al. 1990), Δdis2, Δdsd2 (Ohkura et al. 1989), and Δapp1 (Shimanuki et al. 1993), showed sensitivity like that of the wild type. Cold-sensitive mutants dis1-288, dis2-11, and dis3-54 (Ohkura et al. 1988, 1989, Kinoshita et al. 1991a) did not show hypersensitivity to 0–20 μM okadaic acid. Hence, in examinations performed so far, only the loss of ppa2+ affects the sensitivity to okadaic acid.

Cell size reduction and delay in cytokinesis by okadaic acid

We found that okadaic acid reduces the cell size of S. pombe. As shown in Figure 2a (left), wild-type cells incubated in liquid medium containing 20 μM okadaic acid (indicated by + OA) displayed a ~15% reduction in cell size compared with cells that had not been exposed to the drug (−OA). The stippled bars represent septated cells. The degree of cell size reduction is higher (~20%) for septated cells (from 13.5 in −OA to 11.2 μm in +OA). Examples of DAPI-stained wild-type cells before [indicated by −] and after (+) treatment with 20 μM okadaic acid are shown in Figure 2b. The length of exponentially growing Δppa2 cells was shown previously to be ~20% shorter than that of wild-

Table 1. Effect of okadaic acid on cell division of wild-type and phosphatase-related mutants

| Strains                      | Okadaic acid (μM) |
|------------------------------|-------------------|
|                              | 0     | 5     | 10    | 20    |
| Wild type                    | +     | +     | +     | +     |
| Δppa2                        | +     | +     | +     | +     |
| Δppa2[ppa2+]                  | +     | +     | +     | +     |
| Δppa2[ppa2cs]                | +     | +     | +     | −     |
| Δppa1, Δapp1, Δdis2, Δdsd2    | +     | +     | +     | +     |
| dis1-288, dis2-11, dis3-54   | +     | +     | +     | +     |

S. pombe wild type and mutants were incubated in YPD containing 0–20 μM okadaic acid at 33°C for 12 hr. The number of cells was counted before and after the incubation. (+) The same cell number increase as that of wild type without drug; (+) a reduced cell number increase (~50%); (−) a small cell number increase (~10%). ppa2+ and ppa2cs are multicopy plasmids carrying the ppa2+ or the mutant ppa2cs gene (Kinoshita et al. 1990). dis1, dis2, and dis3 mutants (Ohkura et al. 1988) were examined at the permissive temperature, 33°C.

Figure 2. Cell size reduction, binucleated cell accumulation, and hyperphosphorylation in the presence of okadaic acid. [a] The cell size of S. pombe grown in the absence (−OA) or the presence (+ OA) of 20 μM okadaic acid. Wild-type and Δppa2 cells were incubated in 20 μM okadaic acid at 33°C. The stippled bars represent the septated cells with two nuclei; the open bars indicate total cells. The average cell length for total and septated cells is indicated for each culture. (b) Fluorescence micrographs of wild-type cells stained with DAPI. (+ OA) With 20 μM okadaic acid for 6 hr at 33°C. Bar, 10 μm. (c) Phospholabeled polypeptides in the presence of okadaic acid. S. pombe cells were labeled [32P]H2PO4 [Materials and methods]. [Lane 1] Wild-type labeled for 3 hr without drug; [lane 2] Δppa2 labeled for 3 hr without drug; [lane 3] Δapp2 labeled for 0–3 hr with 20 μM okadaic acid; [lane 4] Δppa2 labeled from 3 to 6 hr with drug. Several protein bands with the altered phosphorylation patterns are indicated with their estimated molecular masses calibrated by standard markers. The amounts of loaded samples are identical.
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Type cells [Kinoshita et al. 1990]. Upon the addition of 20 μM okadaic acid, the average length of Δpap2 cells after 8 hr was 18% shorter (Fig. 2a, right). This shortening was not the result of the arrest of division as cells continued to divide. The average cell length of 7.1 μm for Δpap2 in the presence of 20 μM okadaic acid is nearly identical to that of wee1 cells [Fantes 1981]. Thus, the effects of okadaic acid and the deletion of ppa2+ on cell size reduction are additive.

Another feature of okadaic acid-treated cells is the increase of cells with two nuclei, with or without the septum (the stippled bars in Fig. 2a indicate binucleated cells with the septum). The number of septated cells increased two- to threefold in both wild-type and Δpap2 strains in the continued presence of 20 μM okadaic acid. Approximately 50% of Δpap2 cells were binucleate after incubation in 20 μM drug for 6 hr. Note that DAPI-stained cells displayed no abnormality in nuclear morphology in the presence of the drug. No cell resembling mitotic catastrophe [Russell and Nurse 1986, 1987; Lundgren et al. 1991] has been observed.

The DNA content of the haploid mutant Δpap2 cells was analyzed by FACscan (Fig. 3a). Without the drug (−OA), most of the Δpap2 cells showed a DNA profile with the G2 DNA (2C) content (top), like that of growing wild-type haploid cells. Six hours after the addition of 20 μM okadaic drug, two subpopulations were found with approximately half of the cells containing 4C DNA (+OA, 6 hr; bottom), consistent with the increase of cells with two nuclei as described above. Hence, a deletion mutant, Δpap2 in 20 μM okadaic acid undergoes nuclear division and a round of DNA synthesis but retards cell separation. Cells with the G1 DNA are not apparent.

Reversible effect and hyperphosphorylation by okadaic acid

When Δpap2 cells are incubated in YPD medium containing 20 μM okadaic acid, cells wax through one or two divisions before arresting, as shown in Figure 1 (right). The numbers of cells with a septum increased considerably [Fig. 2a]. To examine whether the effect of okadaic acid is reversible, 20 μM okadaic acid was removed from the culture medium after 4 hr (Fig. 3b). The cells were then washed and incubated in fresh YPD without okadaic acid (indicated by the arrow under −OA). The solid line indicates the cell number; the broken line represents the septated cells (%). Cell numbers increased again 2 hr after the removal of okadaic acid. The frequency of septated cells decreased 1 hr after removal of the drug. Cells returned to the normal level of septation 3–4 hr after the removal of the drug. Hence, the in vivo effect of okadaic acid was reversible.

To investigate whether protein phosphorylation is actually affected by okadaic acid, cells were labeled for 3 hr

Figure 3. Increase of binucleated cells and reversible cell division arrest by okadaic acid. (a) FACscan analysis of ppa2-disrupted cells (Δpap2) incubated in the absence (top, −OA) or the presence (bottom, + OA) of 20 μM okadaic acid for 6 hr at 33°C. The profile of Δpap2 cells in the absence of drug (top) is identical to that of wild-type cells [not shown]. The peak position corresponds to that of G2 DNA content [Costello et al. 1986; Kinoshita et al. 1991a]. The okadaic acid-treated cells (bottom) showed a new peak of cells corresponding to the peak position of 4C DNA (determined by diploid cells in G2). The haploid wild-type cells treated with drug also showed two peaks corresponding to 2C and 4C cells. (b) Δpap2 cells were incubated in the presence of 20 μM okadaic acid for 4 hr and then transferred to culture medium without drug. The cell number (indicated by the solid line; logarithmic scale) and the frequency of septated cells (broken line; percent cell plate index) were counted.
by $[^{32}P]H_3PO_4$ in EMM1 medium with or without 20 μM okadaic acid. Phospholabeled polypeptides were detected by autoradiography (Fig. 2c). Control phosphoproteins in wild-type cells growing without okadaic acid are shown in lane 1. The pattern in Δppa2 cells without okadaic acid [lane 2] is different from that of wild type. Specifically, the intensity of the 78-kD band was reduced (molecular masses determined by standard markers; not shown), and the intensities of several polypeptides were increased (45, 100, and 150 kD; positions are indicated). With 20 μM okadaic acid, the level of phosphorylation was increased further for those 45-, 100-, and 150-kD bands and, in addition, a 60-kD band, after 3 hr [lane 3; labeled from 0 to 3 hr] and 6 hr [lane 4, labeled from 3 to 6 hr]. Hence, both okadaic acid and deletion of ppa2+ causes hyperphosphorylation of similar proteins.

Genetic interactions of ppa2+ with the cell cycle regulators, wee1+ and cdc25+

To obtain information on the role of ppa2+ in cell cycle control, double mutants between Δppa2 and cell cycle regulator mutants were made, and their phenotypes examined. We employed wee1-50, cdc25-22, and cdc2-33 mutants in crosses with a Δppa2 deletion mutant. The double mutant Δppa2 wee1-50 was plated on YPD plates with the control strains of wild type, single Δppa2, and wee1-50. Interestingly, the Δppa2 wee1-50 double mutant did not form a colony at 33°C or 36°C, as shown in Figure 4a. The viability of Δppa2 wee1-50 double mutant cells decreased rapidly after the culture was transferred from 26°C to 33°C, indicating that the double mutant cells become lethal at 33°C and 36°C. This synthetic lethality suggests that ppa2+ and wee1+ may share a function essential for viability at 33°C and 36°C.

We constructed the double mutants Δppa2 cdc2-1w and Δppa2 cdc2-3w and found that both could grow, although somewhat reduced in colony formation (Fig. 4b). These single cdc2 alleles produce normal colonies with a wee phenotype, but the double mutants, cdc2-3w wee1-50 and cdc2-1w cdc25-22 are lethal (Fantes 1981; Russell and Nurse 1987).

A genetic interaction exists between ppa2 and cdc25 (Fig. 4c). The phenotype of a temperature-sensitive cdc25-22 mutant is partially suppressed by the deletion of ppa2+. Two pieces of supporting evidence are shown. First, the double mutant Δppa2 cdc25-22 forms normal-sized colonies at 33°C on the YPD plate, whereas the single mutant cdc25-22 forms small colonies. In addition, in liquid YPD cultures, the division rate of the double mutant measured at 33°C was significantly faster than that of the single cdc25-22 mutant (Fig. 4c, left). Second, the double mutant transferred to 36°C went through several rounds of division before arrested (Fig. 4c right); the single mutant cdc25-22 cells did not divide at all after the shift to 36°C. Thus, a temperature-sensitive cdc25-22 mutation was partly suppressed by the deletion of ppa2+. Such a relationship does not exist be-

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**Figure 4.** Genetic interactions of ppa2 and wee1 or cdc25. (a) The double mutant Δppa2−wee1-50 does not form a colony at 33°C or 36°C. [wt] Wild type. The double mutant forms colonies at 26°C [not shown]. (b) The double mutants Δppa2 cdc2-1w and Δppa2 cdc2-3w as well as single mutants produced colonies at 33°C. (c) The double mutant Δppa2 cdc25-22 and single mutant cdc25-22 grown at 26°C were shifted to 33°C [left] or 36°C [right], and their cell numbers were counted at intervals [logarithmic scale]. The double mutant grew faster than the single mutant at 33°C, and at 36°C the double mutant could divide several times while the single mutant did not. (d) The double mutant Δppa2 cdc2-33 and single mutant cdc2-33 grown at 26°C were transferred to 36°C, and their cell numbers were counted [logarithmic scale].
Identification of ppa1 and ppa2 proteins

To identify the ppa1 + and ppa2 + proteins, we raised antibodies against the bacterially made ppa2 fusion protein. Affinity-purified antibodies were made from polyclonal rabbit antisera. The antibodies recognized both ppa1 and ppa2 polypeptides in S. pombe extracts by immunoblot (Fig. 5), consistent with their high amino acid similarity (~80% identity). Two protein bands were observed, an intense one at 39 kD and another weak band at 36 kD in wild type (lane 1). In Δppa1 cell extracts (lane 2), the weak 36-kD band was missing, whereas in Δppa2 extracts (lane 3), the intense 39-kD band was lost (slight leak from other lanes exists). Note that the intensity of the ppa1 band is increased in Δppa2 mutant cells. A multicopy plasmid with the ppa1 + gene caused an increase in the 36-kD band intensity, whereas the upper 39-kD band intensity decreased somewhat (lane 4). The 39-kD band became intensified with a multicopy plasmid carrying the ppa2 + gene (lane 5) or an alcohol dehydrogenase (ADH)-ppa2 + gene under the ADH promoter (lane 6). We therefore concluded that the 39- and 36-kD bands represent the major and minor phosphatase gene products of ppa2 + and ppa1 +, respectively. Both up- and down-regulation of phosphatase protein levels seem to exist to compensate for the change of either deletion or overexpression. A preliminary gel filtration study showed that the bulk of the ppa1 and ppa2 protein eluted in the ~200-kD fraction.

Inhibition of cell division by overexpression of ppa1 + and ppa2 +

To investigate the overexpression phenotype, the fission yeast nmt1-inducible promoter [Maundrell 1990] was used. The nmt1 promoter was ligated in front of the initiation codon of ppa1 + and ppa2 +, and the resulting plasmids were introduced into S. pombe wild-type cells. In the presence of thiamine, the nmt1 promoter is shut off, whereas in the absence of thiamine, expression is induced [Maundrell 1990]. As shown in Figure 6a, wild-type cells carrying a plasmid with the nmt-ppa1 + or nmt-ppa2 + gene hardly produce any colonies in the absence of thiamine (indicated by −T), whereas normal-sized colonies were found in the presence of thiamine (+T).

It is known that expression is observed 10 hr after induction when the nmt1 promoter is used [Maundrell 1990]. Immunoblotting of extracts of derepressed cells (Fig. 6b) showed that the amounts of ppa1 and ppa2 proteins increased after 10–12 hr and reached maximal levels at 14–18 hr. The inhibition of cell division by overexpression was seen by the reduction in the rate of cell number increase after 10 hr in the absence of thiamine [Fig. 6c], resulting in an increase in average cell length (~15% increase from 10 to 14 hr for both ppa1 + and ppa2 + overexpressing cells). Overexpression of ppa1 + and ppa2 + reversibly arrests cell division. It is not a lethal event for the majority of cells (70% and 50%, respectively, at 14 and 22 hr after overexpression, Fig. 6d).

Cells overexpressing ppa2 under the nmt promoter were observed by immunofluorescence microscopy using the anti-tubulin antibody TAT1 [Woods et al. 1989] and DAPI for DNA staining. Cells before (indicated by +T) and after (−T) overexpression of ppa2 + are shown in Figure 6e. Most overexpressing cells contained the abundant microtubule arrays in the cytoplasm characteristic of interphase cells [Hagan and Hyams 1988]. Overexpression of the ppa1 + gene caused a phenotype indistinguishable from that of ppa2 +.

Cytoplasmic localization of ppa2 protein

To determine cellular localization of ppa1 and ppa2 proteins, indirect immunofluorescence microscopy was performed using affinity-purified antibodies against ppa2. As shown in Figure 7, cytoplasmic immunofluorescence with granular signals was seen in growing wild-type cells (middle), whereas fluorescence was hardly visible in Δppa2 mutant cells (top). After overexpression of ppa2 by the NMT–ppa2 plasmid, cytoplasmic immunofluorescence increased significantly (bottom), and staining was often accumulated into a large body or resided near or on the nuclear periphery. However, nuclei were still deficient of antibody staining.

Detection of ppa2 as a minor histone H1 phosphatase

In a previous study [Kinoshita et al. 1990], we showed that ppa2 has phosphorylase phosphatase activity. Phosphorylase is also a substrate for type 1-like dis2 and sds21 phosphatases. To understand the exact role of ppa2 in cell cycle control, it is important to identify the in vivo substrates specific for ppa2. Sola et al. [1991] reported that in rat liver, Xenopus egg, and Saccharomyces cerevisiae cells, most histone H1 phosphatase is like type 2A, inhibited by 2 nm okadaic acid and only a small fraction is inhibited by the specific type 1 phosphatase inhibitor I-2. We therefore attempted to examine
whether \textit{ppa2} has the phosphatase activity for the \textit{cdc2} phosphorylation sites in histone H1. To prepare phosphorylated histone H1, mitotic extracts containing a high \textit{cdc2} kinase activity were used. Extracts of a mutant \textit{nda3-311}, which arrests in mitotic prophase at 20°C [Hiraoka et al. 1984; Moreno et al. ...
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Figure 7. Cellular location of ppa1 and ppa2. Indirect immunofluorescence microscopy of Δppa2 deletion, wild type (WT), and wild-type overexpressing ppa2 (anti–ppa2, 18 hr) was done using anti–ppa2 antibodies (right). The same cells stained by DAPI are also shown. Immunofluorescence was hardly detected in Δppa2 cells, whereas cytoplasmic immunofluorescence was abundant in wild-type cells. Wild-type cells carrying anti–ppa2 plasmid overexpress ppa2 protein in the absence of thiamine for 18 hr (see text). Immunofluorescence was enhanced further in the cytoplasm, often accumulated at the nuclear periphery or within restricted domains. The deformation of chromosomal DNA was also visible in overexpressed cells. Bar, 10 μm.

1989, Kinoshita et al. 1991b), were prepared. Extract supernatants were incubated with p13\textsuperscript{nucl} beads, and the resulting cdc2-associated beads were used for phosphorylation of calf thymus histone H1. As shown in Figure 8a, the H1 kinase activity in the p13\textsuperscript{nucl} beads increased severalfold in extracts of mitotically arrested nda3-311 cells in comparison with growing cells at the permissive temperature. Extracts of the mutant cells, which entered interphase upon their release to the permissive temperature, contained a low H1 kinase activity. The other substrate we used was a synthetic oligopeptide S1 (Yasuda et al. 1990), AAKAKTPKKAKK (containing only one cdc2 consensus underlined). As shown in Figure 8a, the S1 peptide kinase activity also increased ~10-fold in mitotically arrested nda3-311 cells in comparison with those at the permissive temperature.

We used \textsuperscript{32}P-labeled phosphorylated H1, S1, and a control substrate, \textsuperscript{32}P-labeled phosphorylase [Kinoshita et al. 1990]. Another control used was histone H1 phosphorylated by Xenopus MPF (Labbé et al. 1988). The phosphatase activity was assayed by the amount of released \textsuperscript{32}P after 10 min of incubation at 33°C. As shown in Figure 8b, the greater part of the H1/S1 phosphatase activities detected is okadaic acid sensitive, inhibited by 1 μm okadaic acid. Ten nanomolars okadaic acid, which inactivates type 2A-like phosphatases [Kinoshita et al. 1990], inhibited only 10–15% of H1/S1 phosphatases. In contrast, the same concentration of okadaic acid inhibited 40% of phosphorylase phosphatase activity. Inhibitor-1, a specific inhibitor for type 1 phosphatase (the kind gift of Dr. P. Cohen, University of Dundee, Scotland), inhibited 75% of H1/S1 phosphatase and 50% of phosphorylase phosphatase. These results suggest that the H1 and S1 phosphatase activities detected in fission yeast extracts are similar and made up of types 1 and 2A-like phosphatases, the greatest part being derived from a type 1-like phosphatase. The contribution by ppa1 or ppa2 is probably minor, comprising 15–25% of the activity under the experimental conditions employed. Similar results were obtained using substrates phosphorylated by different cdc2 kinases, S. pombe, Xenopus egg, and S. cerevisiae (data not shown).

We then assayed H1 phosphatase activity in phosphatase mutant extracts (Fig. 8c). The deletion mutant Δppa2 showed only a 5% reduction, whereas a reduction of 30% and 60% was found for deletion mutant extracts of Δdis2 and Δsds21, respectively. In cold-sensitive dis2-11 mutant extracts, 70% H1 phosphatase activity was abolished. These results are again consistent with the major role of type 1-like dis2/sds21 in H1/S1 phosphatase activity.

Discussion

In this paper we show that a fission yeast gene ppa2\textsuperscript{+} encoding a type 2A-like phosphatase catalytic subunit determines the sensitivity to okadaic acid in vivo. The loss of ppa2\textsuperscript{+} causes cells to be hypersensitive to the drug. To our knowledge, this is the first report of the genetic analysis of okadaic acid sensitivity in any organism. Owing to the poor penetration of okadaic acid into cells, a high concentration of the drug is required to obtain the in vivo effect. Mutations in other phosphatase genes, including ppa1\textsuperscript{+}, another type 2A-like phosphatase gene, and dis2\textsuperscript{+}/sds21\textsuperscript{+} type 1-like phosphatase genes, did not show any sensitivity change. As the amino acid sequence of ppa1 is 80% identical to ppa2 and they share an essential function for viability [Kinoshita et al. 1990], ppa1 may well be another target of okadaic acid. Then, why is Δppa1 not shown to be an okadaic acid sensitivity locus? This is explained by the large difference in the amounts of ppa1 and ppa2 phosphatases present in a cell: Immunoblot data suggest that ppa2 is much more abundant than ppa1 in cells. This is consistent with previous enzymatic assay data demonstrating that type 2A-like phosphorylase phosphatase activity is greatly reduced in Δppa2 extracts while the change is small in Δppa1 extracts [Kinoshita et al. 1990]. We suppose that okadaic acid targets both ppa1 and ppa2 in
vivo, but only the loss of the major ppa2 phosphatase causes a recognizable change in okadaic acid sensitivity from the wild type, because the remaining minor ppa1 phosphatase will be inactivated by a lower concentration of okadaic acid.

Multiple cellular abnormalities, such as cell length reduction, accumulation of binucleated cells, and hyperphosphorylation of certain proteins, are produced by okadaic acid. Mitotic apparatus abnormality has not been observed so far. In a single deletion Δppa2 strain, cell size reduction and hyperphosphorylation were observed, but the delay in cell separation was not. The loss of additional ppa1+ may be needed for such a phenotype. Alternatively, other phosphatase(s) sensitive to okadaic acid are possibly responsible for the defect in cytokinesis. Thus, we show that type 2A-like phosphatases appear to play an important role in the genetic control of cell length and cytokinesis. This may be compatible with the cytoplasmic localization of the ppa2 phosphatase. The budding yeast CDC55 gene (the gene encodes a polypeptide similar to the regulatory subunit B of PP2A) was reported to have a phenotype in septation and cell separation (Healy et al. 1991).

Immunofluorescence microscopy using anti-ppa2 antibodies suggests that the bulk of ppa2 is found in the cytoplasm. Increased fluorescence is often accumulated near the periphery of the nucleus in overexpressed cells, but the nucleus itself is devoid of immunofluorescence. This is in striking contrast to the location of the type 1-like phosphatase dis2, which is enriched in the nucleus.
phosphatase (e.g., Virshup et al. 1990). Functions are known to be controlled by the type 2A protein phosphatase (PP2A). Okadaic acid-sensitive phosphatases might exist and be responsible for the activation of PP2A. We showed that the major H1 phosphatase in fission yeast extracts behaved as a type I-like dis2/sds21 phosphatase. Nearly 75% of the H1 phosphatase was abolished by the inhibitor 1. Similarly, 70% of H1 phosphatase was missing in dis2-11 mutant extracts. Ppa2 represented a minor H1 phosphatase. We employed two different substrates, calf thymus H1 and synthetic peptide S1, and obtained the same results. We employed a Xenopus MPF for phosphorylation of H1 and obtained results identical to that of the fission yeast cdc2 kinase. The difference from Sola et al. (1991) is not understood. Further biochemical studies on the ppa2- and dis2-dependent H1 phosphatases is needed to establish their physiological significance. It is of interest to examine whether the activity of ppa2-dependent H1 phosphatase alters during the cell cycle.

Materials and methods

Strains

S. pombe haploid strains HM123 [h− leu1], wee1-50, cdc25-22, cdc2-33, cdc2-1W, and cdc2-3W [Nurse et al. 1976], dis1-288, dis2-11, and dis3-54 [Ohkura et al. 1988] were employed. Gene disruptant strains Δppa2-1 [h− leu1 ura3 ppa2::ura4+], Δppa2 [h− leu1 ura4 ppa2::ura4+], Δdis2 and Δdis2-1 were constructed previously [Kinoshita et al. 1990, Ohkura et al. 1989]. The rich YPD medium contains 1% yeast extract, 2% glucose, and 1% polypeptone. The synthetic EMM2 medium was as described [Mitchison 1970]. Two percent agar was added for plating.
Okadaic acid (Wako Co.) was purchased, and 50–100 ml volume of YPD containing the drug was used for liquid culture.

**Plasmids**

A plasmid containing the *S. pombe* ADH promoter (Russell and Hall 1983) was used for overexpression of the *ppa2*+ gene. For construction of *ppa1*− and *ppa2*−-inducible plasmids, four oligonucleotides were made as follows: *PA1−N*, 5′-TTTCTATAT-GTCGCTCATGAGGAAA-3′; *PA1−C*, 5′-TCCGGGATCCC-GTTAGAAGGATATCG-3′; *PA2−N*, 5′-TTTCTATATGCTATAGAAGGA-3′; and *PA2−C*, TCCCCCGGATT-AAAGGAATGATGTGGG-3′. *PA1−N* and *PA2−N* contained the *NdeI* restriction sequence in front of the initiation codon of the *ppa1*+ and *ppa2*+ genes, respectively. *PA1−C* and *PA2−C*, respectively, contained the *BamHI* and *SmaI* sites just after the termination codons of *ppa1*+ and *ppa2*+ in the noncoding strand. Polymerase chain reaction (PCR) amplification in a DNA thermal cycler (Perkin-Elmer/Cetus) was done using these nucleotides as the primers and *ppa1*− or *ppa2*−-containing plasmids as the templates. Thirty cycles at 94°C for 1 min, then 55°C for 2 min, and 72°C for 3 min were performed. The plasmids pPH102 and pUM22 contained the genomic *ppa1*+ gene and the *ppa2*+ CDNA, respectively. The amplified DNA fragments were ligated with pREP1 that contained the promoter of the *nmt1*+ gene (Maundrell 1990). pREP1 had been restricted by *NdeI* and *BamHI* for *ppa1*+ or by *NdeI* and *SmaI* for *ppa2*+. Resulting plasmids pNMT−*ppa1* and pNMT−*ppa2* could express the entire coding regions of *ppa1*+ and *ppa2*+ in the absence of thiamine, respectively. They were introduced into wild-type HM123 cells by transformation (Ito et al. 1983), and resulting transformants were grown in synthetic EMM2 (+ thiamine) medium and then transferred to the same medium lacking thiamine at 33°C.

**In vivo labeling and immunoprecipitation**

EMM1 culture medium [Mitchison 1970] was used for the labeling of polypeptides by [32P]H3PO4. Cultures were labeled for 2–3 hr, at 10 mM NaCl/ml. Immunoprecipitation for [32P]-labeled extracts using anti-*ppa2* antibodies was performed in 50 mM Tris-HCl at pH 7.5 containing 1 mM EDTA, 1 mM 2-mercaptoethanol, 60 mM β-glycerophosphate, 15 mM NaF, 0.1 mM sodium orthovanadate, 0.4 mM NaCl, 0.05 mM NP-40, and 1 mM PMSF. For other extracts, 0.4 mM NaCl TEG (50 mM Tris-HCl containing 1 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mM PMSF) containing 0.05 mM NP-40 was used. Antibodies were mixed with 10 ml of protein A-Sepharose, incubated at room temperature for 2 hr with shaking, and washed three times with the buffer. Extracts derived from ~1 x 10^7 cells were centrifuged at 14,000 rpm for 30 min. Supernatants were mixed with antibody-bound protein A-Sepharose and incubated at 4°C for 3 hr, followed by washing five times with the buffer. They were boiled for 2 min in the sample buffer and run on SDS–polyacrylamide gels.

**Preparation of *S. pombe* extracts**

*S. pombe* cells were disrupted with glass beads in 0.4 mM NaCl TEG buffer. Supernatants after centrifugation (40,000 rpm for 20 min) were used. Gel filtration was performed in 0.1 mM NaCl TEG buffer using Superose 6 [Pharmacia].

**Fluorescence microscopy**

*S. pombe* cells in liquid culture were collected by low centrifugation and stained with the DNA-specific fluorescent probe DAPI by the procedure described [Adachi and Yanagida 1989]. Direct immunofluorescence microscopy was performed by the method described [Hagan and Hyams 1988] using antitubulin monoclonal antibodies (TAT-1, Woods et al. 1989). *S. pombe* cells were fixed in culture medium containing 3.7% formaldehyde and 0.2% glutaraldehyde at 33°C for 1 hr. The cells were digested with 0.6 mg/ml pl Zymolyase 100T [Seikagaku Corp., Tokyo], followed by permeabilization with 1% Triton X-100.

**FAscan analysis**

The procedures described [Costello et al. 1986, Kinoshita et al. 1991a] were followed. Haploid wild-type cells were used as the control. Cells (1 x 10^7 to 5 x 10^7) were stained by propidium iodide (final concentration, 12.5 μg/ml) and analyzed by FAScan [Beckton-Dickinson].

**Preparation of antisera and immunological methods**

A fusion protein was made using the T7 promoter [Studier and Moffat 1986] and a *BamHII* site in the coding region of the *ppa2*+ gene. The resulting carboxy-terminal polypeptide [26 KD] was purified and injected into rabbits by the procedures described previously [Hirano et al. 1988]. Antibodies were affinity purified. Immunoblots were performed according to Towbin et al. [1979].

**Protein phosphatase assay**

Preparation of [32P]-labeled phosphorylase was as described previously [Kinoshita et al. 1990]. Preparation of *Xenopus* MFPI followed the procedures described [Labbé et al. 1988]. The preparation and use of [32P]-labeled histone H1 and peptide S1 as substrates with fission yeast cdc2 and extracts were as follows. Fission yeast extracts were made from prophase-arrested ada3-311 mutant cells, which are high in cdc2 kinase activity; ada3-311 mutant cells were incubated at the restrictive temperature, 20°C for 6 hr in the rich YPD medium [Hiraoka et al. 1984, Kinoshita et al. 1991b]. Cells (~10^7) were disrupted with glass beads in 20 mM Tris-HCl (pH 7.5) containing the phosphatase inhibitors [50 mM β-glycerophosphate, 50 mM NaF, and 0.1 mM orthovanadate], followed by centrifugation at 14,000 rpm for 20 min. p34^cdc2−Sepharose beads [Brizuela et al. 1987] were added to the supernatant, and the mixture was incubated for 10 min at 25°C with mixing. The cdc2-bound beads were washed five times with the same buffer, followed by two washes with the kinase reaction buffer (20 mM Tris-HCl at pH 7.5, 4.5 mM 2-mercaptoethanol, 1 mM EGTA, and 10 mM MgCl2). Then 0.1 mM ATP, [γ-32P]ATP (~1000 cpm/pmole), 100 μg of calf thymus histone H1, and 4 μg of S1 peptide [AAAKKTPKKAKK derived from H1 containing a consensus sequence for cdc2 kinase, a gift of Dr K. Yasuda [Yasuda et al. 1990]] were added to the reaction buffer and incubated followed at 33°C for 30 min. The reaction mixture was centrifuged, and the supernatant was passed through a phosphocellulose column [the bed volume, 0.5 ml]. The column was washed with 20 mM Tris-HCl at pH 7.5 containing 0.3 mM NaCl and eluted with the Trit buffer containing 1 M NaCl. Eluant was passed through Sephadex G25 [bed volume, 5 ml] to remove NaCl. Protein phosphatase assays were performed as described [Kinoshita et al. 1990, Stone et al. 1993]. Briefly, [32P]-labeled substrates and cell extracts (~20 μg of protein) as the sources of phosphatases were mixed in the phosphatase reaction buffer (50 mM Tris-HCl at pH 7.5 containing 1 mM EDTA, and 1 mM 2-mercaptoethanol) and incubated at 30°C for 10 min. The reaction mixture was spotted on phosphocellulose paper P81 [1.5 x 1.5 cm]. The filter was washed four times with the phosphatase assay buffer and then dried. The incorporation of 32P into the filter was measured in a scintillation counter.
times with 20 mM Tris-HCl at pH 7.5, and the radioactivity in the paper was counted.

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[caption image]



Phosphorylation at Thr167 is required for Schizosaccharomyces pombe p34



[caption image]





[caption image]
Mitotic role of fission yeast pp2 phosphatase

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