$phd1^+$, a histone deacetylase gene of *Schizosaccharomyces pombe*, is required for the meiotic cell cycle and resistance to trichostatin A

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Abstract A gene named $phd1^+$ encoding a protein highly homologous to the yeast and human histone deacetylases, such as *Saccharomyces cerevisiae* Rpd3p and human HDAC1, was cloned from *Schizosaccharomyces pombe*. The immune complex isolated from *S. pombe* cells expressing Phd1 fused to the FLAG epitope showed histone deacetylase activity, which was inhibited by trichostatin A (TSA), a specific inhibitor of histone deacetylase. The null mutation of $phd1^+$ resulted in a marked decrease in the total cellular histone deacetylase activity and an increase in the sensitivity to TSA. Although the *phd1* disruptant showed no obvious defect in the mitotic cell cycle or mating, both homothallic haploid and heterothallic diploid cells failed to form spores in the absence of $phd1^+$. These results indicate that $phd1^+$ encodes a histone deacetylase, which is involved in the meiotic cell cycle in *S. pombe*.

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1. Introduction

Transcriptionally active or competent genes are localized preferentially in the acetylated chromatin domains [1,2]. Histone acetylation occurs at the specific lysine residues in the positively charged N-terminal tails that protrude from the histone octamer of the nucleosome cores, which are important for both histone-DNA and histone-non-histone protein interactions. The neutralization of the positive charge by acetylation has been proposed to lead to loosening histone-DNA contacts, which facilitates the accessibility of a variety of factors to DNA [3,4]. We have shown that two structurally unrelated microbial metabolites, trichostatin A (TSA) and trapoxin (TPX), are potent and specific inhibitors of histone deacetylase (HDAC) [5,6]. TSA reversibly inhibits mammalian HDAC, while TPX irreversibly inactivates it via its epoxide moiety. They induce hyperacetylation of core histones at very low concentrations, which is accompanied by characteristic blockage of the cell cycle, induction of differentiation, and morphological reversion of various transformed and tumor cell lines [7]. The cell cycle arrest by these agents was shown to be ascribed to their HDAC inhibition, since a TSA- and

TPX-resistant mutant cell line was found to possess a TSA-resistant HDAC [5]. Thus, histone acetylation control may be involved in cell cycle, differentiation and tumorigenesis. Recently, a human HDAC (HDAC1) was isolated [8] as a protein that binds the irreversible inhibitor, TPX. HDAC1 was significantly similar to the yeast *Saccharomyces cerevisiae* transcriptional regulator, Rpd3p, which is required for full repression as well as full activation of gene expression [9]. Two *S. cerevisiae* HDAC complexes were also isolated, one of which contained Rpd3p and the other contained Hda1p, also related to Rpd3p [10]. At least three other homologs, HOS1, HOS2 and HOS3, are present in the yeast genome, although their enzymatic activities were yet unidentified [10]. These results suggest that HDAC genes constitute a gene family and each gene product plays a different role. Another yeast, *Schizosaccharomyces pombe*, evolutionarily distant from *S. cerevisiae*, is also a good model organism for studies on the cell cycle and differentiation. Recently, it was shown that TSA relieved transcriptional repression in centromeric heterochromatin in *S. pombe* [11]. However, the biological role of HDAC in this organism is still largely unknown.

In this paper, we report that *S. pombe* $phd1^+$, a gene highly homologous to *S. cerevisiae* and human HDAC genes, encodes an active HDAC enzyme. We demonstrate that its null mutation causes TSA supersensitivity and a marked decrease in the total HDAC activity. A *phd1* disruptant was viable but incapable of sporulation, suggesting that $phd1^+$ is non-essential for the mitotic cell cycle but necessary for the meiotic cell cycle and resistance to HDAC inhibitors.

2. Materials and methods

2.1. Strains and media

The *S. pombe* strains used were JY266 (h^+ *leu1-32*), JY741 (h^- *leu1-32 ura4-D18 ade6-M216*), JY746 (h^+ *leu1-32 ura4-D18 ade6-M210*), JY3 (h^{90}), KH10 (h^-/h^+ *leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216 phd1^+/phd1::ura4*), KH11 (h^- *leu1-32 ura4-D18 ade6-M216 phd1::ura4^+*), KH12 (h^+ *leu1-32 ura4-D18 ade6-M210 phd1::ura4^+*), and KH13 (h^{90} *leu1-32 ura4-D18 ade6-M210 phd1::ura4^+*). YE and MM were prepared essentially as described by Moreno et al. [12] with leucine, adenine and uracil supplement. Media used for sporulation were SPA for heterothallic strains and MEL containing 3% malt extracts for homothallic strains [13].

2.2. Genetic methods and PCR cloning

Standard genetic work was carried out according to Gutz et al. [13]. *S. pombe* was transformed by the lithium acetate protocol of Ito et al. [14]. To isolate a gene encoding a *S. pombe* homolog of *RPD3*, we performed degenerate PCR amplification with the *S. pombe* genomic DNA using two synthesized oligonucleotides: 5′-TA(CT)AT(ATC)G-A(CT)AT(ATC)GA(CT)(AG)T(ATGC)CA(CT)CA(CT)GG(ATGC)-GA(CT)GG-3′ and 5′-GC(ATGC)AC(AG)TT(ATGC)C(GT)(ATG-C)A(ATG)(ATGC)GT(AG)TA(ATGC)CC(ATGC)CC-3′, as the primers on the basis of the sequence similarity between *S. cerevisiae* *RPD3* and human HDAC1 cDNA. As a result, a 0.4-kb DNA frag-

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ment encoding part of a putative *RPD3* homolog was obtained. The 2.5-kb *Eco*RI fragment of *S. pombe* genomic DNA hybridized with the amplified 0.4-kb fragment was cloned into pUC19 for sequencing and pDB248′ for expression in *S. pombe* (pDHD1).

2.3. Immunoprecipitation of recombinant Phd1

pREP10-FLAG-*phd1*$^+$ was constructed by subcloning *phd1*$^+$ downstream of the FLAG sequence. The fusion protein was expressed under the control of the thiamine-repressible *nmt1*$^+$ promoter. After the induced cells had been treated with 2 mg/ml zymolyase for 1 h at 37°C, a whole cell extract was prepared by Dounce homogenizing the protoplasts in immunoprecipitation buffer containing 50 mM HEPES (pH 7.2), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 0.1% Tween 20, 10% glycerol, 60 mM β-glycerophosphate, 1 mM NaF, 0.1 mM NaVO$_4$ and complete protease inhibitor cocktail set (Boehringer Mannheim). A portion (20 mg protein) of the extract of each was immunoprecipitated with an anti-FLAG antibody (Santa Cruz Biotechnology) or an anti-IgG control antibody in the presence or absence of the FLAG peptide competitor.

2.4. Gene disruption

For *phd1* disruption, a linear *Eco*RI fragment carrying Δ*phd1*::*ura4*$^+$ was prepared as follows. The 0.76-kb *Eco*RV region of *phd1*$^+$ was replaced by the *Eco*RV fragment containing *ura4*$^+$. The linear *Eco*RI fragment containing *ura4*$^+$ flanked by the *phd1*$^+$ sequence was introduced into appropriate diploid cells, generated by mating of JY741 with JY746 [15]. After the disruption of *phd1*$^+$ had been confirmed by Southern blotting, two stable diploids were used for tetrad analysis.

2.5. Assay of HDAC activity

The yeast nuclear extract was prepared as described [16]. The assay for in vitro deacetylation of histones was basically according to the method described previously [5]. The nuclear extracts (1 mg protein) of the parental strain (JY746) and the *phd1* disruptant (KH11) or the immunopurified FLAG-Phd1 as described above were used as the enzyme sources. After the reaction mixtures had been incubated for 1.5 h at 37°C, the radioactivity of [^{3}H]acetate released from labelled mouse histones was determined. For the assay of TSA inhibition, the immunopurified FLAG-Phd1 was preincubated with various concentrations of TSA for 5 min before the enzyme reaction.

3. Results

3.1. Cloning of an S. pombe gene homologous to RPD3

To identify *RPD3* homologs of *S. pombe*, we used a degenerate PCR-based strategy. A 0.4-kb DNA fragment, which was amplified by PCR with *S. pombe* genomic DNA and a set of primers corresponding to the conserved subdomains, was sequenced and used as a probe for isolation of the entire ORF by Southern hybridization and colony hybridization. The genomic Southern hybridization with several restriction enzymes showed several hybridizing bands, suggesting that *S. pombe* possesses multiple *RPD3* homologs as does *S. cerevisiae* (Fig. 1). The 2.5-kb *Eco*RI fragment of *S. pombe* genomic DNA strongly hybridized with the 0.4-kb amplified fragment was cloned and sequenced. It contained a ORF of 434 amino acids with a calculated molecular mass of 49.4 kDa as well as a truncated ORF encoding part of phenylalanyl-tRNA synthetase. The 434-amino acid protein was highly similar in amino acid sequence to *S. cerevisiae* Rpd3p (52% identity) and human HDAC1 (58% identity). Thus, we refer to the gene as *phd1*$^+$ for the *S. pombe* histone deacetylase-related gene (GenBank/EMBL data bank accession number AB008888).

3.2. phd1$^+$ encodes a TSA-sensitive HDAC

The bacterially produced glutathione-*S*-transferase fusion protein of Phd1 showed no detectable HDAC activity (data
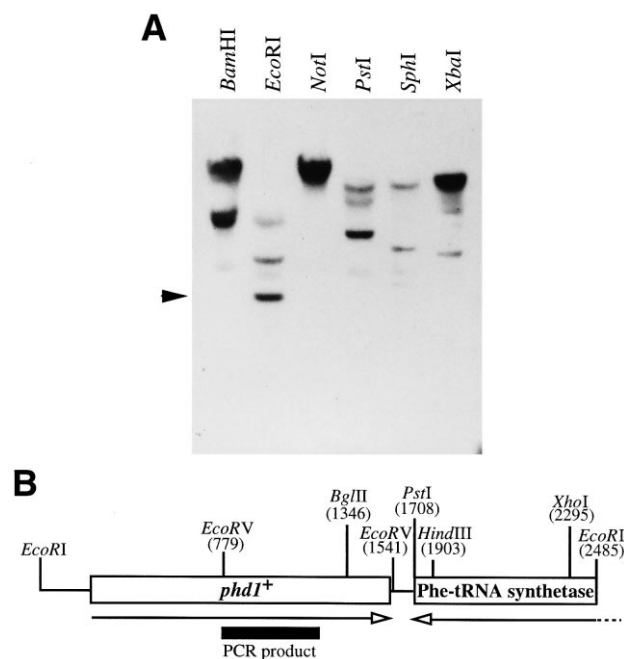

Fig. 1. Southern blot hybridization of the wild-type strain of fission yeast (A). Genomic DNAs were isolated from JY746 and digested with restriction endonucleases as indicated. The hybridization probe used was the PCR product obtained by the degenerated primers. The 2.5-kb *Eco*RI fragment strongly hybridized with the 0.4-kb amplified fragment was cloned and sequenced (indicated by an arrowhead). The restriction map of the cloned 2.5-kb fragment and the position of the PCR product obtained were shown (B).

not shown). We then expressed FLAG-tagged Phd1 in *S. pombe* and analyzed the enzyme activity of the immune complex precipitated with an anti-FLAG antibody (Fig. 2A). Distinct HDAC activity was detected in the immunoprecipitates from cells harboring the FLAG-Phd1 expression vector, while the immunoprecipitates from cells transformed with the control vector showed no HDAC activity (control). The enzyme activity was not recovered when the antibody was saturated with an excess amount of the FLAG peptide (blocking peptide), or when a control antibody was used instead of the anti-FLAG antibody. The enzyme activity of FLAG-Phd1 was inhibited by TSA at nanomolar concentrations in a dose-dependent manner (Fig. 2B). The IC$_{50}$ value of TSA was calculated to be about 43 nM, which is higher than those toward HDAC from higher eukaryotes (e.g. 3 nM for mouse HDAC [5] and 2 nM for human HDAC1 and HDAC3 [17]).

3.3. Disruption of phd1$^+$

To determine whether *phd1*$^+$ is essential for cell viability and other cellular functions, we deleted the 0.76-kb *Eco*RV fragment from the 2.5-kb *Eco*RI fragment (Fig. 1B) and inserted the *ura4*$^+$ gene into the *Eco*RV site to generate *phd1*::*ura4*$^+$ on the chromosome. Appropriate *ura4*-D18 diploid cells were transformed with the linear *Eco*RI fragment carrying the *phd1*::*ura4*$^+$ allele for generating a one-step gene replacement by homologous recombination. The correct gene replacement was confirmed by genomic Southern hybridization (data not shown). Heterozygous diploid *phd1*$^+$/*phd1*::*ura4*$^+$ transformants yielded four haploid spores that germinated and grew on YE agar medium. All the cells with uracil prototroph isolated from each complete tetrad showed
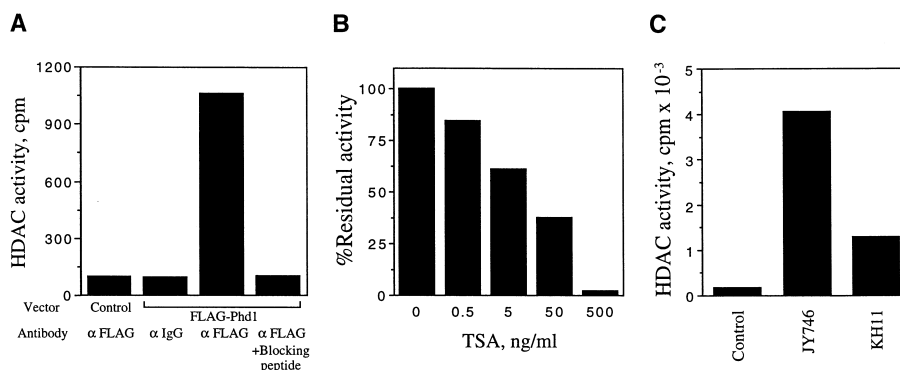
Fig. 2. HDAC activity of recombinant Phd1 and nuclear extract in the absence of Phd1. Enzyme activities of the immune complexes obtained using an anti-FLAG antibody or a control antibody from cell extracts (A). The effect of TSA on HDAC activity of FLAG-Phd1 (B). Total HDAC activity of the nuclear extracts from JY746 and *phd1*-disrupted cells (C). Control indicates the activity of the acid-inactivated JY746 extract.

no obvious change in their morphology or the doubling time, when compared with the parental strain. The null mutant was also able to grow at high (37°C) and low (20°C) temperatures, indicating that *phd1+* is non-essential for cell growth.

To assess the contribution of Phd1 to the total HDAC activity, we compared the total HDAC activity between the parental strain with the wild-type background (JY746) and the *phd1* null mutation (KH11) (Fig. 2C). The enzyme activity of the disruptant extract was reduced to approximately one third of that of the parental strain.

We next analyzed the TSA sensitivity of the disruptant (Table 1). JY266 can grow on the agar plate containing 50 μg/ml of TSA, the highest concentration at which TSA is soluble in medium. On the other hand, the proliferation of the disruptant was completely blocked by 30 μg/ml of TSA at the concentration. The disruptant was also sensitive to TPX, another HDAC inhibitor [6], but not to other antifungal antibiotics. The TSA sensitivity was complemented by introducing *phd1+* on pDB248′ into the disruptant.

3.4. phd1+ is involved in the meiotic cell cycle

TSA inhibited sporulation of the wild-type homothallic (h^{90}) cells in a dose-dependent manner when they were plated on the sporulation medium (Table 2). These observations suggest that the HDAC sensitive to TSA is involved in the process of mating or sporulation. To test whether *phd1+* is involved in mating or sporulation, we constructed a homothallic haploid strain with a null allele of *phd1+* and both mating types of heterothallic haploid strains with the *phd1* deletion, and determined their mating and sporulation

efficiencies. Sporulation of the homothallic disruptant was greatly reduced as observed for the wild-type cells with TSA treatment (Table 2), indicating that *phd1+* is required for mating or sporulation. Although the mating efficiency of the wild-type cells in the presence of TSA significantly decreased, the mating efficiency of the cells with the chromosomal deletion of *phd1+* was essentially the same as that of the wild-type cells without TSA treatment. These results suggest that *phd1+* is not directly involved in the mating process. On the other hand, the heterothallic diploid cells with a homozygous *phd1* deletion sporulated at a markedly decreased efficiency (Table 2). The nuclei stained with DAPI of the homozygous *phd1* disruptant were compared with those of *phd1+* cells. In contrast to the tetrad formation at a high frequency in the wild-type background cells, almost all disruptant cells possessed a single nucleus per cell with slightly deformed morphology (Fig. 3). These results suggest that the *phd1* disruptant is defective in the early meiotic cell cycle.

4. Discussion

The amino acid sequence of Phd1 was the most similar to that of HDAC1, a human HDAC [8], and also highly homologous to those of *RPD3* and *HOS2*, encoding a *S. cerevisiae* HDAC and a putative HDAC, respectively [9,10]. *S. pombe hda1+*, a putative HDAC gene reported very recently [18], was identical to *phd1+*. *hda1+* has been shown to be involved in telomere silencing and spore formation. We showed that

Table 1
Effect of *phd1* disruption on drug sensitivity

Drug	Minimal inhibitory concentrations		
	JY266	KH11 (Δ*phd1*)	
	pDB248′	pDB248′	pDHD1 (*phd1+*)
Trichostatin A (μg/ml)	> 50	30	> 50
Trapoxin A (μg/ml)	> 20	10	> 20
K-252a (μM)	20	20	20
Leptomycin B (ng/ml)	20	20	20
Hydroxyurea (mM)	10	10	10
Cycloheximide (μg/ml)	10	10	10
Valinomycin (μg/ml)	5	5	5
Actinomycin D (μg/ml)	10	10	10

Table 2
Effects of TSA and *phd1* disruption on the efficiency of mating and meiosis

	phd1+			Δ*phd1*
	TSA (μg/ml)			
	0	5	20	
Mating				
Heterothallic	100[a]	23	4.8	83[b]
Sporulation				
Homothallic haploid	100[c]	32	0.47	< 0.01[d]
Heterothallic diploid	100[e]	0.39	< 0.01	< 0.01[f]

[a]JY741×JY746; [b]KH11×KH12; [c]JY3; [d]KH13; [e]JY741/JY746; [f]KH11/KH12.
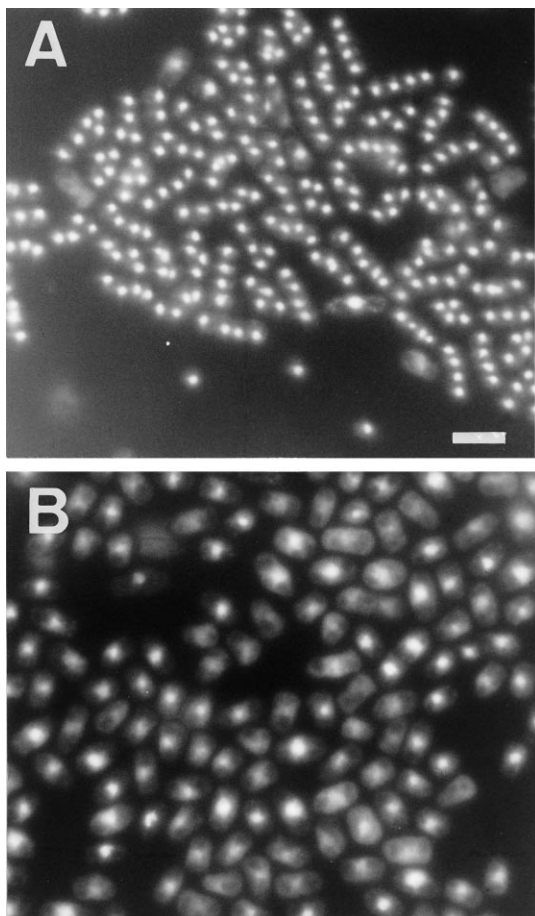Efficiencies were calculated as percentage of the control cultures.

Fig. 3. Effect of *phd1* mutation on meiosis. The nuclear morphology of the diploid cells with the wild-type background (A) or the homozygous *phd1* null allele (B) was visualized by 4′,6-dammidino-2-phenylindole (DAPI) staining after cultivation in sporulation medium at 26.5°C for 2 days. Bar: 10 μm.

FLAG-Phd1/Hda1 expressed in *S. pombe* actually possessed HDAC activity, although the bacterially produced Phd1 was inactive. Since human and *S. cerevisiae* HDAC are known to form a large protein complex associated with other proteins (for review, see [19]), it seems possible that Phd1 without other associated proteins is enzymatically inactive or shows, if any, only very weak enzyme activity. We also showed that the HDAC activity of FLAG-Phd1 was inhibited by TSA. However, the IC_{50} value was significantly higher than those for HDACs of other organisms. This higher IC_{50} value may explain why fission yeast cells are relatively resistant to TSA.

TSA causes arrest of the cell cycle in a wide variety of mammalian cells, suggesting that HDAC is essential for the mitotic cell cycle [7,20]. However, the present study showed the *phd1* disruptant was viable and showed no temperature- or cold-sensitive growth. It is conceivable that the residual enzyme activity in the *phd1* disruptant accounts for the viability of the mutant. Genomic Southern hybridization suggested that *S. pombe* possessed multiple HDAC genes as did *S. cerevisiae*, which we suppose is responsible for the residual enzyme activity. Proliferation of the *phd1* disruptant was more sensitive to TSA than the parental strain, probably due to the marked decrease in HDAC activity in this mutant (Fig. 2C). These results imply redundancy of the HDAC genes and an essential role of HDAC in the mitotic cell cycle in *S. pombe*.

In *S. pombe*, efficiencies of both mating and sporulation were reduced in the presence of TSA. This observation suggests that HDAC is involved in both processes. Even in the absence of *phd1*$^+$, however, mating occurred normally, indicating that *phd1*$^+$ is not directly involved in mating in *S. pombe*. On the other hand, homozygous diploids of the *phd1* disruptants were deficient in sporulation. The nuclear morphology of the *phd1* disruptant suggested that the meiotic cell cycle was arrested before meiosis I in the absence of *phd1*$^+$. Since histone acetylation is believed to regulate transcription and silencing through the structural changes in chromatin [19], it is possible that *phd1*$^+$ plays an important role in the transcriptional regulation for some key gene(s) in the early meiosis. It is obviously important to determine whether *phd1*$^+$ actually regulates the expression of meiosis-related genes. Furthermore, the present study suggests that each HDAC has a different role in cellular function. Characterization of other HDAC genes is important for understanding the mechanism by which the specific gene expression is regulated by histone acetylation.

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