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Cdk1 and *cks* gene homologs are transcriptionally activated during induction of conjugating pairs in mating-type II cells of the ciliate *Blepharisma japonicum*

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SUMMARY

In *Blepharisma japonicum*, conjugation is induced by interaction between cells of complementary mating-types I and II. Cells of both mating-types produce and secrete mating pheromones (gamones). Cells that have received complementary gamones undergo morphological changes (rounding) and start to unite. Nuclear changes, including meiosis, gametic nuclei exchange, fertilization and development of new macro- and micronuclei occur in the conjugants. Although conjugation is such a striking phenomenon, the molecular mechanism of induction of conjugation remains unknown. In order to identify genes that are involved in formation of conjugating pairs, we isolated genes that were expressed specifically in conjugation-induced type II cells, using suppression subtractive hybridization. To induce conjugation, we treated type II cells for 4 hours with a cell-free fluid from type I cells which contained gamone1. During this period, type II cells formed pairs, although these homotypic pairs never entered meiosis. We then purified polyA$^+$RNA and subjected it to cDNA synthesis. This cDNA was then subtracted with cDNA that was prepared from untreated cells. We obtained eight gene fragments. Homology searches revealed that three of these

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fragments showed significant homology to the cdk family (*cdk1* and *cdk2*), 4-hydroxy-phenylpyruvate dioxygenase (*4-HPPD*) and cyclin dependent kinase regulatory subunit (*cks*). Northern hybridization demonstrated that these three genes were specifically transcribed in cells treated with gamone1. We also found that the transcripts had already appeared 2 hours after the onset of gamone1 treatment. Cdk1 and cks are generally involved in cell-cycle regulation, but are here specifically expressed during induction of homotypic type II pairs that undergo neither mitosis nor meiosis.

INTRODUCTION

Conjugation of the heterotrich ciliate *Blepharisma japonicum* is induced by the interaction of type I and type II complementary mating-type cells (Miyake, 1968; Miyake and Beyer, 1973). Vegetative cells continuously divide under nutrient-rich conditions. However, when deprived of nutrients, cells begin sexual reproduction (conjugation). First, type I cells start secreting gamone1, one of the conjugation-inducting substances (mating pheromones), and this stimulates type II cells to secrete gamone2, the other conjugation-inducing substance. Gamone2 promotes gamone1 secretion in type I cells. Both mating-type cells are transformed, ready to join and enter the sexual reproduction process (Miyake, 1981, for review).

Gamone1 and gamone2 have both been isolated and characterized. Gamone1 is a glycoprotein of about 30 kD, and consists of 272 amino acids and seven sugar residues (Miyake, 1981; Sugiura and Harumoto, 2001; Harumoto and Sugiura, 2003). Gamone2 has been identified as calcium-3-(2'-formylamino-5'-hydroxybenzoyl) lactate, which can be chemically synthesized (Kubota, et al., 1973).

Treatment with cycloheximide inhibits conjugating pair formation, which indicates that new protein synthesis is essential for the pair formation (Miyake and Honda, 1976). This suggests expression of various genes during this peroid, but it has not been known which genes are activated by gamones and which genes are involved in the formation of conjugating pairs.

In this study, we used suppression subtractive hybridization in type II cells to isolate genes expressed during induction of conjugation. Suppression subtractive hybridization is an effective method for isolating genes that are differentially expressed in two conditions (Diatchenko, et al., 1999; Fetzer, et al., 2002). Gene expression was compared between conjugation-induced and non-induced cells, and the genes which expressed specifically in gamone1-treated cells were identified. Type II cells are suitable for isolating such genes, because whereas type I cells begin secreting gamone1 autonomously in response to starvation, type II cells usually do not respond this way. They start synthesizing gamone2 and progress toward conjugation only after being stimulated by gamone1. The cDNAs of type II cells before and after treatment with gamone1 were subjected to subtraction, and eight gene fragments were isolated. Homology searches revealed that three of these fragments are homologous to cyclin dependent kinase 1 (*cdk1*), cyclin dependent kinase regulatory subunit (*cks*) and 4-hydroxyphenylpyruvate dioxygenase (*4-HPPD*). We confirmed that these genes are expressed only in type II cells stimulated by gamone1 by northern hybridization. This unusual expression of *cdk1* and *cks* homologs in conjugating pairs of *Blepharisma* may indicate a novel function for these genes.

MATERIALS AND METHODS

1. *Blepharisma* stocks and culture method

We used mating-type II cells of *Blepharisma japonicum* R48 strain. The cells were cultured in diluted extract of wheat grass powder (Pines) with phosphate buffer (pH 6.8) and stigmasterol (5×10^{-4} mg/ml) at 25°C (Tokusumi and Takagi, 2000). *Enterobacter aerogenes* was inoculated, and cultured for 2 days before use. The method of culturing was described in detail by Harumoto and Sugiura (2003).

2. Gamone1 treatment for type II cells

Mating type II cells (1.2×10^6) were collected by centrifugation at 100×*g* and suspended for one day in physiologically balanced solution (modified synthetic medium for *Blepharisma,* SMB; Miyake, 1981; Harumoto and Sugiura, 2003) consisting of 1.5 mM NaCl, 0.05 mM KCl, 0.4 mM $CaCl_2$, 0.05 mM $MgCl_2$, 0.05 mM $MgSO_4$ and 2.0 mM phosphate buffer (pH 6.8). The cell suspension (1,500−2,000 cells/ml) was divided into two samples; one was treated with cell-free fluid from type I cells (CFF1; 10^3 U/ml gamone1) (Miyake and Beyer, 1973; Miyake, 1981), and the other was not treated. Cells were treated with CFF1 for 4 hours to prepare samples for subtraction and northern hybridization, and for 2 or 4 hours for dot hybridization.

Frozen CFF1 was thawed and used in this study. Preparation of CFF1 was described in Sugiura and Harumoto (2001).

3. Total RNA and polyA$^+$RNA isolation

Total RNA was isolated from both gamone1-treated and untreated cells. *Blepharisma* has pigment granules under the cell surface (Giese, 1973), which often prevent recovery of nucleic acids from cells. To remove this pigment, *Blepharisma* cells were subjected to cold shock treatment with SMB at 4°C for 10 minutes. After this treatment, cells discharged massive pigment granules.

Five milliliters of TRIzol Reagent (Invitrogen) was added to about 1×10^5 cells, and total RNA was isolated by the acid guanidinium–phenol–chloroform method. Total RNA isolated from a 3,000-ml culture was 1 mg. PolyA$^+$RNA was isolated with the Oligotex-dT30 [SUPER] kit (Roche Molecular Biochemicals). PolyA$^+$RNA was concentrated by ethanol precipitation and adjusted to a concentration of 1 μg/μl.

4. Suppression subtractive hybridization

Suppression subtractive hybridization was used to isolate differentially expressed transcripts. First and second strand cDNA synthesis, *Rsa*I endonuclease enzyme digestion, adapter ligation, hybridization and PCR amplification were performed according to the manufacturer's protocol for the PCR Select cDNA subtraction kit (Clontech). Subtracted and unsubtracted PCR products were run on a 2% agarose gel to observe the differential band pattern. Specific bands in the subtracted sample were excised, gel-extracted and cloned into pCR2.1-TOPO vector (Invitrogen).

5. Sequencing and homology search

Sequencing was performed by either of the following methods: 1) Big dye terminator v3.1 cycle sequencing kit (Applied Biosystems); the samples were sequenced using ABI3100. 2) Thermo sequence cycle sequencing kit (Amersham Biosciences); sequencing done by DSQ-2000L (Shimadzu). The determined sequences were subjected to homology search through the BLAST server provided by DNA Data Bank of Japan (DDBJ, http://blast.ddbj.nig.ac.jp/top-j.html).

6. Northern hybridization and dot hybridization

Probes were synthesized with a DIG DNA labeling kit (Roche Molecular Biochemicals). In northern hybridization, 10 μg total RNA was subjected to electrophoresis on denaturing 1% agarose gel and blotted onto positively charged nylon membranes (Roche Molecular Biochemicals). In dot hybridization, 5–10 μg total RNA was dropped directly on the membranes.

Table 1. Homology search for eight cDNA fragments obtained by suppression subtractive hybridization in this study.

	fragment size	BLAST search result protein homolog	*E*-value
s1001	432 bp	Cdc2 cyclin dependent kinase (*Pneumocystis carinii*, 104-246)*	$3 \times e^{-50}$
s1002	140 bp	no match domain	
s1003	878 bp	Elongation factor 1 gamma-3, EF-1-γ3 (*Oryza sativa*, 149-304)*	$1 \times e^{-8}$
s1004	710 bp	Hypothetical protein (*Plasmodium berghei*, 73-201)*	1.8
s1005	526 bp	Hypothetical protein (*Emericella nidulans*, 158-244)*	0.82
s1006	343 bp	Glyceraldehyde-3-phosphate dehydrogenase protein (*Blepharisma japonicum*, 228-303)*	$3 \times e^{-35}$
s1007	258 bp	Putative 4-hydroxyphenylpyruvate dioxygenase, 4-HPPD (*Paramecium tetraurelia*, 179-262)*	$4 \times e^{-31}$
s1008	227 bp	Cyclin dependent kinase regulatory subunit, cks (*Tetrahymena thermophila*, 45-96)*	$4 \times e^{-11}$

*Species with highest score is denoted. Numbers indicate amino acid residues that matched this sequence.

RNAs were UV-crosslinked to the membrane, and then hybridized with DIG-labeled DNA probes. The probes were detected by DIG luminescent detection kit (Roche Molecular Biochemicals).

RESULTS

Subtractive hybridization, sequencing and homology search of genes isolated from cells treated with gamone1

To isolate genes expressed in type II cells of *B. japonicum* during the induction of conjugating pair formation by gamone1, we used the suppression subtractive hybridization method. The type II cells had started to form homotypic pairs 2 hours after the addition of CFF1. Fewer than 10% of cells had paired at 2 hours, but the number gradually increased. After 4 hours, most cells had been activated and formed pairs. Suppression subtractive hybridization was performed using cDNAs created from polyA^{+}RNA isolated from gamone1-treated (4 hr) cells as the 'tester' and from untreated cells as the 'driver' (Clontech). Subtracted cDNA fragments were amplified by PCR and electrophoresed. Eight major bands with lengths ranging between about 200 and 900 bp were gel-extracted, cloned and sequenced. One of these bands contained two individual sequences (s1007, s1008). Among these nine sequences, a part of one sequence matched another sequence (s1001). We eventually obtained eight distinct sequences from suppression subtractive hybridization (s1001 – s1008).

Homology searches revealed that one of the sequences (s1006) was glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), a gene already known in *B. japonicum*. The other seven sequences (s1001–s1005, s1007, s1008) were newly isolated in *B. japonicum*. Table 1 shows the result of homology searches for each sequence.

Fragment s1001 had a length of 432 bp and was deduced to encode 143 amino acids (aa). A search revealed high homology to cyclin dependent kinase, *cdk1* (*cdc2*) or *cdk2* in a wide range of organisms. The gene that showed highest similarity (60% identities, *E*-value: 3e-50) was cyclin dependent kinase (*cdc2*) of *Pneumocystis carinii*.

Fragment s1007, a 258 bp (85 aa) sequence, showed high homology to 4-hydroxyphenylpyruvate dioxygenase (*4-HPPD*).

Fragment s1008, a 227 bp (71 aa) sequence, showed homology to cyclin dependent kinase regulatory subunit (*cks*). Alignment of the sequence with human, *Xenopus* and other *cks* homologs revealed that this sequence had a 32-residue overhang at the C-terminus of these known cks proteins. The sequence contains the HXPEPH motif which is conserved in cks-family proteins.

The sequence of s1003 was 878 bp, an estimated 169 amino acid residues. This sequence showed a low homology to elongation factor 1 gamma 3 (*EF-1-γ3*) of *Oryza sativa.*

Sequence s1002 was 140 bp, consisting of repeated GAA. Sequence s1004 was 710 bp, and showed low homology to a hypothetical protein of *Plasmodium berghei* (73–201 aa); and sequence s1005 was 526 bp with low homology to a hypothetical protein of *Emericella nidulans*.

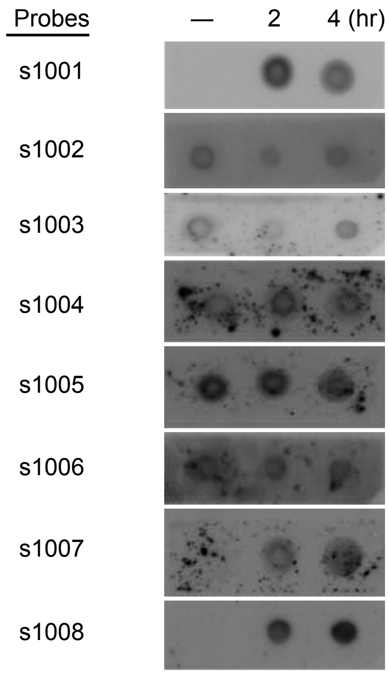

Fig. 1. Dot hybridization performed using total RNA from untreated cells (-), and cells treated for 2 hours (2) and 4 hours (4) with cell-free fluid from type I cells (CFF1). Probes are eight individual cDNA fragments (s1001–s1008) obtained by suppression subtractive hybridization (Table 1). Amounts of applied total RNA were 5 μg for s1002, s1003 and s1006, and 10 μg for s1001, s1004, s1005, s1007 and s1008.

Confirmation of differential expression by dot hybridization analysis

To confirm whether these eight genes were expressed specifically in the formation of conjugating pairs, dot hybridization was performed using total RNA from untreated cells and cells that had been treated with gamone1 for 2 or 4 hours (Fig. 1). Probes were made from the eight sequences (s1001–s1008). Differential expression signals were detected only in gamone1-treated samples probed with s1001 (*cdk1* homolog), s1007 (*4-HPPD* homolog) and s1008 (*cks* homolog). It was also shown that these three genes were already transcribed 2 hours after the onset of the gamone1 treatment. No difference in expression was detected between treated and untreated samples probed with s1002, s1003, s1004, s1005 and s1006.

Northern hybridization analysis

To analyze the transcripts of *cdk1* (s1001) and *cks* (s1008), total RNA from gamone1-treated and untreated samples were electrophoresed, blotted onto nylon membranes, and hybridized with probes (Fig. 2a and 2b). Ethidium bromide staining showed that the amount of RNA applied to the gel was equal. Single bands were detected only in treated samples for both s1001 and s1008. The sizes of the transcripts were about 1,000 nucleotides (s1001, Fig. 2a) and about

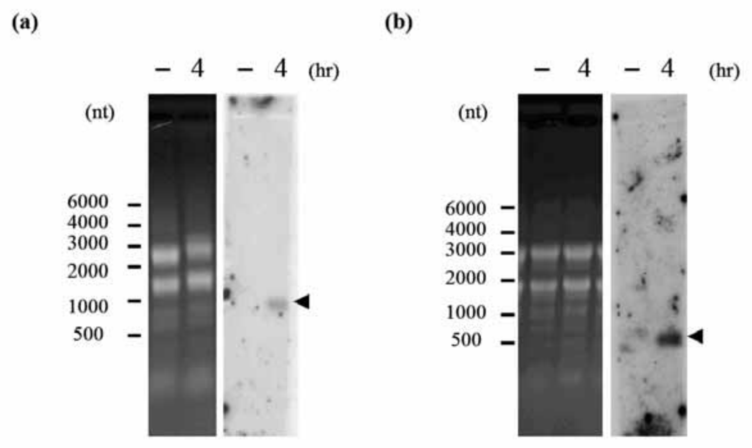

Fig. 2 Northern hybridization probed with s1001 (*cdk1* homolog, a) and s1008 (*cks* homolog, b). Total RNA (10 μg) from untreated cells (-) and cells treated with CFF1 for 4 hours (4) were subjected to electrophoresis and stained with ethidium bromide (left-hand panels), and hybridized with probes (right-hand panels). Arrowheads show positions of detected signals. The size of RNA (nt; nucleotides) is shown on the left.

500 nucleotides (s1008, Fig. 2b). Most cdk1- and cdk2-family proteins are about 280–300 aa. The length of the s1001 transcript detected in this study is consistent with this. When the 500-nucleotide s1008 transcript is translated as a protein with about 150 aa, it is atypically long compared with conventional cks-family proteins (about 100 aa). The 23-residue overhang at the C-terminus of s1008, described above, may be a possible explanation.

DISCUSSION

Using suppression subtractive hybridization, we identified three genes that were expressed specifically in the formation of conjugating pairs of type II cells of *B. japonicum*. *Cdk1*, *cks* and *4-HPPD* homologs were isolated for the first time in *B. japonicum*. Cdk1 is a protein involved in cell-cycle regulation. Cks is a small conserved protein that interacts with cdk, contributing to a family of essential components of the cdk complex that regulates cell-cycle progression.

In this study, suppression subtractive hybridization was applied to gamone1-treated and untreated type II cells. Type II cells are suited to this method because they only start secreting gamone2 and progress toward conjugation after they are stimulated with gamone1. Type II cells were activated and formed conjugating pairs of the same mating type (homotypic pairs). Unlike heterotypic pairs (type I–type II pairs), homotypic pairs (type II–type II) undergo neither meiosis nor nuclear exchange, and macro- and micronuclei remain unchanged. In *B. japonicum*, doublet cells with two mouths have been constructed by treatment with actinomycin S_3 (Miyake, 1975). The doublet can join with two cells, one on either side. Complementary gamone treatment induced a chain consisting of several doublets. Such homotypic chains did not undergo nuclear changes, but meiosis was initiated when a single complementary mating-type cell joined at the end of the chain (Miyake, 1975). Meiosis first started in the cell that united with a complementary cell, and then propagated through the chain. This observation suggests that the nuclear changes are arrested before the onset of meiosis in homotypic pairs, and the signal produced in a heterotypic pair was transmitted along the chain and released the interruption. It also suggests that homotypic pairs are ready for conjugation except for a signal provided by complementary mating-type cells.

Using type II homotypic pairs, we attempted to isolate genes involved in the formation of conjugating pairs; for example, genes related to gamone2 biosynthesis, those involved in signal transduction, or those that regulate morphological changes leading to conjugation. We found that *cdk1*, *cks* and *4-HPPD* homologs were activated by gamone1 in type II cells. Expression of additional genes may also be involved in these processes. These genes might be lost during the procedure of subtractive hybridization or be expressed at levels too low to detect. Significant differential expression is evident in *cdk1*, *cks* and *4-HPPD* homologs, which suggests that they are essential for the processes induced by gamone1 in type II cells. It is known that 4-HPPD is involved in amino acid metabolism. Gamone2 is a small molecule which is synthesized from tryptophan (Miyake, 1981). The *4-HPPD* homolog isolated in this study might be involved in the biosynthetic pathway of gamone2.

It is remarkable that, although *cdk1* and *cks* are cell-cycle related genes, homologs isolated in this study were activated in homotypic pairs which underwent neither mitosis nor meiosis. In *Tetrahymena thermophila*, a *cdk1* homolog was associated with basal body domains, and knockout of the gene resulted in decondensation of macro- and micronuclear chromosomes. Zhang, et al. (2002) suggested that the *cdk1* homolog might be involved in establishing morphogenetic pattern formation and also be associated with linking of histone H1. In *Paramecium tetraurelia*, two *cdk1* gene homologs were isolated (Tang, et al., 1995; Zhang and Berger, 1999). In both *T. thermophila* and *P. tetraurelia*, these genes were expressed during the mitotic cell cycle. The *cdk1* homolog isolated in this study has relatively low homology to these *cdk1* genes, and there has been no report that cdk1 is related to the formation of conjugating pairs in ciliates. The *cdk1* and *cks* homologs isolated in this study may serve unconventional functions.

Cks has been reported to interact genetically and physically with cdk. The function of cks as a cell-cycle regulator has been reported in *Trypanosoma* (Muñoz, et al., 2006), budding yeast (Morris, et al., 2003), *Drosophila* (Swan, et al., 2005), mammalian cells (Spruck, et al., 2003) and a variety of other organisms. However, its function in ciliates has remained unknown. Further study of cdk1 and cks in *B. japonicum* may provide a unique system to investigate not only formation of conjugating pairs but also a wide variety of functions of the cdk–cks complex in ciliates.

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