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Crosstalk of Prolyl Isomerases, Pin1/Ess1, and Cyclophilin A

Fumihiro Fujimori,*[,1] Wataru Gunji,*[,1] Junko Kikuchi,* Tomoko Mogi,* Yoko Ohashi,† Tohru Makino,† Akira Oyama,‡ Koji Okuhara,* Takafumi Uchida,§ and Yasufumi Murakami*[,2]

**Department of Biological Science & Technology, Faculty of Industrial Science & Technology, Science University of Tokyo; †Life-Science Technology Research Center, Olympus Optical Co., Ltd.; ‡Mitsui Knowledge Industry Co., Ltd.; and §Department of Pathology, Institute of Development, Aging and Cancer, Tohoku University, Japan*

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Previous studies have indicated that Ess1/Pin1, a gene in the parvulin family of peptidyl-prolyl isomerases (PPIases), plays an important role in regulating the G_2/M transition of the cell cycle by binding cell-cycle-regulating proteins in eukaryotic cells. Although the *ess1* gene has been considered to be essential in yeast, we have isolated viable *ess1* deletion mutants and demonstrated, via analysis of yeast gene expression profiles using microarray techniques, a novel regulatory role for ESS1 in the G_1 phase. Although the overall expression profiles in the tested strains (C110-1, W303, S288c, and RAY-3AD) were similar, marked changes were detected for a number of genes involved in the molecular action of ESS1. Among these, the expression levels of a cyclophilin A gene, also a member of the PPIase family, increased in the *ess1* null mutant derived from C110-1. Subsequent treatment with cyclosporin A significantly retarded growth, which suggests that ESS1 and cyclophilin A are functionally linked in yeast cells and play important roles at the G_1 phase of the cell cycle.

Key Words: **Pin1; Ess1; peptydyl-prolyl *cis/trans* isomerase; PPIase; cyclophilin A; cell cycle; microarray.**

Peptidyl-prolyl *cis/trans* isomerases (PPIases) catalyze the *cis/trans* isomerization of the peptide bond preceding proline (1–3) and are believed to regulate the activities of mature proteins by promoting assembly or intracellular transportation of their subunits via such isomerizations (4–6). The PPIase family consists of three subfamilies—the cyclophilins, the FK506-binding proteins (FKBPs), and the parvulins, of which the cyclophilins and FKBPs have been the best-characterized.

The parvulin family was originally discovered in bacterial cells (7–11), and homologues of parvulin genes (Pin1 and hPar14) have recently been identified in human tissues (12, 13). Human PIN1 shows strong sequence similarity to a yeast protein, PTF1/ESS1 (14, 15). The ESS1/PIN1 protein has a WW domain at the N-terminus and a PPIase domain at the C-terminus (16), but the prokaryotic parvulin possesses only the PPIase domain. However, the WW domain and the PPIase architecture are conserved in the ESS1/PIN1 homologues of many eukaryotes, including *Saccharomyces cerevisiae* (PTF1/ESS1; 14, 15, 17), *Drosophila melanogaster* (Dodo; 18, 19), *Aspergillus nidulans* (Pin1; 20), mice (Pin1; 21), and *Neurospora crassa* (Ssp1; 22).

The human PIN1 protein was originally isolated through its interaction with the NIMA (never in mitosis gene a)-related kinase and was shown to be essential for the progression of mitosis in HeLa cells (12). The depletion of the PIN1 protein in HeLa cells and the disruption of *ess1* gene function in yeast cells were both shown to inhibit cell growth and induce mitotic arrest (14, 17). PIN1 overexpression is likewise deleterious, retarding growth in budding yeast and causing G_2 arrest in both HeLa cells and extracts of *Xenopus laevis* egg, which suggests that the ESS1/PIN1 gene plays a key role in the initiation of mitosis (12, 20). In budding yeast *ess1* deletion mutants exhibit mitotic arrest, suggesting that the PPIase activity of the PTF1/ESS1 gene product is required for mitotic exit (14, 17). On the other hand, experiments with knockout mice have shown that mice lacking the Pin1 gene develop normally (21), and complete removal of the dodo gene from the *Drosophila* genome yields offspring that are viable and fertile (19). These results indicate that even though Ess1/Pin1 plays an important role in cell cycle

[1] These authors contributed equally to this work.

[2] To whom correspondence and reprint requests should be addressed at Laboratory of Genome Biology Department of Biological Science & Technology, Faculty of Industrial Science & Technology, Science University of Tokyo Yamazaki, Chiba 278-8510, Japan. Fax: +81-471-22-1360. E-mail: yasufumi@rs.noda.sut.ac.jp.

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regulation—especially in mitosis—the existence of functional homologues of these genes and proteins, such as PIN1-Like or hPAR14 (13, 23) implies that the effect of the deletion could in some cases be overcome.

The WW domain of Ess1/Pin1 comprises 35–40 amino acid residues and is known to interact with proline-rich peptides (24, 25). This domain is also capable of binding phosphorylated serine or threonine residues prior to its binding of proline-containing peptides. In experiments carried out *in vitro,* ESS1/PIN1 bound first to residues such as phosphoserine or phosphothreonine and then to a proline residue (12, 16, 20, 26) to create a structure that is recognized by the MPM-2 antibody (27, 28). An important *in vitro* study demonstrated that PIN1 interacts with the mitotic phosphatase CDC25C substoichiometrically (29). PIN1 has also been shown to associate with the phosphorylated tau protein and with microtubules in brain extracts (30). The functional relevance of these interactions, however, is still unclear. And although a PPIase activity of ESS1 has been demonstrated *in vitro* (31), a role for Ess1/Pin1 in controlling the function of cell cycle regulators by direct interaction is also unclear.

Several Pin1 functions have been reported in addition to its interaction with cell cycle regulating proteins. In yeast, Ess1 has been found to be involved in transcriptional regulation as well as in RNA processing (15, 17, 32, 33), and a mutation in Ess1 (Ptf1) has also been proposed to cause a defect in mRNA 3′-end formation (15). In mammalian cells, PIN1 weakly activates transcription when bound to DNA via GAL4 (34). PIN1 depletion in *Xenopus* oocyte extract experiments indicated that PIN1 activity is required for the DNA replication checkpoint. When embryonic fibroblast cells derived from Pin1 knockout mice were synchronized in G_0 by serum starvation, such cells did not enter the cell cycle, even when they were supplemented with normal amounts of serum (21). Ess1/Pin1 has been shown to be important for cell viability and seems to target multiple sites, such as Sin3-Rpd3 histone deacetylase (35, 36), RNA polymerase II CTD, CDC25C, and others.

DNA microarray hybridization analysis is a powerful tool for identifying new targets of transcription factors in yeast. This approach has been recently validated by studies of genes repressed in deletion mutants (37, 38). Here we report a genomic approach to understanding Ess1/Pin1 transcriptional regulation. First, mRNA-expression profiles in various yeast hosts are compared in order to understand the expression background. Then mRNA-expression levels are analyzed in deletion mutants to determine how they differ from wild type, and cell cycle analyses are performed with isolated mutants. Based on these results, we propose a possible role for Ess1 in cell cycle regulation at the G_1 phase involving the concerted action of the gene product with cyclophilin A.

MATERIALS AND METHODS

Yeast host strains. The haploid strains RAY1-13D (*MATa ura3 leu2 his3 trp1*) (39), W303-1a (*MATa ade2-1 ura3-1 his3-11 trp1 leu2-3 112 can1;* kindly provided by Dr. H. Uemura), and S288C (*MATa mal gal2*) were used to prepare mRNA for microarray analysis. The diploid strains C110-1 (*MATa/MAT leu2-3/leu2-3 leu2-112/leu2-112 ura3-52/ura3-52 his6/HIS6;* provided by Dr. H. Uemura) and RAY3A-D (*MATa/MAT ura3/ura3 leu2/leu2 his3/his3 trp1/trp1*) were used for the gene disruption experiment as well as for microarray analysis.

Media for yeast cell growth and disruption of ess1. Cell growth conditions with synthetic complete (SC) and YPD media were as previously described (40, 41). Disruption of the yeast open reading frame (ORF) for *ess1* was performed using a PCR-based gene replacement approach (Fig. 1) with *KanMX6* as the selection marker (42, 43). A pair of oligonucleotides (forward and reverse primers) was constructed. The forward primer (90-mer) contained the 70-nucleotide sequence located immediately upstream from the first ATG codon of the Ess1 ORF (5′-CCT ATT TAT TAT TAC ATC ACC TTT CAC GAG CGG GTA TCA TAC CTT TTT CCC TTC GAT CTA AGT AGA AAA A-3′) plus a 3′-tail of 20 nucleotides (5′-CGT ACG CTG CAG GTC GAC GG-3′) corresponding to the 5′-sequence of *KanMX6.* The reverse primer contained the 70-nucleotide sequence external to the stop codon of the Ess1 ORF (5′-TTC CGC TCG CAG CGA AGA ACA GCG CCA TTT AAC TAT GTG ACT AGT TAA TTT TTT TTC TTT TCA TGG GTT C-3′) plus a 3′-tail of 20 nucleotides (5′-CATCGATGAATTCGAGCTCG-3′) corresponding to the 3′-sequence of *KanMX6.* A PCR was performed with these primers, and approximately 2 μg of the PCR product was used to transform yeast cells treated with lithium acetate. The transformed cells were then plated on YPD agar plates containing 300 μg/ml geneticin disulfate (G418, Sigma).

The G418-resistant transformants were tested by PCR and Southern blot analysis to verify the correct replacement in the targeted locus. In the PCR, oligonucleotides OF (5′-AAT CCC TCC CAC GTA TAA ACA C-3′) and OR (5′-ATT ACT GAG TCA TCT GGA GAG G-3′) were synthesized to correspond to the 5′ and 3′ sites, respectively, flanking this ORF. Oligonucleotides K2 (5′-CGG GCG ACA GTC ACA TCA TG-3′) and K3 (5′-CCC AGA TGC GAA GTT AAG TG-3′) were similarly generated from the *KanMX6* sequence. Colony PCR with the OF and K2 primers or the OR and K3 primers generated products of predicted sizes in the presence of genomic DNA standards (Fig. 1).

Southern analysis. The probe region was amplified with a set of primers [Primer 1 (5′-CTG GAA CAA GAT GCT GCT ACG AAC GG-3′) and Primer 2 (5′-CGC CAT CTC TAG AGC AGT CTG GCG GG-3′)]. Restriction fragments of expected sizes were detected in the genomic DNA samples from both wild type and disruptant.

Tetrad analysis. For tetrad analysis, two independently disrupted clones (RAY3A-D and C110-1) were cultured in 1% potassium acetate (w/v) for 4 to 5 days at 30°C to germinate spores that were then dissected under a microscope. To assess viability dissected spores were incubated on YPD or SC agar plates containing G418 at 25, 30, and 37°C for 2 to 3 days.

Yeast mRNA preparation and labeling of Cy3 and Cy5. Total RNA was prepared, and fluorescence-labeled cDNA was purified and hybridized as previously described (37, 44). Reverse transcriptase (Superscript II; Pharmacia) was used to label cDNAs with Cy3-dUTP or Cy5-dUTP (Amersham). Reaction mixtures were concentrated to less than 10 μl using Microcon-30 microconcentrators (Amicon). A set of labeled cDNA (Cy3-labeled and Cy5-labeled) was resuspended in hybridization solution [3× SSC, 0.2% SDS (w/v)] and hybridized to probes spotted onto microarrays for 6 h at 63°C under the conditions specified by the manufacturer (GeneChip Research). Hybridization signals were detected by a microarray scanner (Scanarray 4000) and normalized to the signal obtained with the *Act1* gene as an internal standard.

Analysis of drug response. To test for drug sensitivity of these yeast strains, clones of exponentially growing yeast cells maintained in liquid culture with either YPD or SC medium were serially diluted and inoculated onto agar plates containing various drugs. Agar plates containing different drug concentrations were prepared by adding the substances from stock solutions to 4 ml of YPD or SC agar chilled to 50°C after autoclaving. Serial dilutions of exponentially growing (A_{600} = 1.0) cells were prepared by first diluting the cultures to 25,000 cells/μl and diluting serially to a final concentration 40 cells/μl. Plates were spotted with 1 μl of each serial dilution, and growth of yeast cells was monitored after incubation of the cells for 48 h at 25°C. The immunophilin drugs cyclosporin A (Sigma), FK506 (Calbiochem), and rapamycin (Sigma) as well as the cell cycle inhibitors hydroxyurea (Sigma), etoposide (Sigma), aphidicolin (Sigma), radicicol (Sigma), nocodazol (Sigma), and demecoline (Sigma) were each dissolved in dimethyl sulfoxide.

Flow cytometry analysis. Propidium iodide was added to a final concentration of 20 μg/ml to yeast cell suspensions (A_{600} = 1.0) grown in YPD medium with drugs (above). Stained cells were then analyzed by flow cytofluorometry using the FACScan (Becton–Dickinson). α-Factor (Sigma) and hydroxyurea (Sigma) were used to synchronize at the G_1 and S phases, respectively. After blocking at the G_1 or S phases by inhibitors, the medium was changed to drug-free one then the cells were released from G_1 or S phases.

RESULTS

Isolation of ess1 Null Mutants and Analysis of Their Phenotypes

To test the viability of *ess1* null mutants, we constructed gene deletions using RAY3A-D and C110-1 strains as host cells. Since the *ess1* allele was successfully replaced with a *KanMX6* gene in these diploid constructs (Fig. 1B), the viabilities of transformant spores could be examined by tetrad analysis. The *ess1* disruptant constructed from RAY3A-D produced no viable spores (Fig. 1E), an observation that is consistent with previously reports (14, 15, 17). In *ess1* disruption experiments with the C110-1 strain, however, colonies of these *ess1* null mutants were viable, but they were substantially smaller than wild type (Fig. 1D).

Southern blot and PCR analyses were performed to confirm that the chimeric gene was, in fact, integrated into the desired chromosomal region (Figs. 1B and 1C). Colony PCR analysis in the presence of genomic DNA with either the OF and K2 primers or the OR and K3 primers yielded products of the expected size (Fig. 1B). Southern blots also detected restriction fragments of the expected sizes from both wild-type and recombinant strains (Fig. 1C). These results indicate that the *ess1* disruption causes a slow growth phenotype in the C110-1 strain.

To examine the growth properties of slowly growing *ess1* deletion cells, the viabilities of dissected spores were tested on YPD and SC plates at several temperatures (25, 30, and 37°C; Fig. 2). All haploid spores of the *ess1* null mutant of C110-1 were viable at all temperatures and on both media, although they grew more slowly on SC agar plates than on YPD (Fig. 2). A similar effect was observed for liquid media. The delet-

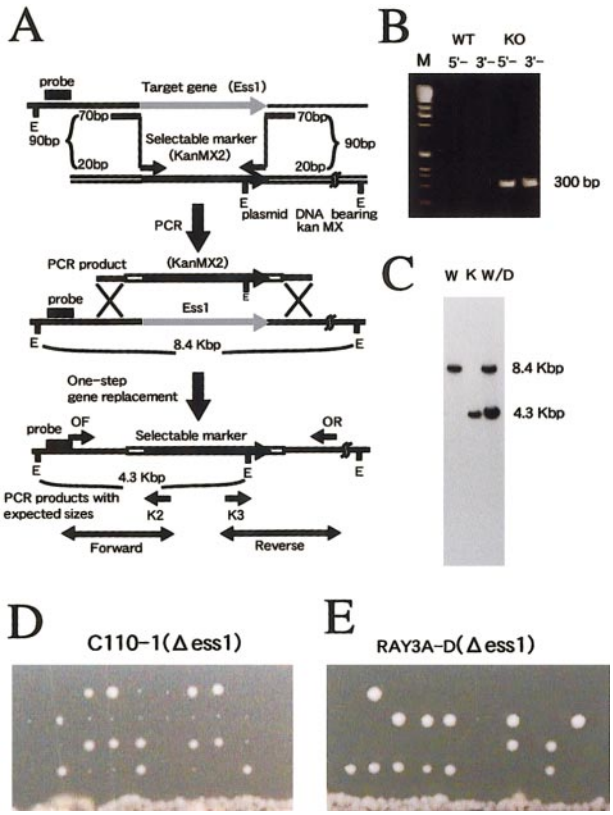

FIG. 1. Outline of the *ess1* disruption procedure. (A) The entire region of the targeted ORF (Ess1) was deleted and replaced with the *KanMX6* by PCR-mediated gene replacement. PCR with a pair of chimeric oligonucleotides (99-mer each) was performed to prepare the DNA fragment containing the selectable marker *kanamycin* used for transforming the yeast diploid RAY3A-d or C110-1. (B) 5′- and 3′-PCR with OF and K2 primers and OR and K3 primers to identify ± diploid cells. (C) Southern blot analysis of genomic DNA extracted from yeast strain C110-1. DNA was digested with *Eco*RI and hybridized with the probe (black bar). The sizes of wild-type (WT) and disrupted (KO) alleles are shown; the yeast genotypes are indicated above the lanes. (D and E) Tetrad analysis results of the *ess1* mutation in the two strains. Each strain, C110-1 or RAY3A-D, was targeted by replacement with the *kanamycin* gene; the hetero knockout diploid was then examined by tetrad analysis. Each tetrad was cultured on YPD medium at 30°C, for 2 days.

ant grew almost as fast as wild type at the two lower temperatures (25 and 30°C) in YPD liquid medium but grew much slower than wild type at 37°C. The *ess1* deletion mutants constructed in W303-1a, S288C, and RAY1-13D strains exhibited lethal phenotypes (data not shown).

Comparison of Expression Profiles in Yeast Host Strains

To understand why *ess1* disruption in RAY3A-D cells yielded a lethal phenotype while disruptants prepared with C110-1 were viable, albeit slowly growing, we analyzed expression profiles by a microarray technique with YFL007w and YFL039c as internal standards. In

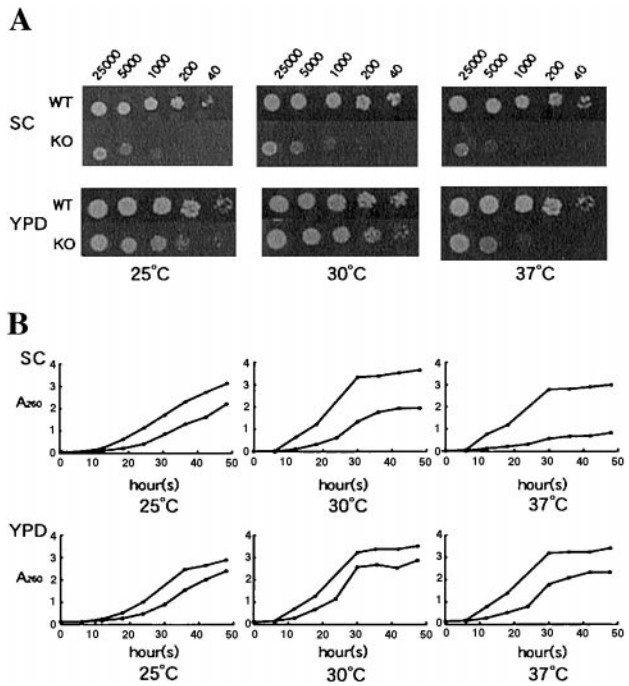

FIG. 2. Growth conditions for *ess1* mutants. (A) Growth of wild type and *ess1* mutants at 25, 30, and 37°C on solid media. Cells were grown to log phase in liquid YPD or SC medium, and serial 1:5 dilutions were spotted onto YPD agar or SC agar plates and incubated for 2 days at the indicated temperatures. (B) Growth curves of wild type and *ess1* mutants at 25, 30, and 37°C on liquid medium. Cells were grown to log phase in liquid YPD or SC medium, then 1/10 vol cultures were transferred into fresh YPD or SC liquid medium, respectively, and incubated for 48 h at the indicated temperature.

a previous study we had selected YFL007w and YFL039c as internal standards for Northern analysis of the ORFs on yeast chromosome VI (41). The YFL007w transcript is of a size that allows its band to be readily distinguished from most other transcripts on Northern blots; YFL039c (*ACT1*) is a commonly used internal control in quantitative Northern analysis. After using these two genes' data to normalize expression levels, the microarray data were found to be highly reproducible (data not shown).

To further evaluate the suitability of the microarray system, expression profiles were determined for all yeast genes prepared from yeast cultures grown in the presence of either glucose or galactose. Among highly expressed genes, the expression levels of GAL-related mRNAs were also elevated (data to be published elsewhere). These results suggest that the accuracy of this DNA microarray is sufficient to support the conclusions described below.

The two host cells were first analyzed for differences in expression levels of all yeast genes. Samples of mRNA were prepared from exponentially-growing haploid cells of C110-1 and RAY3A-D isolated from tetrad analysis. Gene chip analysis indicated that expression levels of most genes were not drastically changed in these host cells (Fig. 3A). Although changes in expression levels were observed for some genes, no significant changes were observed for the genes hypothesized to be involved in cell cycle regulation or cell proliferation (Table 1). The copper-binding metallothioneins (CUP1-1, CUP1-2) were upregulated significantly in the C110-1 strain, compared to RAY3A-D (Table 1) and strains W303-1a and S288C (data not shown); however, this quality appears to be a characteristic of the C110-1 strain itself. YGR234w (YHB1) in C110-1 is a downregulated gene but the meaning of this phenomenon is not clear. The haploid host cells of strains W303-1a, S288C, and C110-1a (all clones isolated by tetrad analysis) were also analyzed by the microarray method. No significant changes in expression level were observed (data not shown).

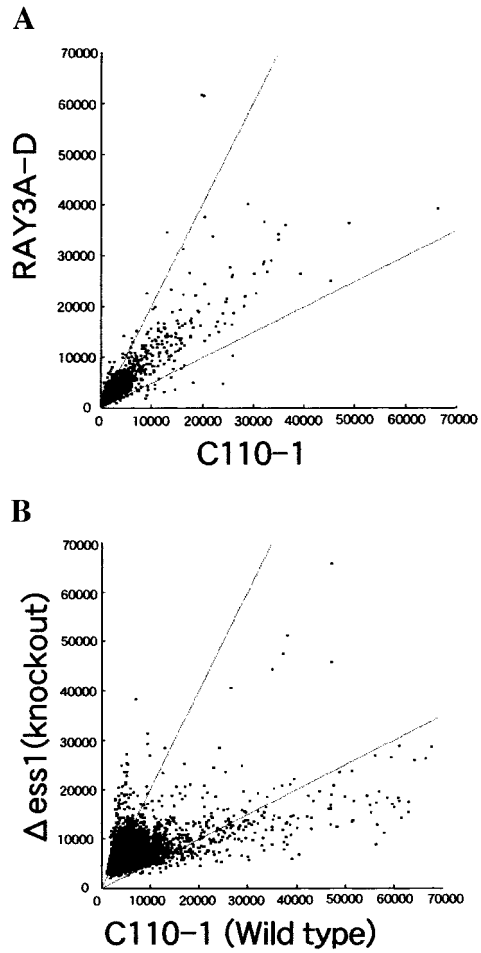

FIG. 3. Microarray analysis results. (A) Plot of the expression ratio for each ORF derived from the C110-1 versus RAY3A-D cells harvested from cultures in YPD liquid medium. ORFs that were induced or repressed in both experiments are shown as dots. (B) Plot of $\log_{10}$ of the expression ratio for each ORF derived from the *ess1* mutant versus wild type harvested from YPD liquid medium. ORFs that were induced or repressed in both experiments are shown as dots.

TABLE 1

ORFs Derived from the C110-1 versus RAY3A-D Cultured in YPD Liquid Medium

Upregulated genes					Downregulated genes				
ORF	Standard name	Function	Difference	Fold	ORF	Standard name	Function	Difference	Fold
YHR055C	CUP1-2	Copper-binding metallothionein	1349.0	3.10	YGR234W	YHB1	Stress response	−626.2	5.06
YHR053C	CUP1-1	Copper-binding metallothionein	1332.8	3.04	YNL143C	Hypothetical ORF		−517.2	2.58
YAR009C	Ty ORF	TyB Gag-Pol protein	693.4	2.65	YLR162W	Hypothetical ORF		−430.1	3.69
YDR050C	TPI1	Riosephosphate isomerase	427.2	2.44	YNL185C	MRPL19	Large ribosomal subunit	−359.6	4.16
YKL060C	FBA1	Aldolase	258.3	2.12	YLR053C	Hypothetical ORF		−302.3	3.96
YJL153C	INO1	L-Myo-inositol-1-phosphate synthase	242.1	2.21	YJR047C	ANB1	Translation initiation factor	−267.2	2.07
YCL064c	CHA1	Catabolic serine dehydratase	229.1	4.90	YEL033w	Hypothetical ORF		−257.0	2.33
YDR233C	Hypothetical ORF		209.7	2.03	YGL009C	LEU1	Isopropylmalate isomerase	−226.3	4.73
YAL008W	FUN14	Product of gene unknown	199.6	3.20	YNL179C	Hypothetical ORF		−224.9	2.86
YKL001C	MET14	Adenylylsulfate kinase	196.2	2.36	YLL053C	Hypothetical ORF		−216.9	3.80

Differences in mRNA Expression Level between Wild Type and Disruptant

To identify genes whose expression levels had changed in the *ess1* null mutant, mRNA expression levels of the mutant were compared to those of wild type C110-1 cells growing exponentially in YPD medium at 25°C. Comparison was assisted by the fact that the growth rate of the *ess1* null mutant approximated that of wild type under these conditions, which effectively eliminated the confounding effects of differences in growth rate per se.

Many genes exhibited lower expression levels in the deletion mutant, although we had tried to minimize the effects of the slowly growing phenotype. Table 2 lists genes that were upregulated in the *ess1* deletant. These were primarily the stress-related genes YBR072w (HSP26), YDR115c (CPH1), YNL064c (YDJ1), and YJR147w (HMS2). Interestingly, the mRNA level of

TABLE 2

ORFs Derived from the *ess1* Mutants versus Wild Type (C110-1) Cultured in YPD Liquid Medium

Upregulated genes					Downregulated genes				
ORF	Standard name	Function	Difference	Fold	ORF	Standard name	Function	Difference	Fold
YBR072W	HSP26	Heat shock protein	323.8	5.35	YGR234W	YHB1	Stress response	−855.4	18.61
YMR209C	Hypothetical ORF		228.5	5.23	YCR005c	CIT2	Citrate metabolism	−838.6	12.99
YDR155C	CPH1	Cyclophilin (PPIase)	225.7	3.24	YOR153W	PDR5	Pleiotropic drug resistance	−688.2	14.44
YMR204C	Hypothetical ORF		215.7	6.07	YOR317W	FAA1	Acyl:CoA synthetase	−499.1	6.45
YMR289W	Hypothetical ORF		209.0	4.85	YCL064c	CHA1	Catabolic Ser/Thr dehydratase	−356.3	6.14
YJR158W	HXT16	Hexose permease	206.4	4.73	YLR198C	Hypothetical ORF		−311.9	4.81
YNL064C	YDJ1	Heat shock protein	205.3	3.05	YOR129C	Hypothetical ORF		−306.5	4.30
YJR043C	POL32	Nucleotide-excision repair	195.7	4.93	YGL008C	PMA1	Plasma membrane H^+-ATPase	−296.1	4.06
YJR051W	OSM1	Osmotic growth protein	189.8	4.46	YKR042W	UTH1		−275.8	4.00
YJR147W	HMS2	Heat shock protein	181.6	4.31	YGR065C	VHT1	H^+-biotin symporter	−272.2	4.48

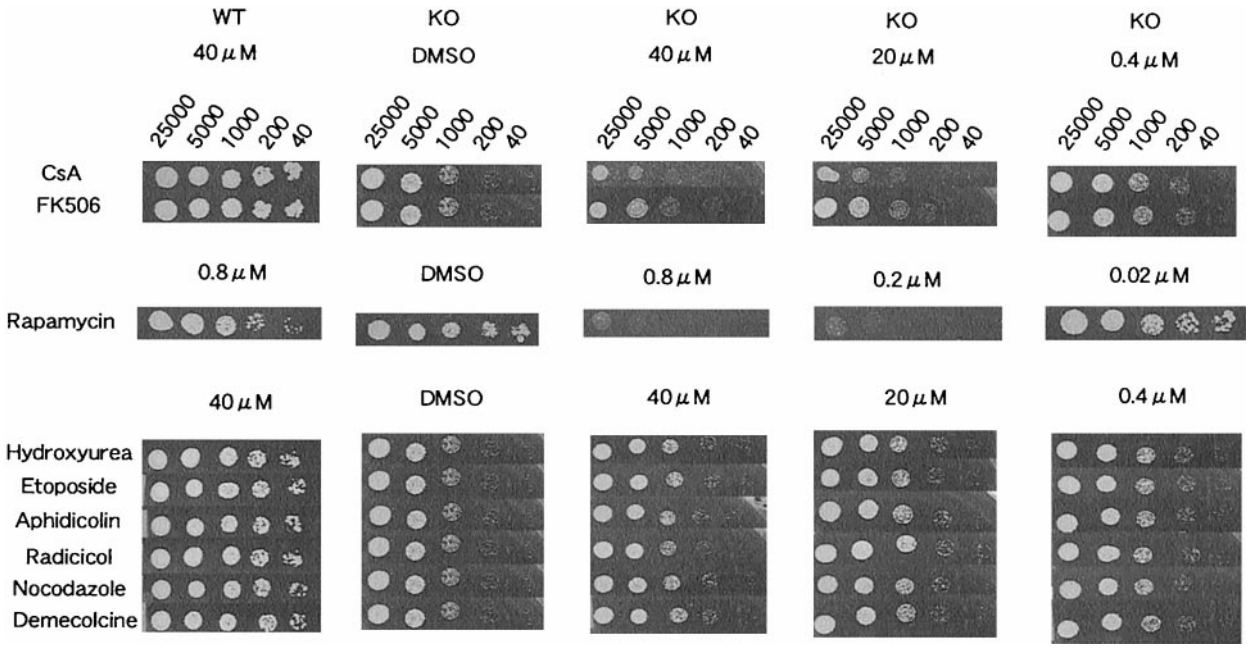

FIG. 4. Deletion of the *ess1* gene in strain C110-1 and its sensitivity to concentrations of several PPIase inhibitors. Each spot was inoculated onto YPD agar plates and then incubated at 25°C for 4 days. Indicated numbers of cells (40 to 25,000) were inoculated onto the YPD agar plates. CsA, cyclosporin A.

CPH1 (cyclophilin A) increased by more than threefold (a difference of 225.7 in arbitrary units). Since cyclophilin A is one of the PPIases, the increase in CPH1 transcripts may be a compensatory effect of the deletion of ESS1. The mRNA levels of other PPIase genes, such as the FKBPs and cyclophilins, were not appreciably changed (data not shown). Most downregulated genes were for proteins related to metabolism (Table 2). That the *ess1*-disrupted mutant causes slow growth via downregulation of genes related to metabolism is difficult to explain; however, the data may provide some hints. For example, some proteins related to metabolism are controlled by related transcriptional factors, and our data may point to the identification of new roles for some transcriptional factors. YOR153w (PDR5) is already known to be multi drug resistant as a controlled by PDR1 and PDR3. Such relationships will be discovered with further study.

Sensitivity of the ess1 Disruptant to Cell Cycle Inhibitors and Immunosuppressants

Gene chip analysis indicated that mRNA levels of cyclophilin A were elevated in the *ess1* null mutant. To determine if an increased level of the gene is important in producing a viable *ess1* null mutant, we attempted to inhibit the activity of cyclophilin A by adding the specific inhibitor, cyclosporin A and measuring its effect on the cellular growth of the deletant. Other immunophilin inhibitors, FK506 and rapamycin, were also tested. As expected, the *ess1* disruptant became hypersensitive to cyclosporin A and slightly less sensitive to FK506. Rapamycin strongly inhibited the cell growth of both the null mutant and wild type (Fig. 4). Whereas a low concentration (0.4 μM) of cyclosporin A did not affect cell viability, concentrations of 20 and 40 μM had substantial inhibitory effects. To determine if this hypersensitivity was specific for immunophilins, we compared the sensitivity of wild-type cells and the *ess1* disruptant to various inhibitors, including hydroxyurea, etoposide, aphidicolin, radicicol, nocodazol, and demecoline (Fig. 4). The *ess1* null mutant was not hypersensitive to these inhibitors.

Cell Cycle Analyses of ess1 Null Mutant in the Presence of Cyclosporin A

To understand the mode of action of cyclosporin A in the *ess1* mutant, a flow cytometry analysis was performed using FACScan (Fig. 5). Relatively small numbers of *ess1*-disrupted cells were arrested at the G_2/M phase in the absence of cyclosporin A (Fig. 5A), and when these cells were treated with the cyclosporin A in plate culture, growth of the *ess1* disruptants were almost entirely inhibited (Fig. 4). However, flow cytometry exhibited no marked increase of cells at G_2/M phase. This result suggests that the mutant can normally exit from the M phase (Fig. 5B). Because of the difficulty in using logarithmically growing cells to determine the effect of cyclosporin A on the transition from the G1 to S phase, synchronized cells were used for cell cycle analysis. Cells were treated with sufficient concentrations of hydroxyurea and α-factor to ensure

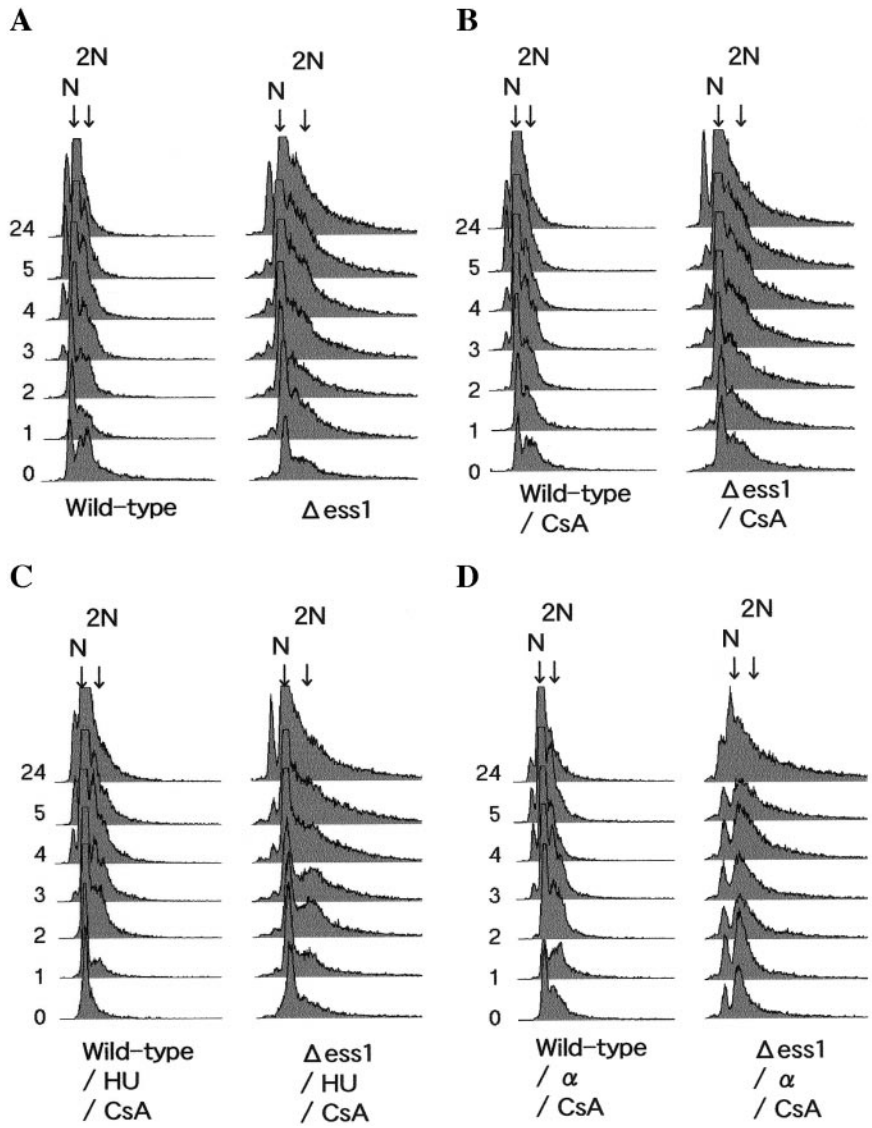

FIG. 5. The *ess1* mutant treated with cyclosporin A cells and arrested in G_1. The wild-type and *ess1*-disrupted mutant cells were grown to midlog phase. Wild type (left side of each panel) and the *ess1* deletant (right side of each panel) were grown on (A) normal YPD medium, (B) YPD with cyclosporin A (50 μM), (C) block and released after hydroxyurea treatment, and (D) block and released after α-factor treatment. Numbers at the left indicate sampling time points in hours for the FACScan analysis. N indicates G_1 phase, and 2N indicates G_2/M phase.

arrest of the cell population at the S phase or the G_1 phase, respectively. The medium was then changed to YPD containing cyclosporin A. Although the cell cycle distribution of *ess1* null mutant cells was almost the same as that of wild type cells in the absence of cyclosporin A after synchronization, the cell cycle progression after release from the G_1 phase block was markedly different between these two cell lines in the presence of cyclosporin A (Fig. 5D). In contrast, the cells that had been synchronized at the G1/S boundary (cells treated with hydroxyurea) entered into the S phase after release from blockage and progressed in both cases toward the G2 and M phases. This result suggests that the *ess1* null mutant has no defects in ability to proceed to the M phase from G1/S boundary, even in the presence of the cyclosporin A (Fig. 5D) and indicates that Ess1 and cyclophilin A function at the G_1 phase in a concerted manner. Treatment with FK506 or rapamycin had no on influence the cell cycle distribution of the *ess1* null mutant cells (data not shown).

DISCUSSION

The evidence is abundant that the phosphorylation of cell cycle regulating proteins plays important roles in the mechanisms of cell cycle regulation (5). Recent reports suggest that PPIase also regulates the activity of these cell cycle regulators by specifically enhancing the activity of phosphorylated proteins via conformational changes (20). One PPIase, ESS1, is believed to

work in mitosis by modifying the activity of CDC25C and the Cdc2/CyclinB complex. Since the CDC25C and the Cdc2/CyclinB complex have a crucial role for cell cycle regulation in mitosis, the loss of Ess1 function, which is required at mitosis in yeast cells, should be lethal. This hypothesis is based on the fact that both PIN1 and ESS1 possess a strong affinity for proteins involved in the cell cycle regulation of G_2/M. However, analyses carried out with embryonic fibroblasts derived from a Pin1 knockout mouse (21) clearly indicated that Pin1/Ess1 has some role in cell cycle regulation at the G_1 phase. Two recent reports also have suggested some roles for Ess1 in cell cycle regulation at the G_2 phase and at phases other than G_2 (35, 36). Analyses of protein-protein interactions with ESS1 indicated that ESS1 interacts with SIN3, a factor that works as a cell cycle regulator in conjunction with SAP30 and Rpd3 (35). This report also suggested that cyclophilin A has a strong affinity for SAP 30, which is one member of a protein complex formed with SIN3. All these lines of evidence indicate that Ess1 may well have some role in G_1 phase cell cycle control, but virtually all these conclusions are based on data obtained from *in vitro* experiments. Thus, the role of the Ess1 molecule at the G_1 phase remained a hypothesis because no *in vivo* evidence existed.

Here we report results that strongly support the role for Ess1 in the G_1 phase of cell cycle regulation. Moreover, these findings also suggest a concerted action by ESS1 and cyclophilin A: A viable yeast *ess1* deletion mutant and a specific inhibitor for cyclophilin A enabled the successful induction of the G_1 arrest phenotype.

It is important in expression profile analysis to minimize the secondary effects that are caused by growth retardation. For example, the secondary effects of slowing growth for temperature-sensitive mutants inevitably confound analysis of expression profiles. Since conditions were discovered in this study under which the *ess1* null mutant has almost the same growth capability as wild type cells, the evidence obtained from the microarray study should reflect primarily the effect of *ess1* disruption. Development of an *ess1* null mutant enabled us not only to characterize Ess1 but also to demonstrate the existence of a molecule that complements the deletion of the *ess1* gene. The expression level of cyclosporin A is markedly upregulated in the *ess1* deletion mutant, a result consistent with the notion that ESS1 and cyclosporin A are somehow biochemically linked.

Microarray analyses provide additional information on the role of Ess1. ESS1 has been shown to have some affinity for the CTD region of RNA polymerase II (36). Interestingly, the expression levels of numerous genes are downregulated in the *ess1* knockout mutant (Fig. 3B), and although the changes are not dramatic, such decreases in expression were observed repeatedly. The syngenic C110-1 host strain had been chosen for these experiments in gene disruption to minimize artifacts caused by variation in genetic backgrounds. Because the cyclophilin A gene is not upregulated in the parent cells, there must be some preceding events in order for the cyclophilin A gene to be upregulated in the *ess1* deletant. It is possible that cyclophilin A regulates transcription as well as the ESS1 level. Loss of interaction between ESS1 and the CTD domain of RNA polymerase II induces changes in global transcriptional regulation. If both ESS1 and cyclophilin A interact with the CTD region of RNA polymerase II, it is then likely that increased expression of the cyclophilin A gene may complement the deletion of the *ess1* gene. Since viable *ess1* null mutants were obtained only from specific yeast cell lines, transcriptional regulation in the cell may have some diversity and *ess1* deletion induced expression of gene by unknown mechanism. Although a precedent for this kind of an induction mechanism is not known, our data seem to support the supposition that Ess1 and cyclophilin A are functionally related.

TABLE 3

PatMatch[a] Results of PPIase and WW Binding Motifs

	PPIase binding motif [RYIFKMG]-[TS]-P-[RFY][b]	WW binding motif XPPXY[c]
Total hit/sequences searched	829/6357	261/6357
Different hit/sequences searched	757/6357	249/6357
Cell cycle[d]		
M/G1	22	9
G1	38	18
S	6	2
S/G2	10	4
G2/M	32	4

[a] Yeast Genome Pattern Matching (Stanford).
[b] Supplemental data of Science, Ref. 44.
[c] Ref. 4.
[d] Refs. 8 and 9.

We have searched the yeast genome for genes with domains that could serve as substrates for PPIase as well as for genes with domains with the potential to interact with the WW domain. This search yielded 757 ORFs as possible substrates for PPIase activity and 249 ORFs as potential WW binding-domain partners (Table 3). Among these ORFs, transcription of 55% of ORFs with a PPIase binding-domain motif were detected at the M/G_1 to G_1 phase, and transcription of 72% of ORFs with WW binding-domain motif were observed at the M/G_1 to G_1 phase (Table 3). Some of these genes are involved in transcription regulation (MCM3, SWI4, SIC1, CDC46, HCM1, PCL2, and others; data not shown). Although the identification of proteins interacting with Ess1 might provide clues to

the functional role of Ess1, inferences from such analyses would be difficult because (i) yeast two hybrid experiments produce many interacting molecules, such results apparently deriving from the fact that WW domains possess high affinity for proline-rich regions; (ii) immunoprecipitation using anti-tag antibodies with Ess1 genes and yeast cells that harbor tagged Ess1-overexpression constructs would detect proteins that are only expressed at very high levels; and (iii) overexpression of Ess1 is itself known to produce a lethal phenotype. In an attempt to overcome these obstacles we have tried to analyze viable deletion mutants of *ess1* for mRNA levels, and have identified only the increased expression of the cyclophilin A gene. Further analysis of the role of the Ess1 protein in the control mechanism of cell cycle progression will likely require more exhaustive analyses of protein–protein interactions. In this vein we are now developing an analytical method based on increasing the specificity of protein-protein interactions.

Finally, our results strongly suggest that Ess1 plays an important role in the cell cycle regulation at the G_1 phase as well as at the G_2/M phase. This conclusion is consistent with our previous study using a mouse Pin1 knockout mutant (21). By analyzing the expression level of the mouse cyclophilin A gene in knockout mice, we may be able to obtain results that support the results described in this paper. Screening for genes in the yeast system that induce synthetic lethality will also be important. Because the cyclophilin A gene has fortunately already been identified as a strong candidate for inducing synthetic lethality, tetrad analysis of an *ess1* and *cyclophilin A* double mutation should provide further evidence. Such analyses are now underway.

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