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The Molecular Chaperone Ydj1 Is Required for the p34^{CDC28}-Dependent Phosphorylation of the Cyclin Cln3 That Signals Its Degradation

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The G_1 cyclin Cln3 of the yeast *Saccharomyces cerevisiae* is rapidly degraded by the ubiquitin-proteasome pathway. This process is triggered by p34^{CDC28}-dependent phosphorylation of Cln3. Here we demonstrate that the molecular chaperone Ydj1, a DnaJ homolog, is required for this phosphorylation. In a *ydj1* mutant at the nonpermissive temperature, both phosphorylation and degradation of Cln3 were deficient. No change was seen upon inactivation of Sis1, another DnaJ homolog. The phosphorylation defect in the *ydj1* mutant was specific to Cln3, because no reduction in the phosphorylation of Cln2 or histone H1, which also requires p34^{CDC28}, was observed. Ydj1 was required for Cln3 phosphorylation and degradation rather than for the proper folding of this cyclin, since Cln3 produced in the *ydj1* mutant was fully active in the stimulation of p34^{CDC28} histone kinase activity. Moreover, Ydj1 directly associates with Cln3 in close proximity to the segment that is phosphorylated and signals degradation. Thus, binding of Ydj1 to this domain of Cln3 seems to be essential for the phosphorylation and breakdown of this cyclin. In a cell-free system, purified Ydj1 stimulated the p34^{CDC28}-dependent phosphorylation of the C-terminal segment of Cln3 and did not affect phosphorylation of Cln2 (as was found in vivo). The reconstitution of this process with pure components provides evidence of a direct role for the chaperone in the phosphorylation of Cln3.

In *Escherichia coli*, mitochondria, and the cytosol of eukaryotic cells, members of DnaJ family of molecular chaperones function together with Hsp70s in protein folding and translocation across membranes (12). Recent studies have demonstrated that the chaperones are also essential for the rapid degradation of certain abnormal proteins in both prokaryotes and eukaryotes (16, 24). These short-lived proteins appear to associate with DnaJ and other chaperones prior to their degradation (16, 24). Since chaperones bind specifically to unfolded polypeptides, they may participate in the recognition of abnormal proteins by the degradative machinery. The present studies were undertaken to determine whether molecular chaperones may also be important in the degradation of regulatory proteins.

To examine this possibility, we studied whether a DnaJ homolog in *Saccharomyces cerevisiae*, Ydj1, functions in the rapid ubiquitin-dependent breakdown of the G_1 cyclin Cln3. Cln3 is an important regulator of the Start checkpoint in the yeast cell cycle (7). Cln3 associates with and activates p34^{CDC28} kinase in early G_1 phase, which leads to the synthesis of other G_1 cyclins (11, 24a, 25), thus allowing the transition from G_1 to S phase. This interaction occurs through the N-terminal segment of Cln3, which contains the cyclin box, a region conserved among different cyclins. Moreover, this N-terminal segment of Cln3 alone is sufficient for the stimulation of the kinase. p34^{CDC28} in turn phosphorylates Cln3, which triggers its ubiquitination, leading to the rapid degradation of Cln3 (8, 28). This critical phosphorylation occurs within a defined C-terminal segment of Cln3 (residues 404 to 580) (28) which is necessary for the regulation of Cln3 breakdown. We present here genetic and biochemical evidence that the phosphorylation and, thus, the regulated degradation of Cln3 require the function of the Ydj1 chaperone.

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MATERIALS AND METHODS

Strains and growth conditions. In this work, the following congenic yeast strains were used: W3031b (*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*) and *ydj1* mutant ACY17b (*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ydj1-2*::*HIS3 LEU2*::*ydj1-151*), kindly provided by A. Caplan (Mount Sinai Hospital). For determination of the half-life of Cln3, the Cln3–β-galactosidase (β-gal) fusion, and Cln2, pulse-labeling of yeast cells was carried out as described by Bachmair et al. (1), with minor modifications. Yeast cells carrying fusion constructs whose expression is under the control of galactose were grown overnight at 30°C in raffinose-containing minimal medium (0.67% yeast nitrogen base, 2% raffinose, amino acids, and adenine). Then 2% galactose was added to the medium to induce the cloned proteins. After 4 to 6 h, cells in mid-log phase (optical density at 600 nm, 0.5 to 1.0) were either maintained at 30°C or shifted to 38°C for 30 min. The cells were collected, resuspended at a density of 2×10^8/ml, and labeled with 200 mCi of Tran^{35}S-label for 5 min. The cells were then transferred to a chase medium containing methionine (0.5 mg/ml) and cycloheximide (0.5 mg/ml). At different times, aliquots of cells were collected by centrifugation and resuspended in cold IP buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing a number of protease inhibitors (aprotinin, E-64, chymostatin, phosphoramidon, *N*-ethylmaleimide, pepstatin A, and phenylmethylsulfonyl fluoride, each at 50 mg/ml) (28). Cells were then lysed by vortexing with glass beads. Immunoprecipitation was carried out as described previously (9), using antibodies indicated in the text and figure legends.

Isolation of Cln3–β-gal–Ydj1 complexes. Immunoaffinity chromatography with anti-β-gal immunoglobulin G as the affinity ligand was carried out as follows. Yeast cells carrying different β-gal constructs and growing exponentially at 30°C were shifted to 38°C for 30 min. Cells were collected by centrifugation, and cell extracts were prepared as described above. The extracts (3 mg of protein) were loaded onto 1-ml anti-β-gal immunoglobulin G affinity columns preequilibrated with the same buffer and washed with 20 volumes of IP buffer. The material that bound to this column was then eluted with 3 ml of 0.1 M acetic acid (pH 2.5). The eluted proteins were precipitated with trichloroacetic acid and analyzed by Western blotting (immunoblotting) with an anti-Ydj1 antibody.

Histone kinase activity. Wild-type and *ydj1-151* mutant strains with hemagglutinin (HA) epitope-tagged p34^{CDC28} kinase (21, 26) genes integrated into the

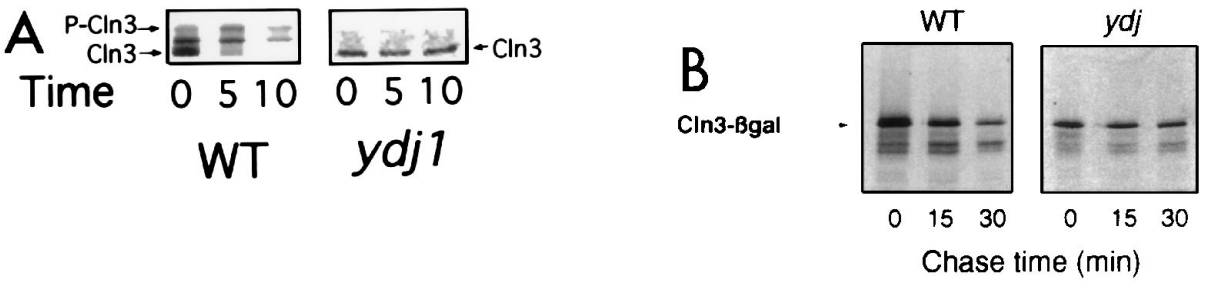


FIG. 1. *YDJ1* is required for degradation of Cln3. (A) The *ydj1-151* mutation stabilizes native Cln3 and inhibits its phosphorylation. Cln3 tagged with the HA epitope (11, 25, 26) was expressed in wild-type (WT) and *ydj1-151* mutant cells, and its degradation was measured by pulse-chase analysis, as described for panel B, and then immunoprecipitation with an anti-HA antibody. Time is in minutes. (B) Cln3(404–580)–β-gal is stabilized in the *ydj1* mutant. Cln3(404–580)–β-gal was expressed in the wild-type and in the *ydj1-151* mutant, and cells grown at 30°C were shifted to 38°C for 30 min prior to labeling with ^{35}S-amino acids for 5 min (28). At intervals during the 38°C chase incubation with an excess of unlabeled methionine and cycloheximide, cells were lysed and Cln3(404–580)–β-gal was immunoprecipitated with an anti-β-gal antibody and resolved by SDS-PAGE (28).

TRP1 locus or HA-tagged Cln3 were grown as described above. Cells in mid-log phase were lysed in ice-cold buffer N (50 mM Tris [pH 7.5], 100 mM NaCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 10 mM NaF, 60 mM β-glycerophosphate, phenylmethylsulfonyl fluoride, aprotinin, E-64, chymostatin, pepstatin A). Then HA-tagged p34^{CDC28} or Cln3 was immunoprecipitated with anti-HA 12CA5 antibody and washed with buffer N, followed by a wash with kinase buffer (10 mM Tris [pH 7.5], 10 mM $MgCl_2$, phenylmethylsulfonyl fluoride, protease inhibitors). The histone H1 kinase reaction was started by addition of 50 ml of kinase buffer plus 5 μM ATP, histone H1 (10 mg/ml), and 10 μCi of [γ-^{32}P]ATP. After a 10-min incubation at 30°C, phosphorylated histone H1 was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

In vitro Cln3 kinase activity. To measure the Cln3 kinase activity of p34^{CDC28}, we used Cln3(404–436;458–488) fused to glutathione transferase (GST) as a substrate. This protein was expressed in *E. coli* and purified to homogeneity on a glutathione affinity column. The Ydj1 protein was overexpressed in *E. coli* and purified to homogeneity as described previously (9). HA-tagged Cln2 was expressed in the wild-type yeast strain, and p34^{CDC28} associated with Cln2 was coimmunoprecipitated with anti-HA antibody. Pellets were washed twice with buffer N, followed by a wash with the kinase buffer. The Cln3 kinase reaction was started by addition of 50 ml of kinase buffer plus 5 μM ATP, 1 μg of Cln3(404–436;458–488)–GST, 2 μg of Ydj1, and 30 μCi of [γ-^{32}P]ATP. After a 10-min incubation at 30°C, phosphorylated Cln3(404–436;458–488)–GST was resolved by SDS-PAGE.

RESULTS

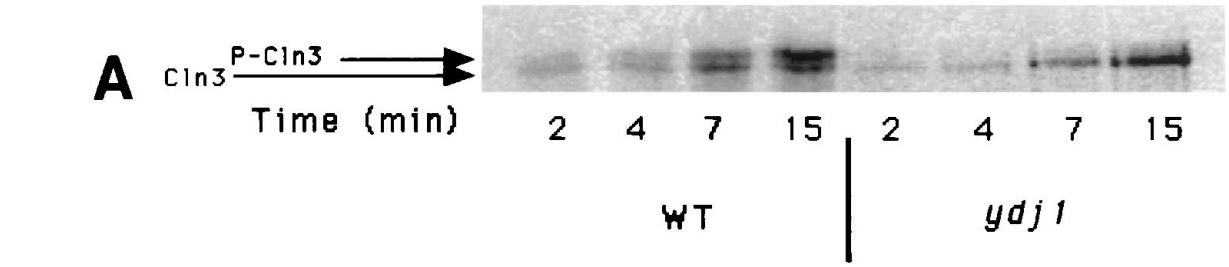
In studies to determine whether Ydj1 is required for the degradation of Cln3, we used a Cln3 derivative tagged with the HA epitope (11, 25, 26). Wild-type cells and temperature-sensitive *ydj1* mutant cells that expressed Cln3-HA were grown at 30°C, and half of each culture was shifted to 38°C, the nonpermissive temperature for *ydj1*. After 30 min, cell proteins were labeled with ^{35}S-amino acids for 5 min, and then the cultures were exposed to an excess of unlabeled methionine and cycloheximide to prevent further incorporation of ^{35}S. The amount of labeled Cln3 was measured after isolation by immunoprecipitation and SDS-PAGE (1, 28). In the wild-type cells at 38°C, newly synthesized Cln3 was rapidly converted to the phosphorylated form, which migrates more slowly on SDS-PAGE, and then was degraded with a half-life of 5 min (28). In the *ydj1* mutant at 38°C, Cln3 was stabilized dramatically and it accumulated in the unmodified form (Fig. 1A). Thus, Ydj1 appeared to be essential for the degradation of Cln3.

To facilitate the study of Cln3 degradation, we have used as a model substrate a protein consisting of *E. coli* β-gal fused to the C-terminal segment (amino acids 404 to 580) of Cln3, which carries the domain that is phosphorylated and targets Cln3 for proteolysis (28). Prior studies showed that this fusion protein is phosphorylated and is rapidly degraded by the same p34^{CDC28}-dependent pathway as native cyclin (28). At 30°C, the rates of breakdown of Cln3(404–580)–β-gal in the wild-type and *ydj1* mutant were similar (half-life, 20 min). At 38°C, in wild-type cells, the half-life of Cln3(404–580)–β-gal was significantly reduced, to about 12 min. By contrast, a marked stabilization of this polypeptide was seen in the *ydj1* mutant (half-life, 50 min) (Fig. 1B).

In mutants defective in ubiquitination of Cln3 or in its subsequent degradation by the proteasome, both Cln3(404–580)–β-gal and native Cln3 accumulate in phosphorylated forms (28), because the phosphorylated form is an intermediate in Cln3 breakdown. However, upon inactivation of Ydj1 in the *ydj1* mutant, Cln3(404–580)–β-gal, like native Cln3, accumulated predominantly as the nonphosphorylated species (Fig. 1B). Thus, Cln3 phosphorylation seems to require Ydj1 function. To test this possibility, we used a mutant with a small deletion (amino acids 437 to 457) in the Cln3 portion of Cln3–β-gal (28). This deletion prevents the breakdown of the fusion protein (28) without affecting its phosphorylation. Thus, this deletion allows the possible effects of Ydj1 on Cln3 phosphorylation to be distinguished from effects on ubiquitination or degradation. In wild-type cells, Cln3(404–436;458–488)–β-gal was rapidly phosphorylated during the 5-min pulse-labeling, as shown by its reduced electrophoretic mobility, and by 20 min this polypeptide was converted almost entirely to the phosphorylated form (data not shown). In the *ydj1* mutant at 38°C, none of the phosphorylated form was seen during the 5-min pulse and little was evident even after 20 min. Thus, even in the absence of degradation, the phosphorylation of this polypeptide requires functional Ydj1. To compare the kinetics of phosphorylation in the wild type and the *ydj1* mutant, we labeled cells for different periods of time and, after immunoprecipitation and SDS-PAGE, quantitated the phosphorylated and nonphosphorylated forms of Cln3(404–436;458–488)–β-gal. As seen in Fig. 2, at 38°C, the *ydj1* mutation dramatically reduced the accumulation of the phosphorylated species. This effect of the *ydj1* mutation was observed when cells were incubated at 38°C for 10, 30, or 90 min prior to labeling to inactivate the mutant Ydj1 protein.

In addition to Ydj1, yeast cells contain multiple members of the DnaJ family, which serve distinct functions in the cell (3). Inactivation of the other major DnaJ homolog in yeast cells, *SIS1*, by shifting a *sis1-85* temperature-sensitive mutant to the nonpermissive temperature had no measurable effect on either the phosphorylation or the degradation of Cln3–β-gal (data not shown). Thus, the effect of Ydj1 on the phosphorylation of Cln3 appeared to be specific.

In protein transport, Ydj1 functions by binding, together with Hsp70, to the polypeptide and maintaining the substrate in a translocation-competent conformation. In a similar way, this chaperone may bind specifically to Cln3 and make it more susceptible to the kinase. Therefore, we tested whether Ydj1



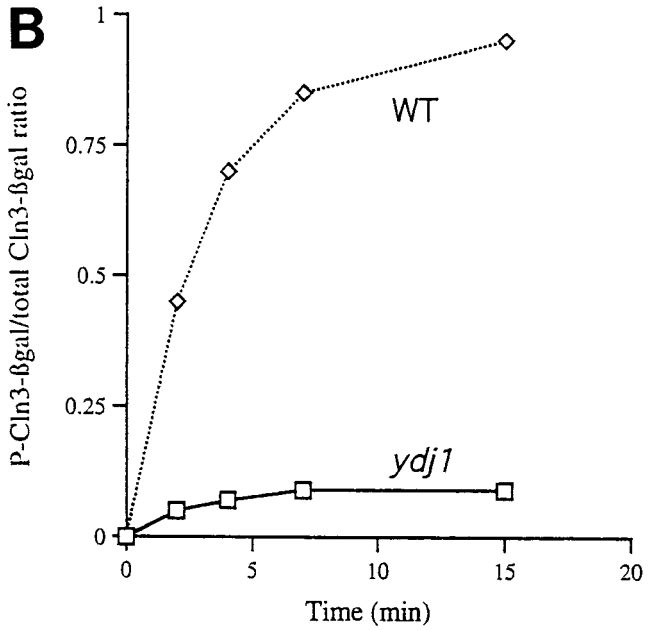


FIG. 2. *YDJ1* is required for phosphorylation of Cln3. (A) Accumulation of the phosphorylated forms of Cln3(404–436;458–480)–β-gal in wild-type (WT) and *ydj1* mutant cells. Cells were labeled with ^{35}S-amino acids in vivo for 2, 4, 7, and 15 min, and Cln3(404–436;458–480)–β-gal was analyzed as described above in the legend to Fig. 1B. (B) Quantitation of the amounts of phosphorylated Cln3(404–436;458–480)–β-gal in the wild type and the *ydj1* mutant from panel A.

associates directly with Cln3 in the cell. Cln3-HA and associated proteins were isolated from cell extracts by immunoprecipitation with anti-HA antibody (26). The bound proteins were resolved by SDS-PAGE and probed with an anti-Ydj1 antibody on a Western blot (Fig. 3A). Ydj1 was present in the bound fraction from the wild-type cell extract but was absent from the *ydj1* mutant extract at the nonpermissive temperature, where phosphorylation is prevented. Furthermore, no Ydj1 was detected in the fraction isolated with the anti-HA antibody from the extract of wild-type cells that did not express Cln3-HA (Fig. 3A).

To determine whether the Ydj1-binding region on Cln3 is close to the site of phosphorylation, we tested whether Ydj1 associates with a truncated version of Cln3–β-gal that includes the site of phosphorylation that is essential for Cln3 degradation. Cln3(404–488)–β-gal and proteins associated with it were isolated by affinity chromatography with an anti-β-gal antibody conjugated to Sepharose. Ydj1 was recovered bound to this fusion protein in extracts of wild-type cells incubated at 38°C that contained Cln3(404–488)–β-gal but was absent from extracts that contained wild-type β-gal (28). In similar experiments with extracts from the *ydj1* mutant cells incubated at 38°C, no Ydj1 was found associated with Cln3(404–488)–β-gal (Fig. 3B). These data suggest that Cln3 phosphorylation may require binding of Ydj1 to the amino acid 404 to 488 domain. It is noteworthy that the state of phosphorylation did not influence the binding of Cln3(404–488)–β-gal to Ydj1. This chaperone was found associated with Cln3(404–488)–β-gal in both the *cdc28* mutant, in which Cln3 is not phosphorylated (28), and the *cdc34* mutant, in which Cln3 accumulated predominantly in the phosphorylated form (28) (data not shown).

A possible explanation of the effects of the *ydj1* mutation on Cln3 phosphorylation is that Ydj1 is essential for the activity of the protein kinase p34^{CDC28}. For example, this chaperone may be required for folding of p34^{CDC28} or for its association with regulatory factors such as cyclins. Alternatively, Ydj1 may be necessary for Cln3 to be maintained in the phosphorylation-susceptible conformation. To distinguish between these possibilities, we studied whether p34^{CDC28} in the *ydj1* mutant is able to phosphorylate its other substrates. Cln2, a G_1 cyclin that is functionally and structurally distinct from Cln3, is also phosphorylated in a p34^{CDC28}-dependent fashion (21), which triggers its ubiquitination (10). Therefore, a plasmid encoding Cln2 tagged with an HA epitope was introduced into the wild-type and *ydj1* mutant cells and the phosphorylation and degradation of Cln2 were followed. Inactivation of Ydj1 at 38°C did not have any demonstrable effect on the phosphorylation of Cln2, since the electrophoretically retarded forms of Cln2 were observed in both the wild type and the *ydj1* mutant (Fig. 4A). In addition, the Cln2 degradation rates were similar in the two strains. Furthermore, when the p34^{CDC28} kinase tagged with the HA epitope was isolated by immunoprecipitation with an anti-HA antibody from extracts of the wild type and *ydj1* mutant grown at 38°C, the kinase from the *ydj1* cells showed no defect in its ability to phosphorylate histone H1 (Fig. 4B).

Since neither the histone H1 nor the Cln2 kinase activity of p34^{CDC28} is affected by the *ydj1* mutation, the defect in phosphorylation appears to be specific to Cln3 and cannot be due to a general defect in the p34^{CDC28} kinase or its inability to interact generally with cyclins. The specific defect in Cln3 phosphorylation in *ydj1* mutants, together with the observation that the Ydj1 protein associates with the C-terminal segment (amino acids 404 to 488) of Cln3, which is in close proximity to the phosphorylation site, strongly suggests that this chaperone affects Cln3 rather than the kinase. Presumably, Ydj1 enhances Cln3 phosphorylation by maintaining this substrate in a conformation that favors its recognition or modification by p34^{CDC28} kinase (e.g., by exposing an otherwise inaccessible phosphorylation site).

Another possibility is that Ydj1 allows phosphorylation only by maintaining Cln3 in a folded form. Thus, in the *ydj1* mutant, Cln3 may not fold properly or may aggregate, which could prevent its phosphorylation and degradation. Therefore, we tested whether Cln3 is properly folded after Ydj1 inactivation, i.e., whether under these conditions it can facilitate the kinase

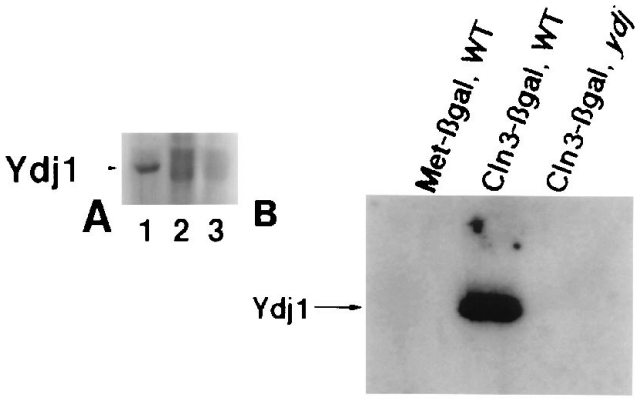


FIG. 3. Ydj1 protein associates with Cln3 near the phosphorylation site. (A) Ydj1 associates with Cln3-HA. Cultures growing exponentially at 30°C were shifted to 38°C for 30 min prior to the preparation of extracts. Cln3-HA and associated proteins were immunoprecipitated with the monoclonal anti-HA antibody 12CA5, and the presence of Ydj1 in this fraction was detected by Western blotting with the specific polyclonal anti-Ydj1 antibody (kindly provided by A. Caplan of Mount Sinai Medical Center). This antibody is very specific for Ydj1, and it does not show any nonspecific cross-reacting bands upon immunoblotting. Lane 1, immunoprecipitate from the extract of the wild-type cells expressing Cln3-HA; lane 2, immunoprecipitate from the extract of the wild-type cells that do not express Cln3-HA; lane 3, immunoprecipitate from the extract of the *ydj1* mutant expressing Cln3-HA. (B) Ydj1 associates with the C-terminal segment of Cln3. Cultures expressing Cln3(404–488)–β-gal or wild-type (WT) β-gal were grown exponentially at 30°C and then were shifted to 38°C for 30 min prior to the preparation of extracts. Cell extracts were loaded onto an anti-β-gal immunoglobulin G column. Proteins associated with Cln3(404–488)–β-gal were eluted with acid and analyzed by Western blotting with an anti-Ydj1 antibody (2).

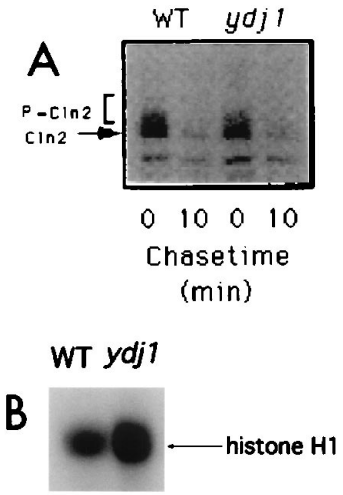


FIG. 4. *YDJ1* is not required for phosphorylation of Cln2 or histone H1 by $p34^{CDC28}$ kinase. (A) Phosphorylation and degradation of Cln2 tagged with HA epitope. Cln2 was expressed in the wild type (WT) and in the *ydj1* mutant, and its degradation was measured by pulse-chase analysis, as described in the legend to Fig. 1A, by immunoprecipitation with an anti-HA antibody. In the wild type and the *ydj1-151* mutant, the slowly migrating, phosphorylated forms of Cln2 are observed in the 5-min pulse, and then this polypeptide disappeared within 10 min in both strains. (B) Histone H1 kinase activity of $p34^{CDC28}$ is not affected by the *ydj1-151* mutation. An HA epitope-tagged derivative of the *CDC28* gene was integrated into the genome of wild-type and *ydj1-151* mutant cells. After incubation at 38°C for 30 min, $p34^{CDC28}$ was isolated from both strains by immunoprecipitation with an anti-HA antibody, and its histone H1 kinase activity was measured (21).

activity of $p34^{CDC28}$. The wild-type and *ydj1* mutant cells were incubated at 38°C for 30 min; then the $p34^{CDC28}$ associated with Cln3-HA was isolated from these cell extracts by immunoprecipitation with an anti-HA antibody, and the histone kinase activity was measured. As shown in Fig. 5, the degree of phosphorylation of histone H1 was about 50% higher in the *ydj1* mutant than in the wild type. This slight increase in the histone H1 kinase activity associated with Cln3-HA in the *ydj1* mutant correlated with a 50% increase in the Cln3-HA content as measured by immunoprecipitation after the uniform labeling of proteins in the cell with [^{35}S]methionine (data not shown). Although Cln3 was stabilized in the *ydj1* mutant, the rather small extent of increase was due to the reduction of the rate of synthesis of this cyclin. No histone H1 kinase activity was detected in the extracts which did not contain Cln3-HA upon immunoprecipitation with the anti-HA antibody (Fig. 5). Thus, folding of Cln3 into the active form is not significantly affected by the *ydj1* mutation.

To test whether Ydj1 influences the proper folding of the Cln3(404–488)–β-gal fusion polypeptide, β-gal activity was measured in the wild-type and *ydj1* mutant extracts prepared from cells incubated at 38°C for 30 min. Insoluble proteins and cell debris were removed from these extracts by centrifugation for 20 min at 14,000 × *g*. Neither the wild-type particulate fraction nor that of the mutant strain contained any Cln3(404–488)–β-gal, as determined by Western blotting with an anti-β-gal antibody. In the soluble fraction from wild-type extracts, 220 U of β-gal per mg of protein was found, and in the *ydj1* mutant, 550 U was detected. The higher activity in the mutant reflects the nearly twofold-higher content of Cln3(404–488)–β-gal, which is due to the greater stability of this protein in this strain. The content of Cln3(404–488)–β-gal was measured after immunoprecipitation of this polypeptide from the uniformly labeled cell extract with anti-β-gal antibody. These data indicate that Ydj1 does not simply serve to maintain the substrate in a soluble, folded form.

To further test whether Ydj1 affects phosphorylation of Cln3 directly, we studied in vitro the effect of purified Ydj1 on this process. HA-tagged Cln2 was expressed in the wild-type strain, and $p34^{CDC28}$ kinase associated with and activated by Cln2 was isolated with anti-HA antibody. As a substrate, we used a polypeptide consisting of Cln3(404–436;458–488) fused to GST. This polypeptide was expressed in *E. coli* and purified by affinity chromatography with glutathione beads. Cln2-activated $p34^{CDC28}$ phosphorylated Cln3(404–436;458–488)–GST poorly, while the associated Cln2 was phosphorylated efficiently (Fig. 6). Addition of Ydj1 protein purified from *E. coli* (9) to the reaction mixture stimulated phosphorylation of Cln3(404–436;458–488)–GST by 7- to 10-fold. GST protein by itself could not be phosphorylated under these conditions (data not shown). It is noteworthy that Ydj1 had little or no effect on phosphorylation of Cln2 in the same reaction mixture (Fig. 6). Thus, the stimulation by Ydj1 of phosphorylation was substrate specific

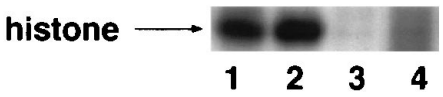


FIG. 5. *YDJ1* is not required for stimulation of $p34^{CDC28}$ kinase by Cln3. Cln3-HA was expressed in wild-type and *ydj1-151* mutant cells. After incubation at 38°C for 30 min, Cln3-HA was isolated from both strains by immunoprecipitation with an anti-HA antibody, and the histone H1 kinase activity of the associated $p34^{CDC28}$ was measured at 38°C (21). Lane 1, immunoprecipitate from the extract of the wild-type cells expressing Cln3-HA; lane 2, immunoprecipitate from the extract of the *ydj1-151* mutant expressing Cln3-HA; lane 3, immunoprecipitate from the extract of the wild-type cells expressing Cln3-HA but without addition of histone H1; lane 4, immunoprecipitate from the extract of the wild type that does not express Cln3-HA.

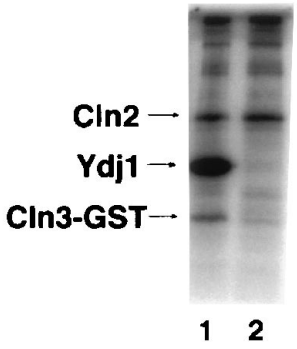


FIG. 6. Purified Ydj1 stimulates phosphorylation of Cln3 by p34^{CDC28} in vitro. Cln2-HA was expressed in the wild-type strain. Cln2 was isolated by immunoprecipitation with an anti-HA antibody, and the Cln3 kinase activity of the associated p34^{CDC28} was measured with Cln3(404–436;458–488)–GST as a substrate. Lane 1 (control), Cln3(404–436;458–488)–GST and Ydj1 were added to immunoprecipitated Cln2-p34^{CDC28}; lane 2, Cln3(404–436;458–488)–GST was added to immunoprecipitated Cln2-p34^{CDC28}. It is noteworthy that purified Ydj1 by itself is an in vitro substrate of Cln2-p34^{CDC28} kinase.

both in vivo and in vitro; i.e., this chaperone facilitated the phosphorylation of Cln3 but not of Cln2. These biochemical data indicate that the effects of Ydj1 on phosphorylation of Cln3 were direct. At present, we are trying to dissect the mechanism of Ydj1-dependent stimulation of Cln3 phosphorylation by the use of this in vitro system.

DISCUSSION

Ydj1, together with the major Hsp70s of the Ssa family, functions in the translocation of protein into the endoplasmic reticulum and mitochondria (2, 3). Cln3 phosphorylation may also require one of these Hsp70s, as well as Ydj1. In fact, we have found that the Hsp70s Ssa1 and Ssa2, like Ydj1, are associated with Cln3(404–488)–β-gal which has been isolated by affinity chromatography (data not shown). By contrast, very little if any of these chaperones was bound to wild-type β-gal. Unfortunately, studies which would show definitively, by genetic methods, that the Ssa proteins have a role in Cln3 phosphorylation and degradation in vivo are complicated by the presence of multiple members of this gene family with overlapping, redundant functions (6, 20). Moreover, minor members of this gene family are induced in strains lacking the major Ssa proteins. Therefore, biochemical studies with pure components will be necessary to clarify whether Hsp70s function together with Ydj1 in Cln3 phosphorylation.

The involvement of a chaperone in Cln3 phosphorylation provides a novel biochemical mechanism of appreciable enzymological interest. In addition, it may be important in the regulation of the cell cycle, especially during prolonged heat shock. High production of Ydj1 and Hsp70s under these conditions may enhance Cln3 phosphorylation and breakdown (28) and thus may cause heat shock-induced G_1 delay, which is known to be suppressed by Cln3 overexpression (22).

Several recent studies have also implicated chaperones in signal transduction mechanisms (23). For example, Ydj1 has been shown to activate steroid hormone receptors and pp60$^{v\text{-}src}$ (4, 15). In addition, mutations in DnaJ or DnaK (Hsp70) in *E. coli* reduce the phosphorylation of threonyl-tRNA synthetase and glutamyl-tRNA synthetase (14, 27) by mechanisms that have not yet been clarified. In mammalian cells, Hsp70 was found to associate with the heme-dependent serine/threonine kinase and to influence its activity during heat shock in response to the accumulation of abnormal proteins (17–19). In this case, the chaperones affect the kinase rather than the polypeptide substrate. However, with Cln3, the chaperones may serve to drive the substrate into a conformation that is capable of being modified by the protein kinase. The present findings indicate a new function for molecular chaperones of the DnaJ family in facilitating substrate modification by protein kinases.

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