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Involvement of the Molecular Chaperone Ydj1 in the Ubiquitin-Dependent Degradation of Short-Lived and Abnormal Proteins in *Saccharomyces cerevisiae*

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In *Escherichia coli* and mitochondria, the molecular chaperone DnaJ is required not only for protein folding but also for selective degradation of certain abnormal polypeptides. Here we demonstrate that in the yeast cytosol, the homologous chaperone Ydj1 is also required for ubiquitin-dependent degradation of certain abnormal proteins. The temperature-sensitive *ydj1-151* mutant showed a large defect in the overall breakdown of short-lived cell proteins and abnormal polypeptides containing amino acid analogs, especially at 38°C. By contrast, the degradation of long-lived cell proteins, which is independent of ubiquitin, was not altered nor was cell growth affected. The inactivation of Ydj1 markedly reduced the rapid, ubiquitin-dependent breakdown of certain β-galactosidase (β-gal) fusion polypeptides. Although degradation of N-end rule substrates (arginine–β-gal and leucine–β-gal) and the B-type cyclin Clb5–β-gal occurred normally, degradation of the abnormal polypeptide ubiquitin–proline–β-gal (Ub-P-β-gal) and that of the short-lived normal protein Gcn4 were inhibited. As a consequence of reduced degradation of Ub-P-β-gal, the β-gal activity was four to five times higher in temperature-sensitive *ydj1-151* mutant cells than in wild-type cells; thus, the folding and assembly of this enzyme do not require Ydj1 function. In wild-type cells, but not in *ydj1-151* mutant cells, this chaperone is associated with the short-lived substrate Ub-P-β-gal and not with stable β-gal constructs. Furthermore, in the *ydj1-151* mutant, the ubiquitination of Ub-P-β-gal was blocked and the total level of ubiquitinated protein in the cell was reduced. Thus, Ydj1 is essential for the ubiquitin-dependent degradation of certain proteins. This chaperone may facilitate the recognition of unfolded proteins or serve as a cofactor for certain ubiquitin-ligating enzymes.

In all eukaryotic cells, molecular chaperones of the DnaJ family function together with an Hsp70 in protein folding and translocation. In *Escherichia coli*, the molecular chaperones DnaJ and DnaK (an Hsp70 homolog) are also essential for the rapid degradation of certain normal and abnormal proteins (22, 30, 32). For example, the short-lived mutant form of alkaline phosphatase PhoA61, which cannot be transported across the cell membrane, is hydrolyzed by an ATP-dependent process that requires DnaK, DnaJ, and GrpE, as well as protease La (*lon*) (30). This degradative process appears to involve an association of PhoA61 with these chaperones and the protease (30). DnaK, DnaJ, and GrpE are involved in the transport of wild-type alkaline phosphatase across the cell membrane (37). These findings suggest that if molecular chaperones fail to promote the transport or folding of a protein, they can facilitate its rapid degradation by cellular proteases. DnaK, DnaJ, and GrpE are also necessary for the rapid breakdown of σ^{32}, the specific transcription factor for heat shock genes (33, 34), and in yeast mitochondria, homologs of these chaperones and of protease La are essential for degradation of certain unfolded proteins (36). Since the chaperones bind selectively to unfolded proteins (12, 17), they may aid in substrate recognition or may function as enzymatic cofactors, which help maintain the protein substrate in a soluble, unfolded conformation that is particularly susceptible to proteolytic attack.

In the cytoplasm of *Saccharomyces cerevisiae*, one of the two major DnaJ homologs, Ydj1 (Mas5), has been shown to function with certain Hsp70s (of the Ssa family) in protein translocation across membranes (7). In this process, Ydj1 interacts directly with Ssa1 and stimulates its ATPase activity (3, 8). The other DnaJ homolog in the yeast cytosol, Sis1, plays an essential role in polysome assembly and the initiation of translation (39). It is not known whether these translational events also require the activity of certain Hsp70 proteins.

Because these chaperones are highly conserved in evolution, we have tested whether they may function as recognition elements or cofactors in the degradation of short-lived proteins in the cytosol of eukaryotic cells, through their capacity to bind to unfolded proteins. In eukaryotes, most proteins to be rapidly degraded first undergo covalent modification by conjugation to multiple ubiquitin molecules (18, 35). This process, which involves several enzymes (E1, E2, and E3), marks proteins for rapid hydrolysis by the 26S proteasome (13, 18, 26). Ubiquitin-dependent degradation has been best characterized genetically in *S. cerevisiae*, where many short-lived substrates have been defined, and a large number of mutants that affect ubiquitination or the proteasome are known.

The present study was undertaken to test whether, in the yeast cytosol, the DnaJ homolog Ydj1 functions in the degradation of various classes of cell proteins, including the ubiquitin-dependent breakdown of highly abnormal polypeptides and normal short-lived proteins and the breakdown of long-lived cell proteins, which is believed to occur in the yeast vacuole (21). To study the effects of inactivation of this chaperone on the degradation of specific proteins, we measured the rapid breakdown of a variety of β-galactosidase (β-gal) fusion proteins whose degradation requires ubiquitination but in-

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volves distinct E2s and E3s. We found that this chaperone is required for the rapid degradation of specific short-lived and abnormal proteins and determined where in the ubiquitin-proteasome pathway Ydj1 functions.

MATERIALS AND METHODS

Yeast strains, culture media, and transformation. The yeast strains used in this study, W303 (*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*) and ACY17b (W303 and *ydj1-2*::*HIS3 LEU2*::*ydj1-151*), were kindly provided by A. Caplan (Mt. Sinai Hospital, New York, N.Y.). All culture media were prepared as described by Ausubel et al. (1). The plasmids carrying β-gal fusion constructs were gifts from D. Finley (Harvard Medical School), D. Kornitzer (Whitehead Institute), and S. Jentsch (University of Heidelberg). The antibody against the Ydj1 protein was provided by M. Douglas (University of North Carolina). Transformation of yeast cells was performed by using lithium acetate as described by Rose et al. (27). All other methods for yeast manipulation were carried out as described by Ausubel et al. (1) or Rose et al. (27).

Measurement of total protein degradation. Yeast cells were grown exponentially (optical density at 600 nm [OD_{600}], 0.5 to 1.0) at 30°C in synthetic dextrose medium supplemented with the nutrients required for the specific strains. These cells were collected by centrifugation and resuspended (OD_{600}, 2.5 to 3.0) in the same medium but without methionine. To generate abnormal proteins containing amino acid analogs, cells were exposed to the proline analog azetidine carboxylic acid (0.5 mg/ml) for 30 min. To study proteins made at the nonpermissive temperature, cells were preincubated at 38°C for 30 min.

To measure the breakdown of short-lived proteins, these cells were then labelled for 5 min with 100 μCi of [^{35}S]methionine (Tran^{35}S-label; ICN). After two washes, cells were resuspended in fresh synthetic dextrose medium containing methionine (0.5 mg/ml) and cycloheximide (0.5 mg/ml) to prevent reincorporation of radioactive amino acids released from proteins. At different time intervals, aliquots of cells were taken and mixed with 100% trichloroacetic acid to give a final concentration of 10%. After incubation at 4°C for 1 h, the samples were centrifuged and the radioactivity in the trichloroacetic acid-insoluble material (precipitates) was measured. The rate of protein degradation is expressed as the percentage of incorporated radioactivity converted into acid-soluble fragments from the cells during the chase period.

To measure the breakdown of long-lived proteins, the cells were labelled with [^{35}S]methionine for 2 h and then incubated in the chase medium for 12 h to allow degradation of the short-lived proteins. The release of radioactivity from the remaining cell protein was then measured in the presence of an excess of methionine and cycloheximide as described above. These measurements were carried out for up to 3 h, during which the mutant and wild-type cells grew at similar rates (see Results).

Western blot (immunoblot) analysis of ubiquitin-protein conjugates in the cell. To inactivate Ydj1, mutant or wild-type cells in the late log phase (OD_{600}, 1.5) at 30°C were shifted to 38°C for 1 h and then collected by centrifugation. The cells were resuspended in radioimmunoprecipitation assay buffer (110 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris-HCl, pH 8.0) and lysed by vortexing with a volume of acid-washed glass beads (0.5 mm; Sigma) for 3 min at 4°C. The same amount of extract (20 μg of protein) was applied to an SDS–15% polyacrylamide gel electrophoresis (PAGE) gel and then transferred to nitrocellulose membrane (0.22-μm pore size; Schleicher & Schuell). The membranes were then treated with a 1:1,000 dilution of antiubiquitin serum (Eastern-Acres Biologicals) for 2 h and subsequently incubated with a ^{125}I-labelled protein A solution (2×10^6 cpm/ml; ICN) for 2 h. The ubiquitin-protein conjugates were quantitated with a Phosphor Imager (Molecular Dynamics).

Pulse-chase experiments and immunoprecipitations. For determination of the half-life of each β-gal fusion construct, pulse-labelling of yeast cells was carried out as described by Bachmair et al. (2), with some modifications. Yeast cells carrying a β-gal fusion construct whose expression is under the control of the galactose promoter were grown overnight at 30°C. The cells were then transferred to galactose-containing, methionine-free minimal medium for induction of the β-gal fusion protein. After 4 to 6 h, cells in the mid-log phase (OD_{600}, 0.5 to 1.0) were collected and resuspended at a density of 2×10^8/ml. Following a 30-min preincubation at 30 or 38°C (for the temperature-sensitive mutant), the cells were labelled with 200 μCi of [^{35}S]methionine for 5 min. The cells were then transferred to the 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 immunoprecipitation (IP) buffer (50 mM Tris-HCl [pH 7.5], 110 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 μg/ml). Cells were then lysed by vortexing with 400 μl of acid-washed glass beads for 3 min at 4°C. After centrifugation, the supernatants were recovered and the radioactivity incorporated into proteins was measured by trichloroacetic acid precipitation.

For IP, cell extracts (2.0×10^6 cpm per sample) were incubated with 0.5 μl of anti-β-gal antibody (Promega). After 1 h at 4°C, the insoluble material was removed by centrifugation and the supernatants were mixed with 15 μl of a suspension of protein A-Trisacryl (Pierce). After incubation at 4°C for 2 h, the protein A-Trisacryl beads were washed three times with IP buffer containing 0.1% SDS and boiled in 1× SDS-PAGE sampling buffer. The supernatants were subjected to SDS–8% PAGE and subsequently to autoradiography. The degradation of β-gal fusion proteins was then analyzed with a PhosphorImager.

Anti-β-gal immunoglobulin G immunoaffinity chromatography of cell extract. Immunoaffinity chromatography using anti-β-gal immunoglobulin G as the affinity ligand was carried out as follows. Exponentially growing (at 30°C) yeast cells carrying different β-gal constructs were shifted to 38°C for 30 min, collected by centrifugation, and then resuspended in IP buffer (see above). Cell extracts were prepared by the glass bead method as described above. The extract (3 mg of proteins) was loaded onto 1-ml anti-β-gal immunoglobulin G affinity column preequilibrated with the same buffer and washed with 20 volumes of IP buffer. The material bound to this column was then eluted with 3 ml of 0.1 M acetic acid (pH 2.5). The eluted proteins were precipitated by trichloroacetic acid and analyzed by Western blotting with an anti-Ydj1 antibody.

Other methods. Protein concentrations were determined by the bicinchoninic acid method (Pierce) in accordance with the manufacturer's instruction. The activity of β-gal was measured by using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as described by Rose et al. (27).

RESULTS

Effects of *ydj1-151* mutation on the degradation of different classes of proteins. To test if Ydj1 plays a role in overall proteolysis, we pulse-labelled cell proteins and then measured the rates of degradation of short-lived and abnormal proteins in isogenic wild-type cells and temperature-sensitive mutant *ydj1-151* cells. After a 5-min pulse of [^{35}S]methionine, the labelled proteins were degraded at 11%/h in wild-type cells but only 5%/h in *ydj1-151* mutant cells, even at the permissive temperature, 30°C (Fig. 1A). Nevertheless, at this temperature, the mutant and wild-type cells grew at similar rates (Fig. 2) despite the differences in proteolysis rates. The defect in overall degradation was much larger upon a shift to the nonpermissive temperature, 38°C, at which the rate of protein breakdown was enhanced by 50% to reach 18%/h in wild-type cells but remained about 6%/h in *ydj1-151* mutant cells (Fig. 1B). Thus, loss of Ydj1 function seemed to prevent the rapid elimination of heat-damaged polypeptides. After the first hour at 38°C, the rates of proteolysis decreased in both strains but remained much higher in the wild-type strain. Thus, the total extent of breakdown of these short-lived components was much less in the *ydj1-151* mutant. At the nonpermissive temperature, the mutant and wild-type cells grew at similar rates for at least 3 h (Fig. 2). Thus, the reduced rates of proteolysis are not a consequence of cytotoxicity. Similar results were also obtained when a pulse of [^{14}C]leucine was used to measure rates of protein degradation.

To evaluate the capacity to degrade abnormal proteins rapidly, wild-type and *ydj1-151* mutant cells were allowed to incorporate an amino acid analog which prevents normal folding of many polypeptides (14). Because of the presence of a *can1-100* mutation in these strains, we could not use the arginine analog canavanine, which is commonly used in such experiments. Instead, these cells were exposed to the proline analog azetidine carboxylic acid. Polypeptides synthesized in the presence of azetidine carboxylate were degraded at 30°C in the *ydj1-151* mutant at approximately 50% of the rate in the wild-type cells (Fig. 1C). Thus, the *ydj1-151* strain has a clear defect in the degradation of various abnormal proteins (e.g., heat-damaged proteins or those containing amino acid analogs).

A 5-min pulse of radioactive amino acids preferentially labels short-lived cell proteins, but it also labels many stable proteins (especially if they are expressed at high levels). To test whether Ydj1 also affects the degradation of such long-lived cell proteins, a different labelling procedure was utilized. Cells growing at 30°C were shifted to 38°C, exposed to [^{35}S]methionine for 2 h, and then incubated in the presence of high concentrations of nonradioactive methionine for 12 h to allow

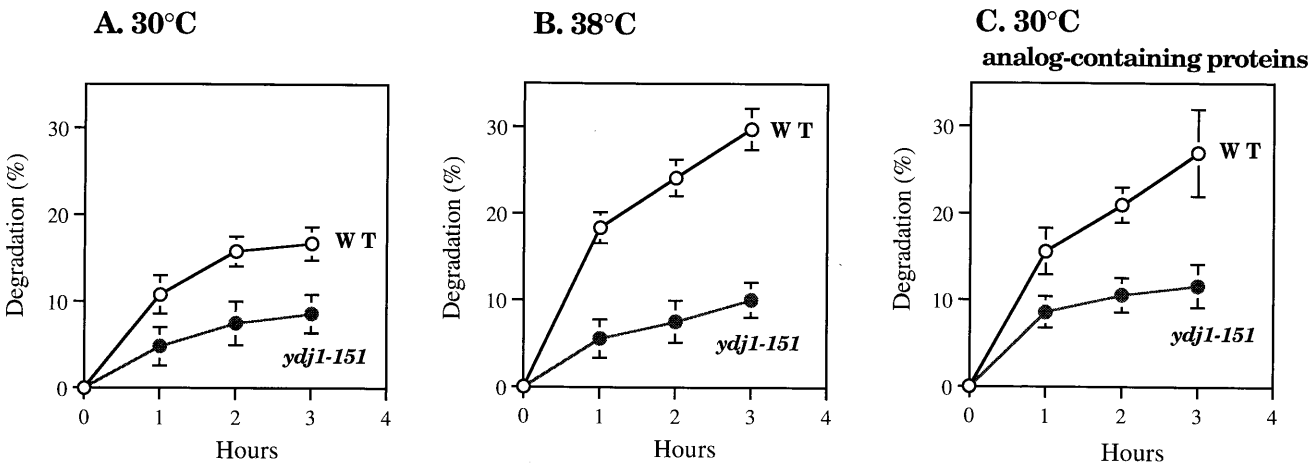

FIG. 1. Degradation of pulse-labelled short-lived normal proteins and of abnormal (azetidine carboxylate-containing) polypeptides in wild-type (WT) and *ydj1-151* mutant strains. Cells were pulse-labelled with 100 μCi of [^{35}S]methionine for 5 min to preferentially label short-lived polypeptides at 30 or 38°C (as indicated) in the presence or absence of the proline analog azetidine carboxylate (0.5 mg/ml), and then the rate of degradation was measured during the chase period. (A) Degradation of proteins at 30°C. (B) Degradation of proteins at 38°C. (C) Degradation of azetidine-carboxylate-containing polypeptides at 30°C. The data shown here are the mean values and standard errors obtained in four independent experiments.

complete degradation of the short-lived radioactive proteins. The breakdown of the remaining long-lived polypeptides was then measured. The rate of degradation of these stable components in the *ydj1-151* mutant appeared to be the same as in wild-type cells (about 2%/h) (Fig. 3). Most short-lived and abnormal proteins in *S. cerevisiae* and other eukaryotic cells are degraded by the ubiquitin-proteasome-dependent pathway (19, 28), while the breakdown of long-lived proteins, which was not affected in this chaperone mutant, seems to occur in the vacuole (21). These findings, together, suggest that Ydj1 is specifically involved in degradation by the ubiquitin-proteasome-dependent pathway.

Effects of the *ydj1-151* mutation on degradation of model substrates. To further define the role of Ydj1 in proteolysis, we tested the effects of the *ydj1-151* mutation on the degradation of individual short-lived polypeptides, which are known to be substrates of the ubiquitin-dependent pathway. We transformed wild-type and mutant cells with plasmids expressing different short-lived fusion proteins containing β-gal and monitored their breakdown by IP with an anti-β-gal antibody. The degradation of an abnormal fusion polypeptide, ubiquitin–proline–β-gal (Ub-P-β-gal) (2, 20), like the breakdown of analog-containing proteins, requires the ubiquitin-conjugating enzyme Ubc4 (20). The degradation of this protein was very rapid in wild-type cells (half-life, 7 to 10 min) but was completely blocked in the *ydj1-151* mutant at the nonpermissive temperature, 38°C (Fig. 4). In contrast, at the permissive temperature (30°C), the half-life of Ub-P-β-gal was 7 to 10 min in both the wild-type strain and the *ydj1-151* mutant (data not shown).

It is of particular interest that the *ydj1-151* mutation affected

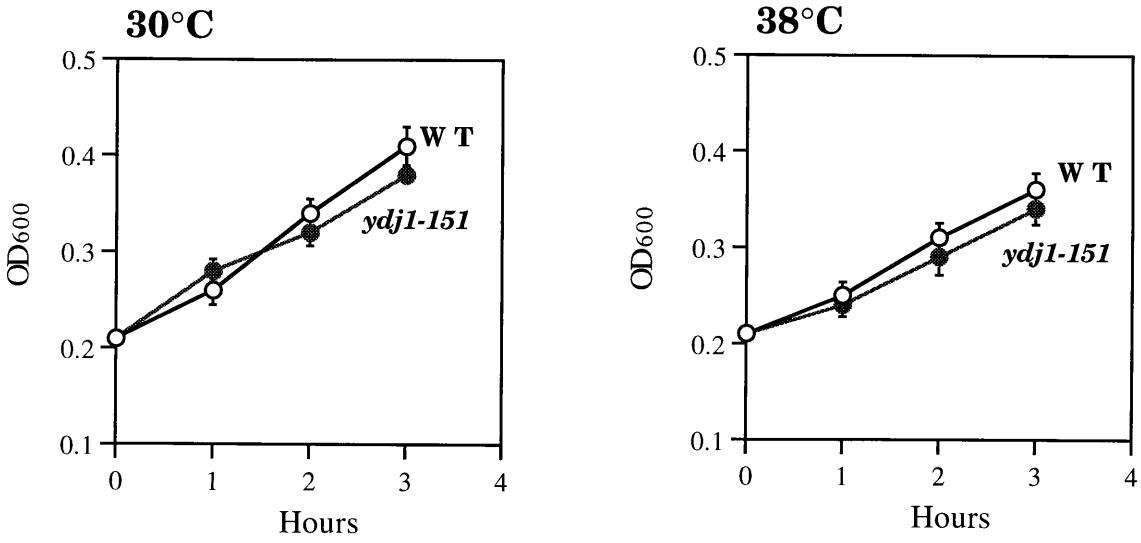

FIG. 2. Growth of wild-type (WT) cells and *ydj1-151* mutant cells. Cells growing exponentially at the permissive temperature (i.e., 30°C) were either kept at the permissive temperature or shifted to the nonpermissive temperature and incubated for another 3 h. The growth of cells at both temperatures was monitored by measuring the OD_{600} of cultured cells. The growth of cells was monitored for only 3 h, the time period used in all of these studies of proteolysis. The data shown here are the mean values and standard errors obtained in three independent experiments.

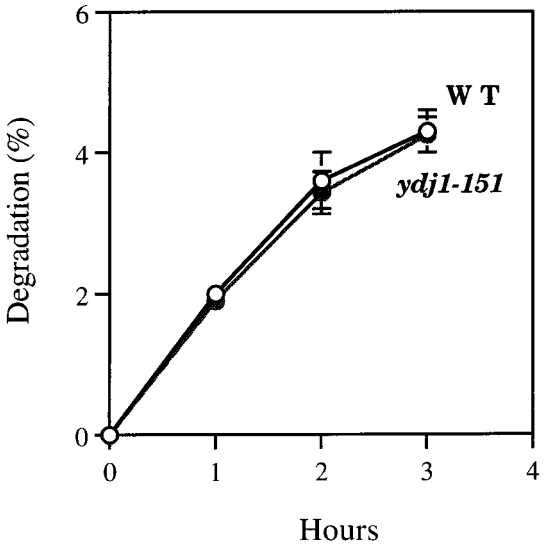

FIG. 3. Degradation of long-lived proteins in wild-type (WT) and *ydj1-151* mutant strains. To measure the degradation of long-lived proteins, cells were labelled with 100 μCi of [^{35}S]methionine for 2 h at 30°C and then incubated in medium containing an excess of nonlabeled methionine for 12 h to allow degradation of the short-lived proteins. After this incubation period, the temperature was shifted to 38°C for 30 min to inactivate Ydj1 function and then cells were resuspended in fresh chase medium with excess methionine and cycloheximide. The rate of degradation of the remaining labelled proteins was then measured at 38°C. The data shown here are the mean values and standard errors obtained in three independent experiments.

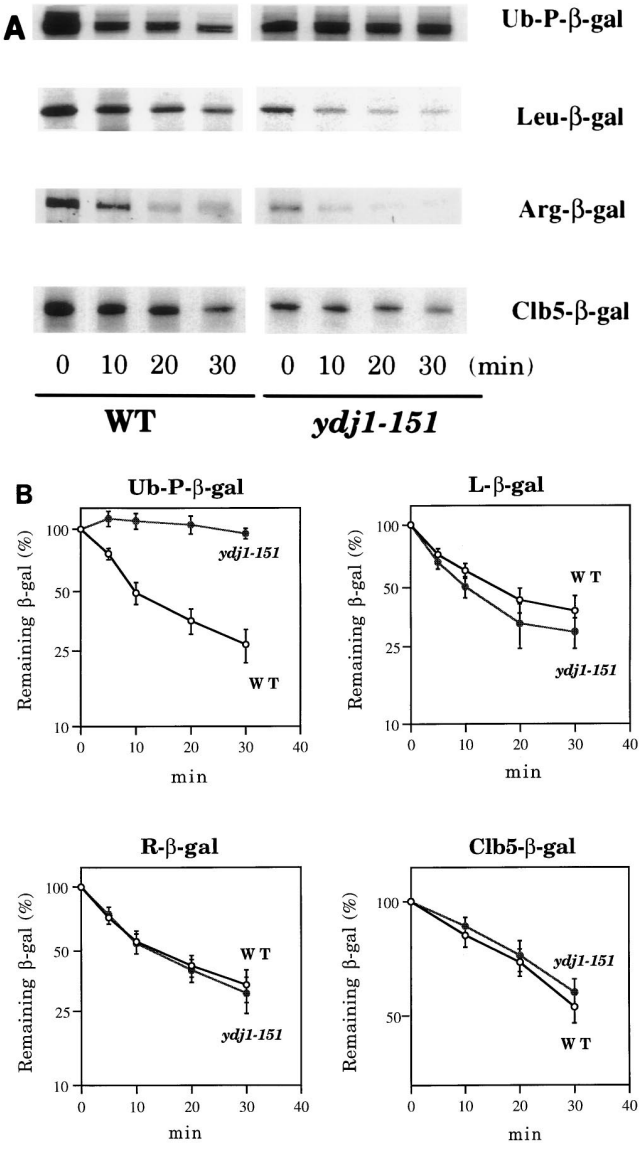

FIG. 4. Pulse-chase analysis of the breakdown of several different short-lived fusion polypeptides in wild-type (WT) and *ydj1-151* mutant strains. Cells were shifted to the nonpermissive temperature (38°C) for 30 min prior to labelling with 200 μCi of [^{35}S]methionine for 5 min. During the subsequent chase period, the degradation of the different β-gal fusion polypeptides was measured by quantitative IP with an anti-β-gal antibody. (A) Degradation of Ub-P-β-gal, L-β-gal, R-β-gal, and Clb5–β-gal. The data shown here were taken from one of three experiments, all of which showed similar results. (B) Quantitation of the degradation rates of these different β-gal constructs. The data shown here are the mean values and standard errors obtained in three independent experiments.

the rate of degradation of only certain polypeptides which are substrates for the ubiquitin-proteasome pathway. In contrast to the breakdown of Ub-P-β-gal, the very rapid hydrolysis of the N-end rule substrates leucine–β-gal (L-β-gal) and arginine–β-galactosidase (R-β-gal), which have half-lives of 3 to 5 min (2, 35), was not reduced in repeated experiments with the *ydj1-151* mutant, even at the nonpermissive temperature (Fig. 4).

Degradation of other model substrates. To determine whether Ydj1 also affects the ubiquitin-dependent degradation of normal short-lived cell proteins, we studied the rapid breakdown of the B-type cyclin Clb5 by using the fusion construct Clb5–β-gal, which is rapidly degraded by a mechanism similar to that by which the wild-type Clb5 protein is degraded (28). This process was also not affected by the *ydj1-151* mutation (Fig. 4). In addition, we tested the effect of the *ydj1-151* mutation on the rapid breakdown of the transcriptional activator Gcn4 by using a Gcn4–β-gal fusion construct. The degradation of this fusion protein also requires ubiquitin and the proteasome and involves a mechanism similar to that of the breakdown of Gcn4 (24). In the *ydj1-151* mutant, the degradation of Gcn4–β-gal was reduced two- to threefold at the nonpermissive temperature (Fig. 5). This partial stabilization was seen repeatedly in three distinct experiments and was clearly different from the complete stabilization seen with Ub-P-β-gal. It is noteworthy that there are two pathways for Gcn4 degradation (24) and presumably only one of them requires Ydj1 function. In any case, these finding suggest that Ydj1 is important for the rapid breakdown of not only certain abnormal polypeptides but also certain short-lived normal proteins in yeast cells.

It is noteworthy that, at the nonpermissive temperature in the *ydj1-151* mutant strain, the incorporation of radioactive amino acids into cell proteins, including all of these fusion proteins, was about twofold lower than in wild-type cells at the nonpermissive temperature, while there was no consistent difference in protein labelling at the permissive temperature (data not shown). For this reason, all measurements of the rates of protein degradation were normalized to the amount of label incorporated at time zero. The defect in the breakdown of Ub-P-β-gal and of Gcn4–β-gal cannot be related to this reduced incorporation of the radioactive precursor in the *ydj1-151* mutant, since in these same cells other substrates, e.g., L-β-gal, R-β-gal, and Clb5–β-gal, were degraded at a normal rate. Presumably, it is because of the temperature sensitivity of this mutant strain. However, during a 3-h chase period at the nonpermissive temperature (38°C), the growth of cells was not impaired at all, which suggested that the *ydj1-151* strain had

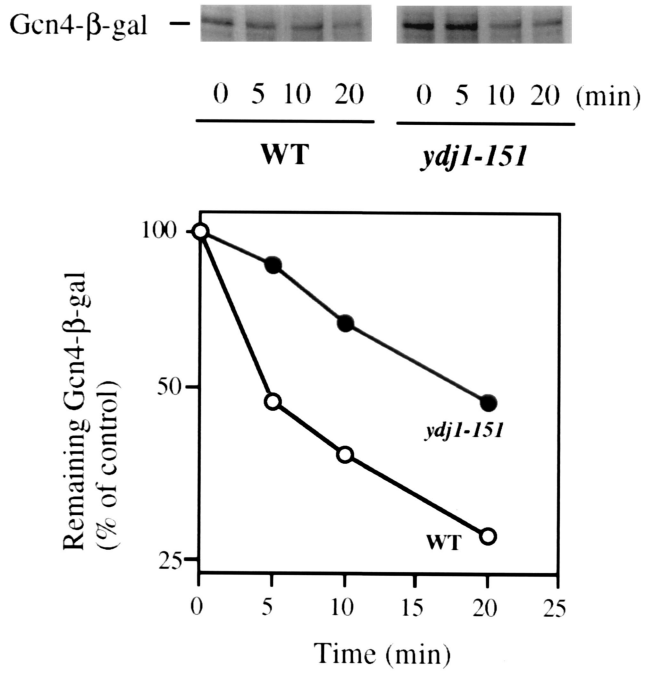

FIG. 5. Degradation of Gcn4–β-gal in wild-type (WT) and *ydj1-151* mutant strains. Pulse-chase analysis of degradation was carried out by IP with an anti-β-gal antibody as described in the legend to Fig. 4. For quantitation of degradation rates, a PhosphorImager was used. Similar results were obtained in duplicate experiments.

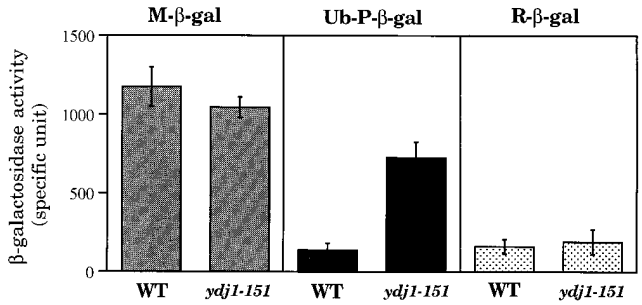

FIG. 6. Levels of different β-gal activities in wild-type (WT) and *ydj1-151* mutant strains. Cells carrying different β-gal constructs, M-β-gal, Ub-P-β-gal, and R-β-gal, were grown in the presence of galactose (final concentration, 2%) for at least 6 h to induce the β-gal fusion proteins regulated by the galactose promoter. The cells were shifted to the nonpermissive temperature (38°C) for 1 h and then collected. Enzymatic activity of β-gal in the cell extract was measured as described by Rose et al. (27) and is given in specific units (nanomoles of ONPG hydrolyzed per minute per milligram of protein). The data shown here are mean values and standard errors obtained in three independent experiments.

normal cellular function despite the lack of Ydj1 function, at least during this period (Fig. 2).

Measurement of Ub-P-β-gal, R-β-gal, and M-β-gal content in the cells. To obtain an independent estimate of the degradation rates of the Ub-P-β-gal, R-β-gal, and methionine–β-gal (M-β-gal) fusions by a method not involving isotopes or pulse-chase approaches, we simply assayed β-gal activity in cells carrying these fusion proteins. Reduced degradative capacity should lead to enhanced protein levels and greater activity if enzyme folding can occur. Because of the rapid breakdown of Ub-P-β-gal in wild-type cells, the β-gal activity (measured by the hydrolysis of ONPG) was 10 times lower than in cells carrying the stable wild-type enzyme M-β-gal when it was assayed at the nonpermissive temperature. By contrast, in the *ydj1-151* mutant expressing Ub-P-β-gal, the β-gal activity was four to five times higher than in the wild-type strain and was close to the activity of stable M-β-gal (Fig. 6). These data are consistent with failure of the mutant cells to degrade Ub-P-β-gal at the nonpermissive temperature. Furthermore, the activity of β-gal in both wild-type and mutant cells containing R-β-gal was very low, in confirmation of the rapid degradation of this N-end rule substrate in the mutant strain (Fig. 4). In addition to confirming the stabilization of Ub-P-β-gal proteins in the *ydj1-151* mutant, these findings also suggest that Ydj1 is not required for the folding or assembly of the active form of β-gal, which is a tetrameric enzyme, or for maintaining it in a soluble form.

Ydj1 is associated with Ub-P-β-gal in cells. The capacity of a DnaJ-like protein to bind selectively to a variety of proteins could be important in the recognition of protein substrates or in increasing their susceptibility to the degradative machinery. We therefore tested if Ydj1 associates directly with Ub-P-β-gal. By using an anti-β-gal immunoglobulin G-agarose affinity column, we isolated Ub-P-β-gal and the proteins associated with it from wild-type and *ydj1-151* mutant cells and resolved them by SDS-PAGE. The presence of Ydj1 was then analyzed by Western blotting with an anti-Ydj1 antibody. Figure 7 shows that Ydj1 is associated with Ub-P-β-gal in the wild-type strain (lane 2) but not in the *ydj1-151* mutant strain (lane 3), in which this fusion polypeptide was stable. In this experiment, M-β-gal was present in slightly larger amounts than Ub-P-β-gal, and the amounts of Ub-P-β-gal (as measured with an anti-β-gal antibody by quantitative Western blotting) were the same in the wild-type and *ydj1-151* mutant lanes. Furthermore, no Ydj1 was found in association with the stable wild-type enzyme M-β-gal in the wild-type strain (lane 1). Thus, Ydj1 appears to associate specifically with the short-lived fusion protein and thus may facilitate its degradation.

Effects of the *ydj1-151* mutation on in vivo protein ubiquitination. In the ubiquitin-proteasome pathway, Ydj1 may be

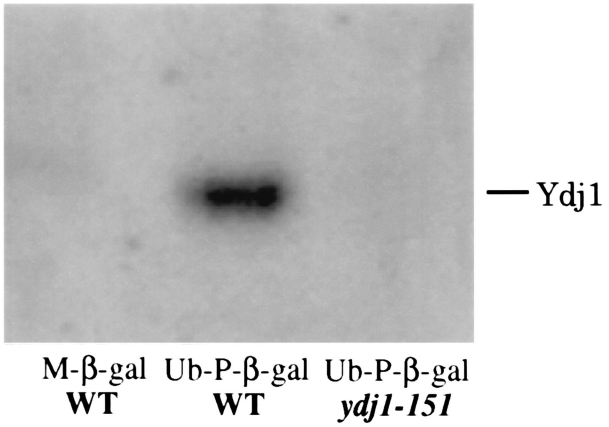

FIG. 7. Association of Ydj1 with Ub-P-β-gal in wild-type (WT) and *ydj1-151* mutant strains. Exponentially growing cells were shifted to the nonpermissive temperature (38°C) for 30 min prior to the preparation of extracts. The cell extracts were loaded onto an anti-β-gal immunoglobulin G column, and the proteins associated with β-gal were eluted with acid and then analyzed by Western blotting with an anti-Ydj1 antibody. Lanes: 1, wild-type cells carrying M-β-gal; 2, wild-type cells carrying Ub-P-β-gal; 3, *ydj1-151* strain carrying Ub-P-β-gal. The levels of these β-gal constructs in the lanes were similar as determined by Western blot analysis with an anti-β-gal antibody. Similar results were obtained in three independent experiments.

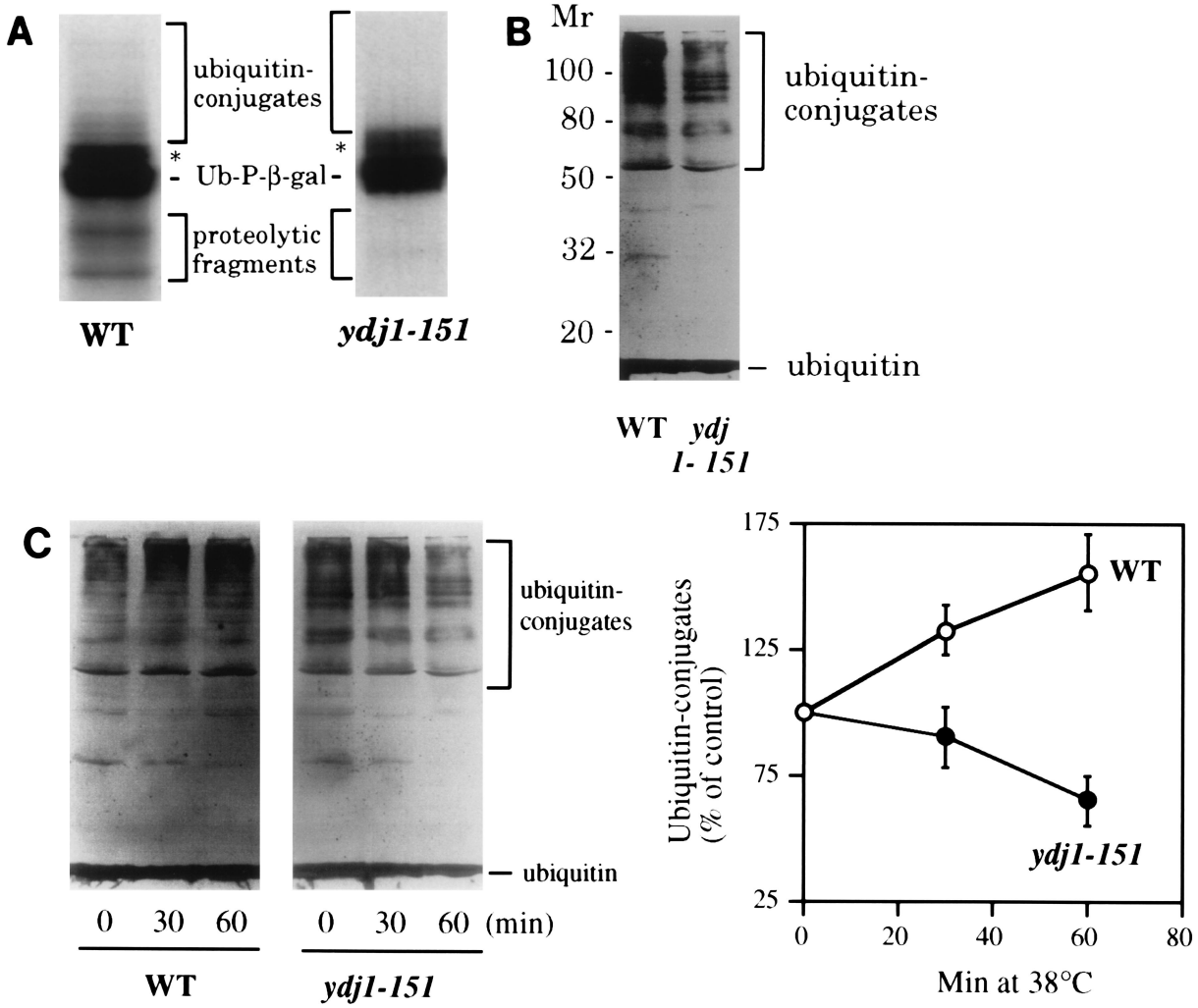

FIG. 8. Ubiquitin conjugation in wild-type (WT) and *ydj1-151* mutant strains. (A) Ubiquitin conjugation to Ub-P-β-gal in wild-type and *ydj1-151* mutant strains. Exponentially growing cells were shifted to 38°C, the nonpermissive temperature, for 30 min and then labelled with 200 μCi of [^{35}S]methionine for 20 min. The immunoprecipitation of Ub-P-β-gal from cell extracts with an anti-β-gal antibody was carried out as described in the legend to Fig. 3. The mono- and diubiquitinated forms of Ub-P-β-gal (20) are marked by asterisks, and the higher-molecular-weight multiubiquitinated adducts and the proteolytic fragments of Ub-P-β-gal are indicated. (B) Content of ubiquitin-protein conjugates in wild-type and *ydj1-151* mutant strains. During exponential growth at 30°C, the cells were shifted to 38°C for 1 h to inactivate Ydj1 molecules. Cell proteins (20 μg per lane) were separated on an SDS–15% polyacrylamide gel and probed with an antiubiquitin antibody. The blot was treated with ^{125}I-labelled protein A and then subjected to autoradiography. Free ubiquitin and high-molecular-weight ubiquitin-protein conjugates are indicated. (C) Changes in the levels of ubiquitin-protein conjugates in wild-type and *ydj1-151* strains. After cells were shifted to 38°C, aliquots of cells were collected at the intervals indicated and cell extracts were prepared. Equal amounts of proteins (20 μg per lane) were applied to an SDS–15% polyacrylamide gel, and the total content of ubiquitinated proteins was measured with an antiubiquitin antibody as described above. Quantitation of ubiquitin-conjugated proteins was done with a PhosphorImager. Similar results were obtained in three independent experiments. The data shown in the graph are mean values and standard errors obtained in three independent experiments.

important either for the conjugation of ubiquitin molecules to certain cell proteins or for the subsequent degradation of the ubiquitin-conjugated polypeptides. To decide between these possibilities, we tested whether the ubiquitination of Ub-P-β-gal was impaired in the mutant strain. After incubation at 38°C for 30 min, Ub-P-β-gal from wild-type and *ydj1-151* mutant cells was quantitatively immunoprecipitated with an anti-β-gal antibody and analyzed by SDS-PAGE. Although multiubiquitinated adducts of Ub-P-β-gal (as well as a large proteolytic fragment) were observed in the wild-type strain, very few conjugates (and no fragment) of Ub-P-β-gal were seen in the *ydj1-151* mutant, where this fusion protein is stable (Fig. 8A). Thus, Ydj1 is critical for the ligation of multiple ubiquitin moieties to this short-lived protein. In both wild-type and mutant cells, a doublet of high-molecular-weight bands (marked asterisks in Fig. 8A) was also observed, which represents mono- and diubiquitinated forms of this fusion protein (20). However, these adducts are not related to Ub-P-β-gal degradation, since they are also present in the E2 mutant (*ubc4* mutation), in which Ub-P-β-gal cannot be multiubiquitinated and is completely stable (20). In contrast to Ub-P-β-gal, the level of ubiquitinated R-β-gal or L-β-gal was not decreased in the *ydj1-151* mutant (data not shown).

As shown in Fig. 1, the degradation of short-lived proteins in the *ydj1-151* mutant was generally reduced but less dramatically than the breakdown of Ub-P-β-gal. To test how general the defect in ubiquitin conjugation in the *ydj1-151* mutant was, we compared the total level of ubiquitinated proteins in wild-type and mutant cells at the nonpermissive temperature (38°C for 1 h) by Western blotting with an anti-ubiquitin antibody

(Fig. 8B). In four independent experiments, the levels of free ubiquitin did not differ, but the amount of ubiquitin-protein conjugates was 50% smaller in the *ydj1-151* mutant (lane 2) than in the wild-type strain (lane 1).

Upon heat shock of cells, the total amount of protein ubiquitination increases because of the buildup of abnormal proteins in the cells (25). To test if this response is defective in the chaperone mutant, we measured the changes in the levels of ubiquitinated proteins in wild-type and *ydj1-151* mutant cells after a shift to the nonpermissive temperature. Upon incubation at 38°C, the amount of ubiquitinated proteins increased in the wild-type cells, but in the *ydj1-151* strain, where overall proteolysis failed to rise (Fig. 1B), the content of ubiquitinated proteins decreased (Fig. 8C). In contrast, the level of free ubiquitin was constant over this period in both the wild type and the *ydj1-151* strain. Therefore, the decrease in the content of ubiquitinated proteins was due to a real defect in the ubiquitination of certain proteins, rather than to a lack of free ubiquitin or some nonspecific defect in protein synthesis.

Apparently, in the *ydj1-151* strain, there is reduced formation of ubiquitin conjugates while their degradation continues. Therefore, the total level of ubiquitinated proteins in the cell decreases. If Ydj1 were involved in the degradation of ubiquitinated proteins by the 26S proteasome, then accumulation of ubiquitin conjugates would have been anticipated in the *ydj1-151* mutant, as has been observed with mutations in certain proteasome subunits (16) and upon treatment with proteasome inhibitors (11). Thus, the overall reduction (about 50%) in ubiquitin conjugation in the *ydj1-151* strain appears to be due to a large decrease in the ubiquitination of certain specific proteins, such as Ub-P-β-gal, which is specifically stabilized in the *ydj1-151* strain. Therefore, Ydj1 seems to promote degradation of a number of cell proteins by facilitating their conjugation to ubiquitin.

DISCUSSION

The present findings indicate that the molecular chaperone Ydj1 is necessary for rapid degradation of certain types of proteins by the ubiquitin-proteasome pathway. The *ydj1-151* mutant generally reduced the rapid breakdown of many short-lived normal proteins and of abnormal proteins containing amino acid analogs or those damaged by heat. Moreover, experiments with model substrates indicated an absolute block in proteolysis for certain short-lived polypeptides, e.g., Ub-P-β-gal, and partial inhibition for the others, e.g., Gcn4-β-gal, which is known to be degraded by two ubiquitin-dependent pathways (24). It is noteworthy that inactivation of Ydj1 did not reduce the very rapid hydrolysis of the N-end rule substrates L-β-gal and R-β-gal or of B-type cyclin Clb5, which also require ubiquitin and the proteasome. In the ubiquitin-proteasome pathway, ubiquitin molecules are first activated to thiol esters, transferred to a ubiquitin carrier protein called E2, and then conjugated to the substrates by a ubiquitin-protein ligase called E3 (5, 18, 19). Although several of these Ydj1-dependent substrates require UBC4 and UBC5 for ubiquitination (e.g., amino acid analog-containing proteins and Ub-P-β-gal) (19), the E3s involved in these processes are unknown. By contrast, N-end rule substrates require UBC2 for their ubiquitination (10). Therefore, it remains possible that this chaperone is necessary only for ubiquitination of substrates which require specific ubiquitination enzymes (e.g., UBC4 and UBC5).

Although Ydj1 appears to be important for the selective breakdown of certain polypeptides, the slower degradation of long-lived cell proteins was indistinguishable in wild-type and *ydj1-151* mutant cells. These stable components constitute the bulk of cell proteins and are believed to be degraded in the yeast vacuole (21). In fact, in related studies, we have found that the degradation of these long-lived components is reduced not by proteasome inhibitors but by inhibitors which block the yeast vacuolar proteases (24a). Because the methods available for preferential labelling of short-lived or abnormal polypeptides necessarily also label long-lived proteins, whose degradation is unaffected in these chaperone mutants, the decrease in the overall breakdown of pulse-labelled proteins in the *ydj1-151* mutant must underestimate the real magnitude of the defect in the breakdown of certain short-lived polypeptides. Therefore, many polypeptides that are rapidly degraded within 3 h in wild-type cells must be degraded only slowly or not at all in *ydj1-151* cells.

Several findings indicate that Ydj1 is important for ubiquitin conjugation only to certain types of short-lived and abnormal proteins. (i) In wild-type cells, Ydj1 was found associated with the short-lived protein Ub-P-β-gal but not with the stable protein M-β-gal. Moreover, in the *ydj1-151* strain, no association of Ydj1 with Ub-P-β-gal was seen (Fig. 7), which may account for its reduced ubiquitination and hydrolysis. Interestingly, we have recently found that in the *ydj1-151* strain at the nonpermissive temperature, the nonfunctional chaperone itself was rapidly hydrolyzed, which may account for the lack of association of Ydj1 and Ub-P-β-gal in this mutant (data not shown). (ii) In the *ydj1-151* strain, there was a very large decrease in ubiquitin conjugation to Ub-P-β-gal (Fig. 8A), whose degradation was blocked selectively, while no such decrease was seen in the level of ubiquitinated R-β-gal or L-β-gal, whose breakdown is independent of these chaperones. (iii) At the nonpermissive temperature, the *ydj1-151* strain showed a reduction in the total cellular content of ubiquitinated proteins (Fig. 8B), which is correlated with and can account for the reduction in overall proteolysis (Fig. 1). By contrast, in wild-type cells, the level of these conjugates increased and overall proteolysis rose upon a shift to 38°C (Fig. 8C).

Unfortunately, we and others have been unable (for reasons that are unclear) to detect ubiquitination of Ub-P-β-gal in yeast lysates, even with Ydj1 added. Presumably, some additional chaperone(s) (e.g., Hsp70 or a functional analog of GrpE) or another factor(s) is limiting in vitro and is necessary for ubiquitination of such substrates. It has been shown that the function of hsc70/hsp40 (a DnaJ homolog) in protein folding in eukaryotes also involves a novel cochaperone, Hip (18a). Such chaperones could similarily play an important role in the ubiquitination of specific substrates, such as Ub-P-β-gal (see below).

Molecular chaperones may enhance ubiquitin-dependent degradation by maintaining the substrate in a conformation which promotes its recognition or modification by the ubiquitination enzymes. The chaperones may do so by functioning as specific recognition factors for E2s and E3s, or alternatively, they may simply prevent aggregation of the substrate. For example, in *E. coli*, DnaK and DnaJ prevent the aggregation of newly synthesized proteins at high temperatures (15). Similarly, Ydj1 has been suggested to function with Hsp70 in protein translocation across membranes by maintaining the substrate in an unfolded, nonaggregated, soluble state (4, 9). However, in the degradation of Ub-P-β-gal, Ydj1 does not seem to be required simply to prevent aggregation of the substrate or to facilitate its folding, because in the *ydj1-151* mutant at the nonpermissive temperature (38°C), undegraded Ub-P-β-gal molecules are soluble, folded normally, and assembled into active tetramers, as shown by the higher β-gal activity (Fig. 6).

It is therefore likely that this chaperone plays a role in substrate recognition or modification by E2 or E3. Accordingly, we have found that Ydj1 associates with Ub-P-β-gal, but not with the wild-type enzyme M-β-gal (Fig. 7). These in vivo experiments cannot absolutely eliminate the possibility that Ydj1 acts indirectly, e.g., by promoting proper folding or by preventing the aggregation of a specific E2 or E3 necessary for ubiquitination of these specific substrates. However, in our experiments, the mutant cells were switched to the nonpermissive temperature for only 30 min before the radioactive pulse. Thus, the ubiquitin-conjugating systems and proteasomes which were synthesized during prior growth at 30°C should still be functional.

Consequently, it seems likely that the association of Ydj1 with certain substrates is important for their ubiquitination and that this chaperone functions as a recognition element or as a cofactor for the ubiquitination enzymes. The chaperones may present the bound polypeptides to E2s and E3s in a soluble, partially unfolded conformation that favors ubiquitination. In *E. coli* (31) and mitochondria (36), the associations of certain abnormal proteins with DnaJ and DnaK and of other polypeptides with GroEL and GroES (22) are critical for their digestion by the ATP-dependent proteases, and in the endoplasmic reticulum, the Hsp70 homolog BiP seems also to function in facilitating hydrolysis of certain abnormal proteins (23). Possibly, Ydj1 and certain Hsp70s have specific sites for interaction with the critical E3s. Alternatively, the binding of the chaperones to the substrates may simply make critical domains available for recognition by E3s. Interestingly, Ydj1 binding has been recently shown to make certain domains of the Cln3 cyclin available for phosphorylation by the Cdc28 kinase (38). Tests of these models are difficult, because the E3s active in breakdown of the abnormal proteins (e.g., analog-containing polypeptides or Ub-P-β-gal) have not been identified.

Since large defects in proteolysis were seen upon inactivation of Ydj1, it seems likely that the other main DnaJ homolog in the yeast cytosol, Sis1 (39), is unable to replace Ydj1's function in proteolysis, although it can suppress the effect of deletion of Ydj1 on cell growth if highly overexpressed on a multicopy plasmid (3). In related studies (24b), we have found that Sis1 is involved in the degradation of certain abnormal proteins, but unlike Ydj1, Sis1 is not required for protein ubiquitination and instead functions later in this pathway in the degradation of the ubiquitinated substrates by the 26S proteasome.

Since Ydj1 appears to function in concert with Hsp70s in protein transport (3), it seems likely that mutations in certain yeast Hsp70s genes would also affect the degradation of certain short-lived proteins. In related studies, we have found that some double mutants of the *SSA* and *SSB* families (*ssa1 ssa2* and *ssb1 ssb2* mutants) (6) show a clear reduction in the overall breakdown of short-lived and abnormal proteins but not a defect in the degradation of long-lived proteins, like the *ydj1-151* mutant (24b). Although these data strongly suggest that Ydj1 functions in proteolysis together with Hsp70, rigorous conclusions based on studies of Hsp70 mutants are difficult because there are multiple, homologous members of the Hsp70 family, and also mutations in one or two members of these families can stimulate the compensatory expression of other Hsp70s. In addition, mutations in more than two members of the *SSA* family result in lethality (6). Therefore, we have focused here on the role of Ydj1 in protein breakdown, which can be more rigorously studied by using the temperature-sensitive mutant.

Molecular chaperones can play multiple roles in intracellular proteolysis, presumably because of their capacity to bind selectively to unfolded proteins. By functioning in the ubiquitin-proteasome pathway, Ydj1 may serve as a quality control mechanism for the cell's protein folding and translocation machinery. If the chaperone fails to catalyze these processes successfully, as may occur frequently with mutant or damaged polypeptides, they may promote the rapid degradation of the abnormal polypeptides. Heat shock, oxygen radicals, and other environmental stresses can damage cell proteins, and accumulation of such abnormal polypeptides in stressed cells signals the induction of the family of heat shock proteins (31), including molecular chaperones that catalyze the refolding of the damaged polypeptides. This study and related ones with bacteria and mitochondria have established that these same chaperones can also facilitate the rapid degradation of abnormal, perhaps irreparably damaged, polypeptides. In this degradative process, the chaperones appear to function together with other heat shock proteins that are components of the cell's degradative machinery, including ubiquitin, the E2s, UBC4 and UBC5 in *S. cerevisiae* (29), and ATP-dependent proteases in prokaryotes (31).

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