The Role of Ubiquitin Conjugation in Glucose-induced Proteolysis of Saccharomyces Maltose Permease*

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In Saccharomyces, the addition of glucose induces a rapid degradation of maltose permease that is dependent on endocytosis and vacuolar proteolysis (Medintz, L., Jiang, H., Han, E. K., Cui, W., and Michels, C. A. (1996) J. Bacteriol. 178, 2245–2254). Here we report on the role of ubiquitin conjugation in this process. Deletion of DOA4, which causes decreased levels of available ubiquitin, severely decreases the rate of glucose-induced proteolysis, and this is suppressed by the overproduction of ubiquitin. Overexpression of ubiquitin in an endocytosis-deficient end3-ts strain results in the glucose-stimulated accumulation of a larger molecular weight species of maltose permease, which we demonstrate is a ubiquitin-modified form of the protein by utilizing two ubiquitin alleles with different molecular weights. The size of this ubiquitinated species of maltose permease is consistent with monoubiquitination. A promoter mutation that reduces expression of RSP5/NPI1, a postulated ubiquitin-protein ligase, dramatically reduces the rate of glucose-induced proteolysis of maltose permease. The role of various ubiquitin-conjugating enzymes was investigated using strains carrying mutant alleles ubc1Δ, ubc4Δ, ubc5Δ, cdc34-ts2/ubc3, and ubc9-ts. Loss of these functions was not shown to effect glucose-induced proteolysis of maltose permease, but loss of Ubc1, -4, and -5 was found to inhibit maltose permease expression at the post-transcriptional level.

Glucose regulates maltose transport in Saccharomyces at several levels. It blocks transcription of the maltose permease gene by multiple mechanisms cumulatively referred to as glucose repression (2), and it inactivates maltose permease by a process referred to as glucose-induced inactivation or catabolite inactivation (1, 3). Together, these processes allow for the rapid shift from maltose to glucose fermentation. Previously, we showed that glucose-induced inactivation of maltose permease consists of two apparently independent processes: the proteolysis of maltose permease protein and the rapid inhibition of maltose transport activity, which occurs even faster than can be explained by loss of the protein alone (1). Molecular genetic analysis using mutations in END3, REN1/VPS2, PEP4, and PRE1 and PRE2 demonstrated that the proteolysis of maltose permease is dependent on endocytosis, vesicle sorting, and vacuolar proteolysis and is independent of the proteasome.

Studies of a variety of different nutrient transporters suggest that the inactivation and/or degradation of permeases is a generalized mechanism used to respond to changes in nutrient availability from less desirable nutrient sources or starvation conditions to preferred nutrients and rich medium. The general amino acid permease Gap1 protein is inactivated by the addition of ammonium ions to yeast cells growing on proline as the sole nitrogen source (5, 6). Inactivation occurs as a 2-fold process with enzymatic inactivation by phosphorylation preceding degradation of the permease (5, 6). The high affinity Pho84 phosphate transporter undergoes rapid degradation once the supply of phosphate and/or carbon source is exhausted (7). Uracil permease (Pur4p) is phosphorylated on serine residues at the plasma membrane and is rapidly degraded under adverse growth conditions (8). A common feature of the degradation of the maltose, galactose, uracil, and general amino acid permeases is that all are mediated by endocytosis and subsequent transport to the vacuole, the site of degradation. Ubiquitination has been implicated as the mechanism marking these proteins and several others for rapid endocytosis and selective degradation (4, 6, 8–11). We report here that ubiquitination of the maltose permease occurs in response to glucose and explore the cellular components involved in this process.

Ubiquitination of Saccharomyces Ste2 protein, a-factor receptor, is required for its ligand-stimulated endocytosis and vacuolar proteolysis (10). END4 mutations inhibit endocytosis of a-factor and stimulate the appearance of mult ubiquitinated species. A sequence in the C-terminal cytoplasmic domain of Ste2p, SINNDAKSS (12), is sufficient to stimulate endocytosis, but mutation of the Lys in this target sequence to Arg inhibits ligand-stimulated ubiquitination and degradation. These results clearly implicate ubiquitination in receptor targeting to endocytosis. Ubiquitination also is required for endocytosis of yeast uracil permease (8) and probably the galactose transporter, Gal2p (4).

Additional studies also have implicated ubiquitination as a signal for the endocytosis and vacuolar degradation of other plasma membrane proteins including mammalian peptide hormone receptors (reviewed in Ref. 13). The yeast ABC transporter Ste6 accumulates in a ubiquitinated form in the plasma membrane of strains that are deficient in endocytosis (11). In strains that have normal endocytic functions, this protein is generally found associated with internal membranes. Another protein from this same family of yeast transporters, the multidrug transporter Pdr5, is also ubiquitinated prior to endocytosis and degradation in the vacuole, suggesting that ubiquitination may trigger the endocytosis of this short lived protein (14). Similar results have been reported for the human fibroblast growth factor receptor (15). Moreover, many other plasma membrane receptor proteins are found as ubiquitin conjugates.

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including the lymphocyte homing receptor, the platelet-derived growth factor receptor, the c-Kit receptor, and the mammalian immunoglobulin E receptor (16–19).

In this study, we used molecular genetic analysis to explore the role of ubiquitin in the glucose-induced inactivation of the maltose permease. Our results indicate that loss of free ubiquitin, via a DOA4 null mutation, impairs the glucose-induced proteolysis of maltose permease and that the effects of the doa4Δ null mutation can be suppressed by the overexpression of ubiquitin. We demonstrate that the maltose permease exists as a ubiquitinated species and that the amount of this ubiquitinated species increases dramatically upon the addition of glucose to maltose fermenting cells. Rsp5/Npi1 ubiquitin-proteasomal degradation pathway by cleaving and recycling ubiquitin from substrate remnants still bound to protease (24). Although Doa4p is only one of several species of ubiquitin hydrolase enzymes found in Saccharomyces, loss of the DOA4 gene product significantly decreases the rate of ubiquitin recycling and severely decreases levels of available ubiquitin. We used a doa4Δ null mutant strain to explore the dependence on ubiquitin-glucose-induced proteolysis of maltose permease.

MATERIALS AND METHODS

Strains and Plasmids—The Saccharomyces cerevisiae strains used in this study and their relevant genotypes are listed in Table I. Plasmid pDOA4–8 carries the wild-type allele of DOA4. Plasmid YEpl6 (pCUP1-Ub) contains UBI4 encoding ubiquitin expressed from the copper-inducible CUP1 promoter, and Yepl105 (pCUP1-mycUb) contains a myc-tagged ubiquitin allele also expressed from the CUP1 promoter (20, 21). These plasmids were obtained from Mark Hochstrasser (University of Chicago). Plasmid pUN70 serves as a YC vector control (22) as does plasmid yATAg200 (pCUP1-vector), which contains a CUP1 promoter without any fused gene sequence.

Plasmids pRS416-MAL61/HA, pUN70-MAL61/HA, pRS415-MAL61/HA, and pUN30-MAL61/HA all carry the HA-tagged maltose permease under the control of its native promoter (22, 23). Plasmids pUN60-MAL63, pUN30-MAL63, and cYCP60-MAL63 all carry the MAL63 genes, required in many strains for maltose-induced expression of the MAL structural genes.

Plasmid pADH1-MAL61 expressing the MAL61/HA gene from the constitutive ADH1 promoter was constructed as follows. Using in vitro mutagenesis, an XhoI site was introduced into pUN30-MAL61/HA 12 base pairs upstream of the start codon of the permease gene. The promoter sequence of this gene was removed by digestion with XhoI and SacI and replaced with the 400-base pair ADH1 promoter, amplified from plasmid pGAD424 (CLONTECH Inc., Palo Alto, CA) by polymerase chain reaction.

Strain Construction—Strain CMY1025 is a maltose fermenting leucine’ haploid segregant from a diploid obtained by mating strains CMY1001 and PMY270, which carries a doa4Δ::LEU2 disruption (24). Southern analysis using MAL61-specific probes revealed the presence of two maltose permease genes, one at the MAL1 locus (MAL61/HA, derived from CMY1001) and a second (MAL31, derived from PMY270) mapping to the partially functional MAL3 locus encoding MAL31 (maltose permease) and MAL29 (maltase) (25).

Inactivation Assay—The standard inactivation assay protocol was used as described previously (1). Unless otherwise indicated, cells were grown at 30 °C to early log phase (A600 0.1–0.3) in YP (rich) or SM (selection) medium containing 2% maltose, harvested by filtration with cellulose filters, and resuspended in nitrogen starvation medium (1.74 g/liter of yeast nitrogen base without amino acids and ammonium sulfate plus 2% (w/v) carbon source, usually glucose. At selected time intervals, cells were harvested by filtration for Western analysis and maltose transport assays. All values depicted in this study are the average of at least two experiments and were carried out in duplicate. Variation was less than 15%. Growth dilution was calculated as the A600 at time 0 divided by the A600 at time x. Western Analysis and Quantitation of Relative Protein Levels—Cells were harvested, and total protein extracts were prepared by the methods described previously (1, 28). Equal amounts of total protein are loaded per well for comparison of time courses or relative protein levels. SDS-polyacrylamide gel electrophoresis analysis and detection were carried out for the HA-tagged Mal61 maltose permease (1). The intensity of the band was quantitated by scanning films with a BeckmanDU640 spectrophotometer, and relative Mal61/HA protein levels were determined by comparison of the area under the curve. Western blots were done in duplicate on all samples for duplicate experimental cultures, and densitometer quantitation of the relative protein levels was carried out twice for each sample lane (1).

RESULTS

Ubiquitin Is Required for Glucose-induced Proteolysis of Maltose Permease—The yeast DOA4 gene encodes a ubiquitin hydrolase enzyme that functions late in the proteasomal degradation pathway by cleaving and recycling ubiquitin from substrate remnants still bound to protease (24). Although Doa4p is only one of several species of ubiquitin hydrolase enzymes found in Saccharomyces, loss of the DOA4 gene product significantly decreases the rate of ubiquitin recycling and severely decreases levels of available ubiquitin. We used a doa4Δ null mutant strain to explore the dependence on ubiquitin-glucose-induced proteolysis of maltose permease.

Glucose-induced inactivation of maltose permease was characterized in the doa4Δ null strain, CMY1025, and as a control in strain CMY1025 carrying the wild-type DOA4 gene on a CEN plasmid. As is evident from Fig. 1 (top two panels), the doa4Δ mutant strain exhibits a dramatically decreased rate of glucose-induced proteolysis of Mal61/HA permease. The extent that the loss of maltose permease protein parallels the growth of the culture (growth dilution). In comparison, in the doa4Δ strain, maltose permease protein is degraded more rapidly than can be expected from growth alone. Table II indicates that the steady state rate of malose transport in the doa4Δ strain is slightly higher (37%) than that of a strain expressing the wild-type DOA4 gene, which is consistent with the decrease in maltose permease turnover. Interestingly, despite the apparent lack of glucose-induced proteolysis of maltose permease in the doa4Δ strain, glucose stimulates a decrease in maltose transport activity, indicating that the inhibition of transport

### Table I

S. cerevisiae strains used in this study

| Strain          | Genotype                          | Source |
|-----------------|-----------------------------------|--------|
| CMY1001         | MATa MAL61/HA MAL12 MAL13 GAL leu2  | Ref. 1 |
|                 | uro3–52 lys2–801 ade2–101 trp1–83 his3–  |        |
|                 | Δ200 DOA4                          |        |
| CMY1004         | end3-ts (isogenic to CMY1001)     | Ref. 1 |
| PMY270          | MATa doa4Δ::LEU2 his3::Δ200 leu2–3,112 | P. McGraw |
|                 | puro3–52 lys2–801 trp1–1 MAL11 MAL32 |        |
| CMY1025         | doa4Δ::LEU2 his3::Δ200 uro3–32 lys2–801 | This study |
|                 | trp1 MAL61/HA MAL12 MAL13 MAL31    |        |
|                 | MAL32                             |        |
| 23346c          | MATa uro3 NPI1                     | Ref. 6 |
| 27038a          | MATa uro3 npi1                     | Ref. 6 |
| MGG15           | MATa calc34-2ts uro3–52 his3–Δ200  | Ref. 37|
| MHY501          | HIS3::Δ200 leu2–3,112 lyr2–801 trp1–1 | Ref. 34|
| MHY498          | ubc4–Δ1::HIS3 (isogenic to MHY 501)| Ref. 34|
| MHY499          | ubc5–Δ1::LEU2 (isogenic to MHY 501)| Ref. 34|
| MHY509          | ubc1–Δ1::HIS3 (isogenic to MHY 501)| Ref. 34|
| MHY508          | ubc4–Δ1::HIS3 ubc5–Δ1::LEU2 (isogenic to MHY 501) | Ref. 34 |
| MHY519          | ubc1–Δ1::URA3 ubc4–Δ1::HIS3 (isogenic to MHY 501) | Ref. 34 |
| FM394           | MATa his3::Δ200 leu2–3,112 uro3–52 lys2–801 trp1–1 | Ref. 45 |
| FM395           | his3::Δ200 leu2–3,112 uro3–52 lys2–801 trp1–1 | Ref. 45 |
|                 | ubc9::TRP1 leu2::ubc9Pro-Ser::LEU2  |        |

1 The abbreviations used are: E2, ubiquitin carrier protein; HA, hemagglutinin.
Effects of a doa4Δ null mutation on glucose-induced inactivation of maltose permease. Strain CMY1025 (doa4Δ) was transformed with plasmid pDOA4 carrying the DOA4 gene or plasmid pUN70 as a vector control. Transformants were grown in selective medium plus 2% maltose and harvested, and the standard inactivation assay was performed (as described under “Materials and Methods”). Plasmid pCUP1-Ub (yEP96), carrying a c-myc-tagged allele of UBI4 expressed from the copper-inducible CUP1 promoter (21) or the vector control plasmid pCUP1-vector (yATAG200) lacking the UBI4 insert were introduced into strain CMY1025. Transformants were grown to early log phase in selective medium plus 2% maltose, incubated for 4 h with 0.1 mM copper sulfate, harvested, and transferred to nitrogen starvation medium plus 2% glucose. At the indicated times, the A600 was determined, and aliquots of culture were removed for maltose transport assays and the preparation of total protein extracts for Western analysis of Mal61/HA protein levels as described under “Materials and Methods.” Representative Western blots are shown, but the quantitation data used in the graph was obtained from the average of at least two experimental cultures with samples each run on duplicate gels, and scanned twice. The relative levels of Mal61/HA protein (●) and maltose permease transport activity (○) compared with the zero time sample are plotted along with the growth dilution (■). Growth dilution represents the growth of the culture during the course of the experiment and is calculated as the A600 at time 0 divided by the A600 at time x.
activity occurs by a process that is independent of ubiquitin availability.

In order to test the possibility that the ubiquitin deficiency in the doa4Δ strain is responsible for the decreased rate of glucose-induced proteolysis of maltose permease, we determined whether overexpression of ubiquitin could overcome the loss of active ubiquitin recycling. Plasmid pYEP96 (pCUP1-Ub), carrying the ubiquitin gene UBI4 fused to the copper-responsive promoter from CUP1, was introduced into the doa4Δ mutant strain CMY1025 (20). The standard inactivation assay was carried out, except 0.1 mM copper sulfate was added to the culture medium 4 h prior to the transfer to glucose and the initiation of the inactivation assay (29). As can be seen in Fig. 1 (bottom two panels), overexpression of ubiquitin in the doa4Δ (pCUP1-Ub) strain suppresses the loss of DOA4, restoring a more rapid rate of glucose-induced proteolysis of maltose permease than that observed in the doa4Δ (pCUP1-vector) control strain. Table II shows that the steady state transport rate of maltose in a ubiquitin-overexpressing doa4Δ strain, CMY1025 (pCUP1-Ub), is half that seen in the control strain, CMY1025 (pCUP1-vector). This is also consistent with the proposal that ubiquitin is required for rapid turnover of maltose permease.

**Table II**

| Strain | Relevant genotype | Transport rate (nmol/mg (dry wt)/min) |
|--------|------------------|-------------------------------------|
| CMY1025 (pCUP1-vector) | doa4Δ::LEU2 | 1.57 |
| CMY1025 (pCUP1-Ub) | doa4Δ::LEU2 | 0.80 |
| CMY1025 (pUN70) | doa4Δ::LEU2 | 1.27 |
| CMY1025 (pDOA4–8) | doa4Δ::LEU2 | 1.26 |
| 23346c (pRS416MAL61/HA) | RSP5/NPI1 | 3.03 |
| 27038a (pRS416MAL61/HA) | rps5/npi1 | 3.62 |

* Determined following a 4-h incubation in 0.1 mM copper sulfate.

In order to confirm that this higher molecular weight species is indeed a ubiquitinated maltose permease, we utilized the modest molecular weight difference produced by conjugation to c-myc-tagged ubiquitin versus untaged ubiquitin. The difference in size between the product encoded by these two alleles, approximately 1.3–1.5 kDa, previously has been used to verify ubiquitinated substrates such as the Malt2 transcriptional regulator (8, 9, 14, 21).

Strain CMY1004 (pCUP1-mycUb) expressing the Cu²⁺-inducible c-myc-tagged ubiquitin and strain CMY1004 (pCUP1- Ub) expressing the Cu²⁺-inducible untaged ubiquitin were both grown at room temperature to early log phase, and 0.1 mM CuSO₄ was added to the growth media. After 4 h, the cultures were moved to 37 °C for 1 h prior to the addition of 2% glucose. After the glucose was added, cells were allowed to continue growing at 37 °C for ½ h and then harvested for Western analysis of Mal61/HAp.

As is seen in Fig. 3, both strains carrying the different alleles of ubiquitin exhibit the higher molecular weight species of Mal61/HAp protein described above, but in the strain carrying the c-myc-tagged allele of ubiquitin, this species is slightly larger than the corresponding species in the strain carrying the untaged allele of ubiquitin. The c-myc-tagged ubiquitin-maltose permease conjugate also appears to be significantly more abundant than the corresponding untaged species, consistent with reports that the c-myc ubiquitin-conjugated proteins are more stable (21). These results confirm that this higher molecular weight species is indeed a ubiquitinated maltose permease.

**RSP5/NPI1 Plays a Role in the Glucose-induced Proteolysis of Maltose Permease—** RSP5/NPI1 encodes a ubiquitin-protein ligase that participates in the induced degradation of at least two permeases; the general amino acid permease, encoded by GAP1, and the uracil permease, encoded by FUR4 (6). An rps5/npi1 mutant allele was isolated based on its nitrogen repression-resistant phenotype (31) and has since been shown...
to be a Ty1 insertion into the RSP5 promoter (6). Strains carrying this mutant allele synthesize significantly reduced levels of this essential protein that are adequate for cell growth but insufficient for ammonium ion-induced proteolysis of Gap1 permease (32).

To characterize the role of RSP5/NPI1 in glucose-induced proteolysis of maltose permease, a plasmid-borne epitope-tagged maltose permease gene, MAL61/HA, was introduced into isogenic RSP5/NPI1 and rsp5/npi1 strains. The results of inactivation assays carried out on these two strains are shown in Fig. 4. Rapid glucose-induced proteolysis of the Mal61/HA maltose permease is seen in the RSP5/NPI1 strain, but this rate is dramatically reduced (approximately 5–10-fold) in the rsp5/npi1 mutant strain. As Table II shows, the rsp5/npi1 strain also expresses slightly higher maltose transport activity in maltose-grown cells, which is consistent with a decrease in rapid maltose permease turnover. These results indicate that RSP5/NPI1 plays an important role in the glucose-induced proteolysis of maltose permease.

Role of E2 Encoded by UBC1, UBC4, and UBC5 in Glucose-induced Inactivation of Maltose Permease—The E2 enzymes catalyze the covalent attachment of ubiquitin to substrate proteins. At least 13 UBC genes have been identified in yeast, and they function in many diverse aspects of cellular biology including DNA repair, cell cycle, protein degradation, and peroxisome biogenesis (reviewed in Refs. 13 and 33). UBC1, UBC4, and UBC5 are implicated in the bulk degradation of short lived and abnormal proteins and are implicated in the degradation of the transcription factor Mata2, the a-factor receptor Ste2p, and the a-factor receptor Ste3p (9, 10, 13, 33). UBC6 and UBC7 also function in the degradation of the Mata2 repressor through a pathway that is distinct from that of UBC4 and UBC5 (34). The functions of several E2 enzymes, like Ubc4/5 and Ubc6/7, overlap, since the most dramatic results are seen only in double mutants. Ubiquitination, in some cases via the Ubc4/5 E2 enzymes, has been implicated in the signaling of endocytosis and degradation of many yeast membrane proteins including Ste2p, Gap1p, Fur4p, Ste3p, and Pdr5p (6, 9, 10, 11, 14, 33). We explored the role of these E2 enzymes in glucose-induced inactivation of maltose permease.

A series of isogenic ubc mutant strains, carrying ubc1Δ, ubc4Δ, or ubc5Δ alleles, were transformed with CEN plasmids carrying the MAL-activator gene MAL63 and the HA-tagged
maltose permease gene MAL61/HA, and the half-life of maltose permease was determined using the standard inactivation assay. The single mutant strains showed no significant change in half-life of the permease compared with the parental strain: UBC1 UBC4 UBC5 (0.4 h), ubc1Δ (0.5 h), ubc4Δ (0.5 h), and ubc5Δ (0.3 h). Rather unexpectedly, double mutant strains containing either the ubc1Δ ubc4Δ or ubc4Δ ubc5Δ double null mutation expressed no detectable maltose permease protein, suggesting a possible role for these gene functions in maltose permease expression either at the transcription or post-translational level.

Table III compares the level of maltose transport activity, maltase activity, relative maltose permease protein levels, and maltose fermentation in these maltose-grown ubc mutant strains transformed with MAL61/HA (columns 3–6). The single mutant strains exhibit between 20% (ubc1Δ) and 35% (ubc5Δ) of the level of maltose permease protein expressed by the UBC1,4,5 parental strain, and these levels also are paralleled by a decrease in maltose transport activity. All single mutant strains fermented maltose in 1 day. The ubc1Δ ubc4Δ and ubc4Δ ubc5Δ double mutant strains expressed only 5–10% of the parental levels of maltose transport activity and took 7–9 days to ferment maltose. Interestingly, maltase activity in these strains does not correlate with the levels of maltose permease. The maltase gene is divergently transcribed from a shared promoter with the maltose permease gene, and both genes are coordinately regulated. No significant variation from the parental strain is seen in single deletion mutant strains, and maltase levels are actually increased in the ubc1Δ ubc4Δ double mutant about 2-fold above that of the parental strain. Only in the ubc4Δ ubc5Δ strain were the levels of maltase significantly decreased, but not to the same extent of maltose transport levels. Expression from MAL62 promoter-LacZ (MAL62 encodes maltase) and MAL61 promoter-LacZ reporter constructs also were tested in these ubc strains in order to monitor the effects of these mutations on transcription initiation. Results (data not shown) were consistent with the expression levels of maltase, suggesting that the effect of loss of Ubc1/4 or Ubc4/5 ubiquitin-conjugating enzymes is not at the level of transcription initiation.

To investigate this further, Mal61/HAp was expressed in this same series of strains except from the constitutive ADH1 promoter (results in Table III, column 7). Again, reduced levels of Mal61/HA protein are detected in all of the mutant strains but particularly in the ubc1Δ, ubc1Δ ubc4Δ, and ubc4Δ ubc5Δ strains. Thus, these results also suggest a role for Ubc1–4, and -5 in a post-translational process required for maltose permease expression.

In an effort to determine the rate of glucose-induced proteolysis of maltose permease in the ubc1Δ ubc4Δ and ubc4Δ ubc5Δ double mutant strains, we used the strains that express MAL61/HA from the ADH1 promoter. These strains, grown on glycerol/lactate as the carbon source, accumulate levels of maltose permease adequate (approximately 25% of wild type; data not shown) to allow us to carry out an inactivation assay. The parental strain and the ubc1Δ ubc4Δ and ubc4Δ ubc5Δ double mutant strains carrying plasmid pADH1-MAL61/HA were grown to early log phase in selective medium plus 3% glycerol, 2% lactate. A standard inactivation assay was carried out with the exception that 12.5 μg/ml cyclohexamide was added to the 2% glucose inactivation medium at time 0 to stop the continued synthesis of maltose permease. The results shown in Fig. 5 do not demonstrate a significant effect on the rate of proteolysis in the mutant strains. The half-life in both the parental strain and the ubc1Δ ubc4Δ strain is about 0.5 h, and in the ubc4Δ ubc5Δ double mutant it is only increased about 2-fold.

CDC34/UBC3 and UBC9 Do Not Function in the Glucose-induced Proteolysis of Maltose Permease—Jiang et al. (35) identified two glucose-sensing/signaling pathways that stimulate glucose-induced inactivation of maltose permease. Pathway 1 transmits a Rgt2p-dependent glucose signal and utilizes Grr1p as a downstream component. Saccharomyces Grr1p is an F-box protein (36). F-box proteins are substrate-specific adaptor proteins that recruit various substrates to a core ubiquitination complex referred to as the SCF complex because of the presence in the complex of Skp1, Cdc53p, and the F-box protein (36). SCF complexes, along with particular Ubc enzymes, participate in the coordination of many cellular processes through targeted degradation of specific proteins. The yeast CDC34 (UBC3) gene encodes an essential ubiquitin-conjugating enzyme, and is found in the Cdc4p-containing SCF complex required for Sic1p degradation and G1/S transition, DNA replication, and spindle pole body separation (37–41).

The Ubc enzyme that functions with the Grr1p-containing SCF complex has not been identified (42). Given the involvement of Grr1p in pathway 1, we wished to test the possibility that Cdc34p also is involved in glucose-induced inactivation of maltose permease. For this purpose, a strain carrying the cdc34Δ–2ts mutant allele (37) was transformed with plasmids carrying the MAL63 MAL-activator and MAL61/HA. Cells were grown in selective medium plus 2% maltose to early log phase at the permissive temperature, 23 °C, at which time the temperature was raised to 37 °C for 2 h prior to the start of the inactivation assay, which was carried out at 37 °C. The control culture was maintained at 23 °C throughout the experiment. No significant effect is observed on the kinetics of maltose

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**Table III**

| Strain | Relevant genotype | Fermentation rate | Maltase activity | Transport rate | Relative Mal61/Hap levels |
|--------|------------------|------------------|-----------------|---------------|--------------------------|
|        |                  |      |       |                 | MAL61/HA | ADH1proMAL61/HA |
| MHY501 | UBC1 UBC4 UBC5   | 1    | 477   | 3.54           | 100     | 100                |
| MHY509 | ubc1::HIS3       | 23   | 559   | 0.68           | 26      | 17                 |
| MHY498 | ubc4::HIS3       | 1    | 419   | 0.84           | 21      | 56                 |
| MHY499 | ubc5a::LEU2      | 1    | 303   | 2.22           | 23      | 71                 |
| MHY519 | ubc1a::URA3 ubc4Δ::HIS3 | 7–9 | 1144  | 0.34           | ND*     | 9                   |
| MHY508 | ubc4::HIS3 ubc5Δ::LEU2 | 7–9 | 130   | 0.18           | ND      | 40                  |

* p-Nitrophenyl-α-D-glucopyranoside.

b ND, not detected.
permease inactivation and proteolysis (Fig. 6). Maltose transport activity of this strain grown at the permissive temperature is 4.39 nmol/mg (dry weight)/min and is essentially unchanged (4.43 nmol/mg (dry weight)/min) after 2 h at the nonpermissive temperature.

Recent evidence indicates that the ubiquitin-like protein Smt3p of Saccharomyces and SUMO-1, its mammalian homolog, are covalently attached to other proteins posttranslationally (43). UBC9, an essential yeast gene, is required for Smt3 conjugation in vivo (43). This suggests that UBC9 functions as an E2-like protein in a Smt3p conjugation pathway analogous to ubiquitin-conjugating enzymes. Tir1p, an Arabidopsis thaliana F-box-containing homologue of Grr1p is a downstream component in the jasmonate-sensing pathway and functions in the conjugation of Rub1p, another ubiquitin-like homologue, to target proteins (44). For these reasons, we decided to test the possibility that Ubc9p is involved in glucose-induced proteolysis of maltose permease.

Isogenic UBC9 and ubc9-ts strains were transformed with plasmids containing the MAL63 MAL-activator and MAL61/HA genes (45). The resulting transformants were grown to early log phase at room temperature in selective medium plus 2% maltose and equilibrated at 37 °C for 2 h. Standard inactivation assays were carried out at the nonpermissive temperature of 37 °C. The results shown in Fig. 6 demonstrate that loss of Ubc9p function has no significant effect on glucose-induced inactivation of maltose permease. The rate of loss of maltose transport activity and proteolysis of maltose permease protein are comparable in both strains.

Steady state maltose transport activity following assays at 37 °C in both strains also was comparable at 2.82 and 2.81 nmol/mg (dry weight)/min for the UBC9 and ubc9-ts strains, respectively.

**DISCUSSION**

The results described above strongly suggest that ubiquitination of maltose permease is an essential early step in the rapid glucose-induced proteolysis of maltose permease. Several lines of evidence support this conclusion. First, by utilizing a c-myc-tagged allele of ubiquitin that produces more stable and more abundant protein-conjugates than its untagged ubiquitin counterpart (21), we demonstrated the accumulation of a higher molecular weight species of MAL61/HAp in strains that express this tagged ubiquitin allele (Fig. 2). The higher molecular weight species of MAL61/HAp is a ubiquitin-conjugated maltose permease based on a size shift observed when different molecular weight ubiquitin alleles were utilized (Fig. 2). This technique has been used previously to demonstrate ubiquitin conjugates of the Matα2 transcriptional activator, the ABC transporter Ste6, and the Pdr5 multidrug transporter (11, 14, 21). We also show that the abundance of this ubiquitinated species of Mal61/HAp dramatically increases upon the addition of glucose to the growth medium (Fig. 3).

Second, the rate of glucose-induced proteolysis of Mal61p is slowed in a doa4Δ strain (Fig. 1). DOA4/UBP4 encodes a ubiquitin-hydrolase that is localized to the 26 S proteasome and appears to play an important role in maintenance of free unconjugated ubiquitin pools (33). Loss of Doa4p affects a variety of physiological functions, suggesting decreased levels of available ubiquitin, at least in certain compartments (24). The Doa4p deficiency in glucose-stimulated Mal61p turnover can be overcome by overproduction of ubiquitin (Fig. 1), indicating that the slow rate of proteolysis in this strain is directly attributable to the depletion of available ubiquitin.

Third, reduced levels of Rep5p/Npi1p dramatically decrease the rapid rate of glucose-induced proteolysis of maltose permease (Fig. 4). The ubiquitin-protein ligase encoded by RSP5/NPI1 has previously been shown to be necessary for the induced degradation of the general amino acid and uracil permeases (6, 8) as well as for the internalization of Ste2p.² RSP5/NPI1 may be associated with the membrane at certain

² R. Dunn and L. Hicke, personal communication.
times (6), a fact that is consistent with its role in ubiquitination of maltose permease at the plasma membrane.

The following findings are also consistent with the conclusion that ubiquitination of maltose permease marks this protein for degradation. Ubiquitin-conjugated maltose permease appears to be in relatively low abundance compared with the...
level of unubiquitinated maltose permease. This is similar to results demonstrated for the α-factor receptor and the yeast uracil permease (8, 10). Additionally, evidence exists to support the proposal that conjugation of ubiquitin to maltose permease takes place at the plasma membrane prior to endocytosis. The MAL61/HAP-ubiquitin conjugate accumulates in an end3-Δs strain that is deficient for endocytosis at the nonpermissive temperature, and this strain even accumulates MAL61/HAP at the membrane at the permissive temperature (1).

The ubiquitin-conjugated maltose permease species observed in Figs. 2 and 3 has an apparent molecular mass approximately 7–8 kDa higher than the nonubiquitinated maltose permease when viewed on SDS-polyacrylamide gels. This is likely to correspond to a monoubiquitinated form. Studies of a truncated allele of Ste2p demonstrated a ligand-induced monoubiquitination sufficient for internalization and vacuolar degradation (10, 46). These authors suggested that a single ubiquitin moiety, as opposed to the polyubiquitin chains preferred by the proteasome, is recognized by the endocytotic machinery. Studies using the Lys to Arg mutant alleles of ubiquitin unable to form polyubiquitin chains do not indicate a decreased efficiency of degradation (46). This is in contrast with results reported for the uracil permease. The target sites in both Ste2p and Fur4p have been identified, and both appear to require phosphorylation of key serine residues prior to ubiquitination. Our published results demonstrated that Mal61/HAP is phosphorylated and that the level of phosphorylation is increased by glucose (1). Therefore, we are also exploring the role of phosphorylation in the glucose-induced proteolysis of maltose permease and are attempting to identify the kinase(s) involved.

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