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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, I., 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 *ubc1*Δ *ubc4*Δ, *ubc4*Δ *ubc5*Δ, *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 (Fur4p) 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, α-factor receptor, is required for its ligand-stimulated endocytosis and vacuolar proteolysis (10). *END4* mutations inhibit endocytosis of α-factor and stimulate the appearance of multiubiquitinated 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 endocytotic functions, this protein is generally found associated with internal membranes. Another protein from this same family of yeast transporters, the multidrug transporter Pdr5, also is 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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TABLE I
S. cerevisiae strains used in this study

Strain	Genotype	Source
CMY1001	*MAT***a** *MAL61/HA MAL12 MAL13 GAL leu2 ura3–52 lys2–801 ade2–101 trp1-Δ63 his3-Δ200 DOA4*	Ref. 1
CMY1004	*end3-ts* (isogenic to CMY1001)	Ref. 1
PMY270	*MATα doa4Δ1::LEU2 his3-Δ200 leu2–3,112 ura3–52 lys2–801 trp1–1 MAL31 MAL32*	P. McGraw
CMY1025	*doa4Δ1::LEU2 his3-Δ200 ura3–52 lys2–801 trp1 MAL61/HA MAL12 MAL13 MAL31 MAL32*	This study
23346c	*MAT***a** *ura3 NPI1*	Ref. 6
27038a	*MAT***a** *ura3 npil*	Ref. 6
MGG15	*MAT***a** *cdc34–2ts ura3–52 his3-Δ200*	Ref. 37
MHY501	*MAT***a** *his3-Δ200 leu2–3,112 lys2–801 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	*MAT***a** *his3-Δ200 leu2–3,112 ura3–52 lys2–801 trp1–1(am)*	Ref. 45
FM395	*MATα his3-Δ200 leu2–3,112 ura3–52 lys2–801 trp1–1(am) ubc9Δ::TRP1 leu2::ubc9Pro-Ser::LEU2*	Ref. 45

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 *doa4*Δ 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-protein ligase is implicated in the proteolysis of maltose permease. Mutations in *UBC1*, *UBC4*, and *UBC5* encoding ubiquitin conjugation enzymes UBC (ubiquitin carrier proteins; E2[1] enzymes), in combination were found to dramatically decrease the level of maltose permease expressed, apparently by affecting a post-transcriptional process but not glucose-induced proteolysis.

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 YEp96 (pCUP1-Ub) contains *UBI4* encoding ubiquitin expressed from the copper-inducible *CUP1* promoter, and YEp105 (pCUP1-mycUb) contains a c-*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 yCP 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 pUN90-MAL63, pUN30-MAL63, and YCP50-MAL63 all carry the *MAL63 MAL*-activator gene, 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 *Xho*I site was introduced into pUN30-MAL61/HA 12 base pairs upstream of the start codon of the permease gene *MAL61/HA*. The promoter sequence of this gene was removed by digestion with *Xho*I and *Sac*I 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 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 *MAL32* (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 (A_{600} 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 A_{600} at time 0 divided by the A_{600} at time x.

Maltose Transport Assay and Maltase Assay—Maltose transport was measured by the uptake of 1 mM [^{14}C]maltose as described previously (1, 26). Transport assays were done in duplicate on at least duplicate cultures. Maltase activity was determined as described previously (27). Maltase activity describes the nmol of *p*-nitrophenol α-D-glucopyranoside cleaved per mg of protein per min as measured spectrophotochemically.

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 signal was quantitated by scanning films with a Beckman DU640 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 *doa4*Δ null mutant strain to explore the dependence on ubiquitin of glucose-induced proteolyis of maltose permease.

Glucose-induced inactivation of maltose permease was characterized in the *doa4*Δ 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 *doa4* mutant strain exhibits a dramatically decreased rate of glucose-induced proteolysis of Mal61/HA permease, to the extent that the loss of maltose permease protein parallels the growth of the culture (growth dilution). In comparison, in the *DOA4* strain, maltose permease protein is degraded more rapidly than can be expected from growth alone. Table II indicates that the steady state rate of maltose transport in the *doa4*Δ 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 *doa4*Δ strain, glucose stimulates a decrease in maltose transport activity, indicating that the inhibition of transport

[1] The abbreviations used are: E2, ubiquitin carrier protein; HA, hemagglutinin.

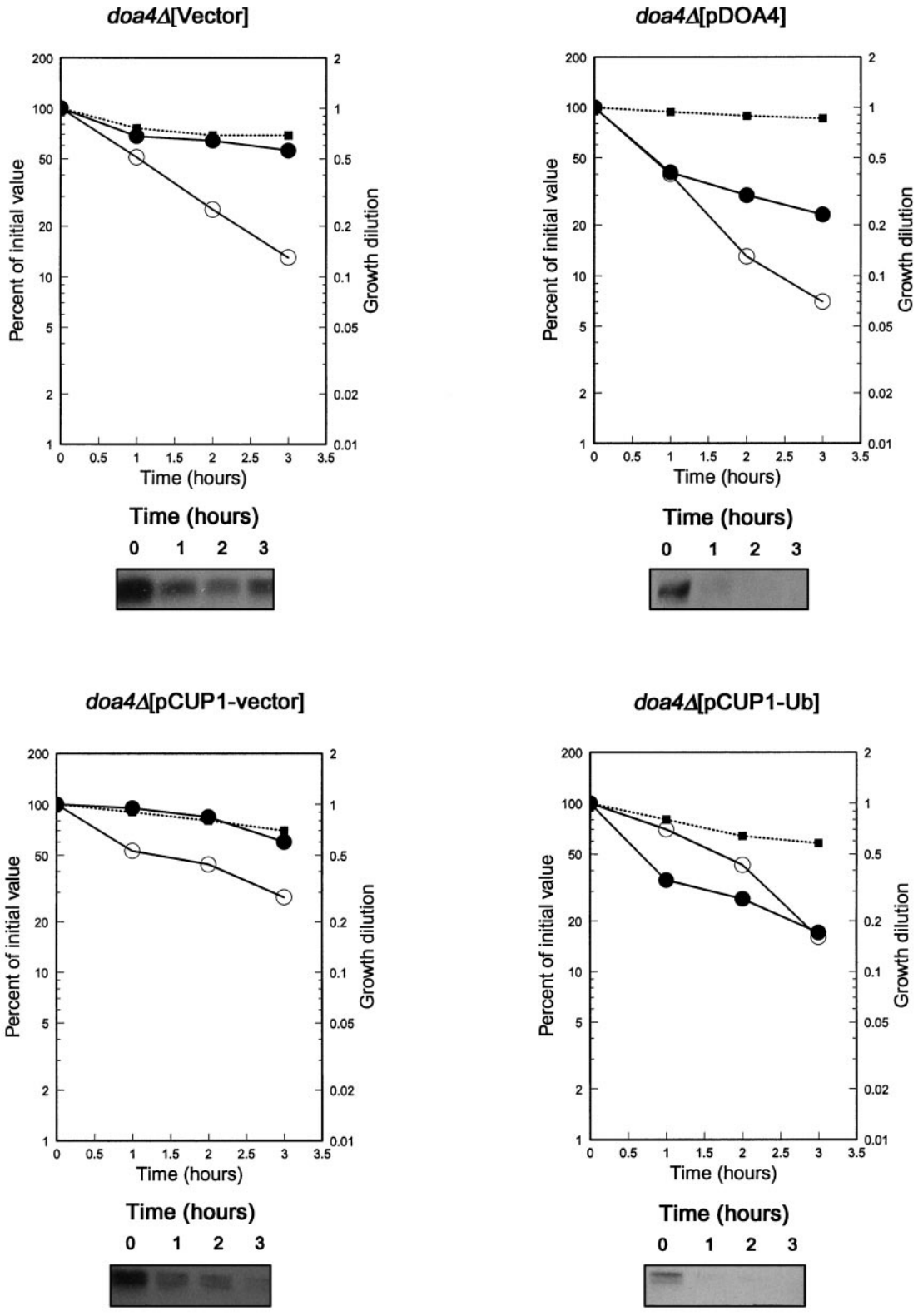

FIG. 1. **Effects of a *doa4*Δ null mutation on glucose-induced inactivation of maltose permease.** Strain CMY1025 (*doa4*Δ) was transformed with plasmid pDOA4 carrying the *DOA4* gene or plasmid pUN70 as a vector control. Transformants were grown in selective medium plus 2% maltose and harvested, and 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 A_{600} 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 A_{600} at time 0 divided by the A_{600} at time x.

TABLE II
Maltose transport rates of doa4 and rsp5/npi1 *mutant strains*

All strains were grown in rich medium with 2% maltose to early log phase at 30 °C. Maltose transport rates were determined as described under "Materials and Methods." See "Materials and Methods" for description of plasmids pCUP1-Ub (yEP96) and pCUP1-vector (yATAG200). All determinations are from two separate cultures, each assayed in duplicate. Variation is less than 15%.

Strain	Relevant genotype	Transport rate
		nmol/mg (dry wt)/min
CMY1025 (pCUP1-vector)	*doa4Δ::LEU2*	1.57[a]
CMY1025 (pCUP1-Ub)	*doa4Δ::LEU2*	0.80[a]
CMY1025 (pUN70)	*doa4Δ::LEU2*	1.72
CMY1025 (pDOA4–8)	*doa4Δ::LEU2*	1.26
23346c (pRS416MAL61/HA)	*RSP5/NPI1*	3.03
27038a (pRS416MAL61/HA)	*rsp5/npi1*	3.62

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

activity occurs by a process that is independent of ubiquitin availability.

In order to test the possibility that the ubiquitin deficiency in the *doa4*Δ 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 yEP96 (pCUP1-Ub), carrying the ubiquitin gene *UBI4* fused to the copper-responsive promoter from *CUP1*, was introduced into the *doa4*Δ 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 *doa4*Δ (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 *doa4*Δ (pCUP1-vector) control strain. Table II shows that the steady state transport rate of maltose in a ubiquitin-overexpressing *doa4*Δ 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.

Maltose Permease Is Ubiquitinated in Response to Glucose—In order to determine whether maltose permease is ubiquitinated directly, we used strain CMY1004, which contains a temperature-sensitive allele of *END3* to slow down endocytosis and degradation of maltose permease and thereby enhance the levels of any putative ubiquitinated species (1). *END3* is an early function in the endocytosis process (30). We have shown that endocytosis and the subsequent proteolysis of Mal61/HA maltose permease are completely inhibited at the nonpermissive temperature, in *end3-ts* strains, and that, even at the permissive temperature, maltose permease protein accumulates to higher levels in the plasma membrane (1). Plasmid pCUP1-mycUb (yEP105), which encodes a c-*myc*-tagged allele of ubiquitin, was introduced into strain CMY1004.

Strain CMY1004 (pCUP1-mycUb) was grown to very early log phase (A_{600} 0.2–0.3) in selective media plus 2% maltose at room temperature, and 0.1 mM $CuSO_4$ was added to induce expression of c-*myc-Ub* (29). After a 5-h ubiquitin induction period, the culture was transferred to 37 °C to inhibit endocytosis, and then after an additional 1 h glucose was added to a final concentration of 2%. Samples were collected at time points throughout this process, and the level of Mal61/HA protein was determined by Western blotting. Published reports indicate that substrate proteins that are conjugated with a c-*myc*-tagged allele of ubiquitin are more stable than untagged ubiquitin-substrate protein conjugates and accumulate to a significantly higher level (21).

As is evident in Fig. 2, overexpression of ubiquitin during the

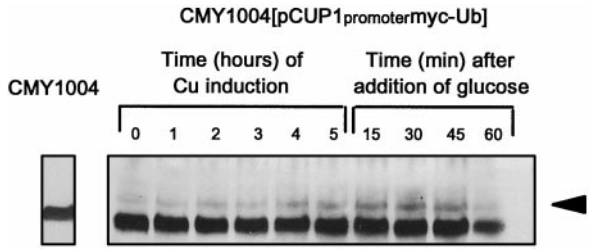

FIG. 2. **Overexpression of a c-*myc*-tagged ubiquitin allele in an *end3-ts* strain.** Strain CMY1004, containing an *end3-ts* allele, was transformed with plasmid pCUP promoter-mycUb (yEP105) encoding a c-*myc*-tagged allele of ubiquitin on a copper-inducible promoter, *CUP1*. The resulting strain was grown to early log phase at room temperature and then incubated with 0.1 mM copper sulfate for 5 h. At the 4th h of incubation, the medium was moved to 37 °C to inhibit endocytosis. After a total of 5 h of incubation, 2% glucose was added to the growth medium. At the selected time points, aliquots were collected for Western analysis as described under "Materials and Methods." The *lane* labeled *CMY1004* depicts Western analysis of that strain grown in maltose lacking the pCUP1-mycUb (yEP105) plasmid.

Cu^{2+} induction period results in the accumulation of a larger molecular weight species of Mal61/HA protein in the *end3* strain even prior to the addition of glucose. Thus, it appears that a small amount of a putative ubiquitinated species of maltose permease is present during growth on maltose. The addition of the 2% glucose to the growth medium causes an increase in the abundance of this larger molecular weight band, which peaks at about 30–45 min.

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* untagged 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 Matα2 transcriptional regulator (8, 9, 14, 21).

Strain CMY1004 (pCUP1-mycUb) expressing the Cu^{2+}-inducible c-*myc*-tagged ubiquitin and strain CMY1004 (pCUP1-Ub) expressing the Cu^{2+}-inducible untagged ubiquitin were both grown at room temperature to early log phase, and 0.1 mM $CuSO_4$ 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/HA 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 untagged allele of ubiquitin. The c-*myc*-tagged ubiquitin-maltose permease conjugate also appears to be significantly more abundant than the corresponding untagged 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. The size of this ubiquitinated species of maltose permease is increased by approximately 6–7 kDa, compared with the major species of Mal61/HAp, and is consistent with a monoubiquitinated 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 *rsp5/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.

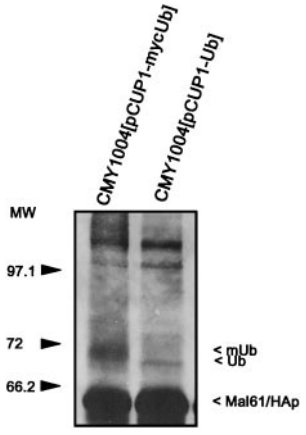

FIG. 3. **Western blot analysis of an *end3-ts* strain expressing two different molecular weight alleles of ubiquitin.** Strain CMY1004 was transformed with plasmid pCUP1-Ub (yEP96) carrying *UBI4* expressed from the *CUP1* promoter or pCUP1-mycUb (yEP105) encoding a c-*myc*-tagged allele of ubiquitin also expressed from the *CUP1* promoter. Both strains were grown to early log phase at 30 °C in selective media plus 2% maltose and then incubated in 0.1 mM copper sulfate for 4 h, the last hour at 37 °C to inhibit endocytosis. 2% glucose was added for ½ h prior to harvesting the cells for Western analysis of Mal61/HA protein.

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 Matα2, the α-factor receptor Ste2p, and the **a**-factor receptor Ste3p (9, 10, 13, 33). *UBC6* and *UBC7* also function in the degradation of the Matα2 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 *ubc1*Δ, *ubc4*Δ, or *ubc5*Δ alleles, were transformed with *CEN* plasmids carrying the *MAL*-activator gene *MAL63* and the HA-tagged

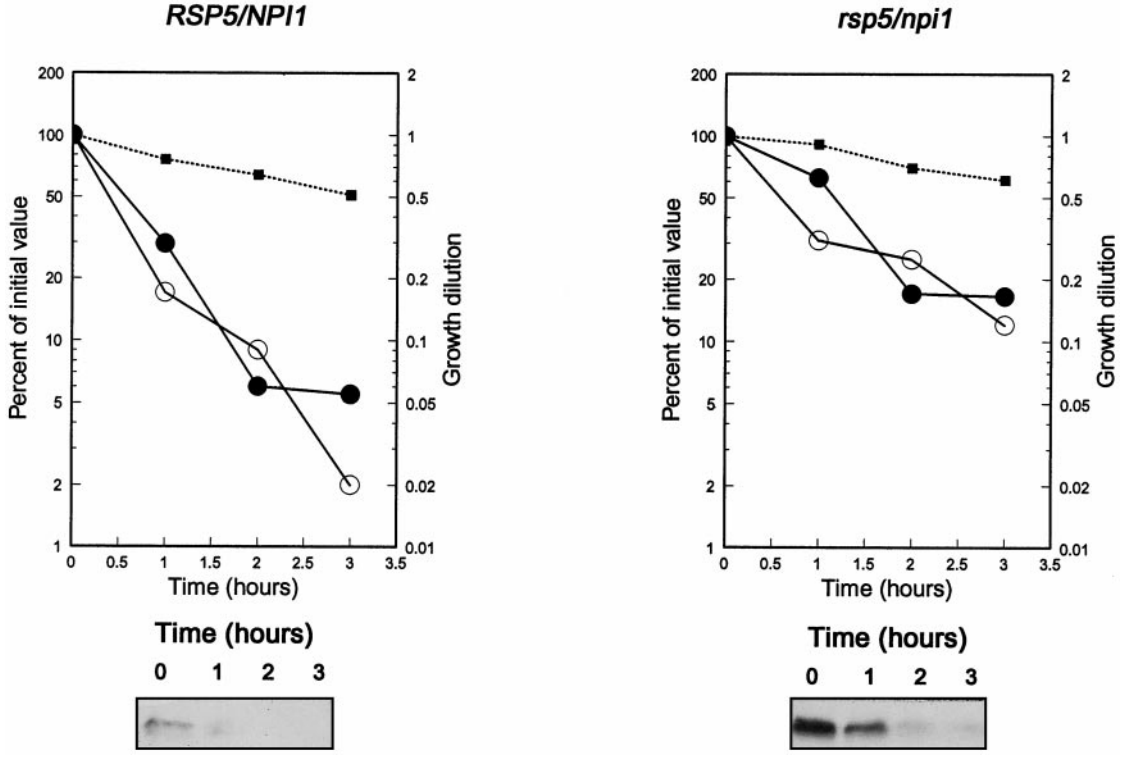

FIG. 4. **Effects of reduced expression of Rsp5/Npi1 ubiquitin-protein ligase on glucose-induced inactivation of maltose permease.** Isogenic *RSP5*/*NPI1* and *rsp5*/*npi1* strains were transformed with a plasmid expressing the *Mal61*/*HA* gene. A strain carrying the *npi1* mutation isolated by Grenson (31) was utilized that expresses significantly reduced levels of Rsp5/Npi1 protein (32). The standard inactivation assay protocol was carried out. The relative levels of Mal61/HA protein (●) and maltose transport activity (○) compared with the zero time sample are plotted along with the growth dilution (■) as described in the legend to Fig. 1.

TABLE III
Effect of ubc1, ubc4, and ubc5 mutations on MAL gene expression

Strains listed in column 1 were transformed with plasmid pUN30-MAL63 encoding the *MAL*-activator and either pRS415-MAL61/HA or pADH1-MAL61/HA carrying *MAL61/HA* expressed from the *ADH1* constitutive promoter (results in column 7). All strains were grown to early log phase at 30 °C in selective medium with 2% maltose (pRS415-Mal61/HA transformants) or 3% glycerol, 2% lactate (pADH1-MAL61/HA transformants). Maltose transport rates and maltase activity were determined as described under "Materials and Methods." Relative protein levels were determined by Western blot analysis.

Strain	Relevant genotype	Fermentation rate in maltose	Maltase activity	Transport rate	Relative Mal61/HAp levels	
					MAL61/HA	ADH1proMAL61/HA
		days	*nmol PNPG[a]/mg protein/min*	*nmol/mg (dry wt)/min*	*%*	
MHY501	*UBC1 UBC4 UBC5*	1	477	3.54	100	100
MHY509	*ubc1Δ::HIS3*	1	559	0.68	26	17
MHY498	*ubc4Δ::HIS3*	1	419	0.84	21	86
MHY499	*ubc5Δ::LEU2*	1	303	2.22	23	71
MHY519	*ubc1Δ::URA3 ubc4Δ::HIS3*	7–9	1144	0.34	ND[b]	9
MHY508	*ubc4Δ::HIS3 ubc5Δ::LEU2*	7–9	130	0.18	ND	40

[a] *p*-Nitrophenol α-D-glucopyranoside.
[b] ND, not detected.

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* (O.4 h), *ubc1*Δ (0.5 h), *ubc4*Δ (0.5 h), and *ubc5*Δ (0.3 h). Rather unexpectedly, double mutant strains containing either the *ubc1*Δ *ubc4*Δ or *ubc4*Δ *ubc5*Δ 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% (*ubc1*Δ) and 35% (*ubc5*Δ) 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 *ubc1*Δ *ubc4*Δ and *ubc4*Δ *ubc5*Δ 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 *ubc1*Δ *ubc4*Δ double mutant about 2-fold above that of the parental strain. Only in the *ubc4*Δ *ubc5*Δ 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 *ubc1*Δ, *ubc1*Δ *ubc4*Δ, and *ubc4*Δ *ubc5*Δ 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 *ubc1*Δ *ubc4*Δ and *ubc4*Δ *ubc5*Δ 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 *ubc1*Δ *ubc4*Δ and *ubc4*Δ *ubc5*Δ 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 *ubc1*Δ *ubc4*Δ strain is about 0.5 h, and in the *ubc4*Δ *ubc5*Δ 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 Sk1p, 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 G_1/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 *cdc34–2ts* 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

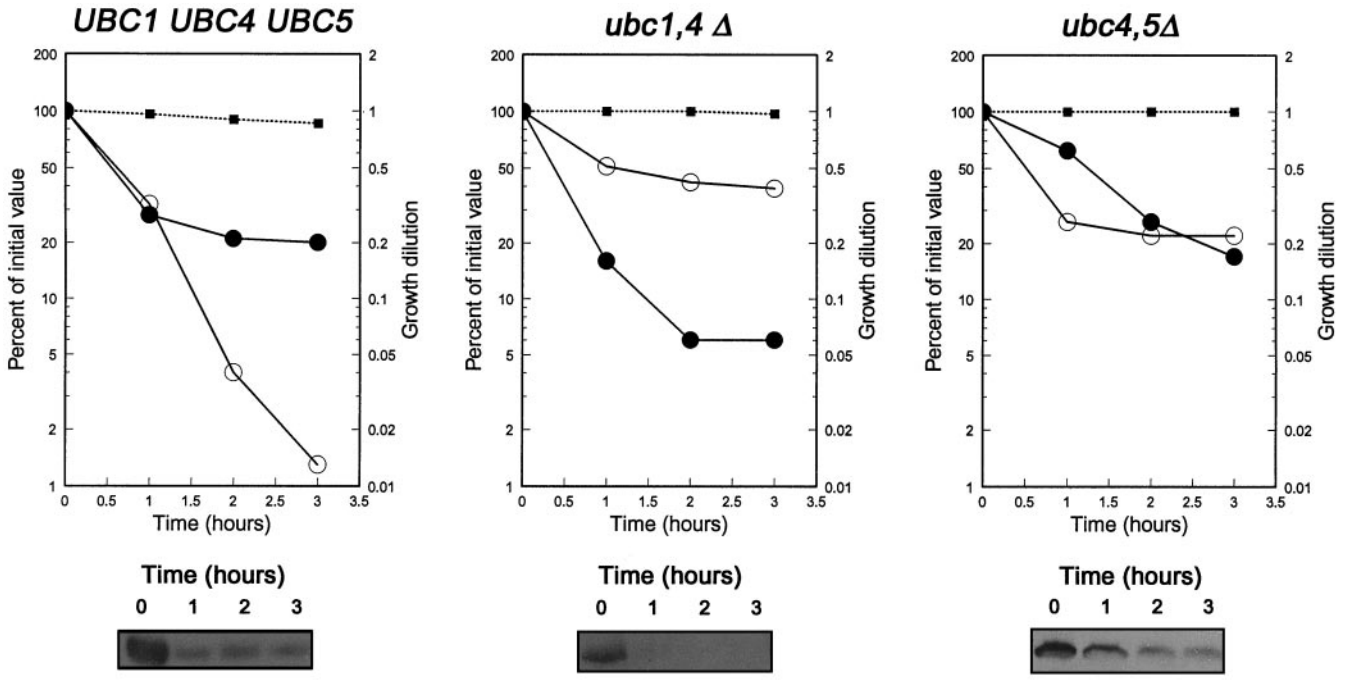

FIG. 5. **Effects of glucose-induced inactivation on *ubc1,4*Δ and *ubc4,5*Δ strains that express *MAL61/HA* on a constitutive *ADH1* promoter.** Strains were grown in selective media plus 3% glycerol, 2% lactate to early log phase. After harvesting, cells were resuspended in inactivation media supplemented with 12 μg/ml of cyclohexamide, and the standard inactivation assay was carried out as described in the legend to Fig. 1 and under "Materials and Methods." The relative levels of Mal61/HA protein (●) and maltose transport activity (○) compared with the zero time sample are plotted along with the growth dilution (■).

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 *doa4*Δ 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 Rsp5p/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.

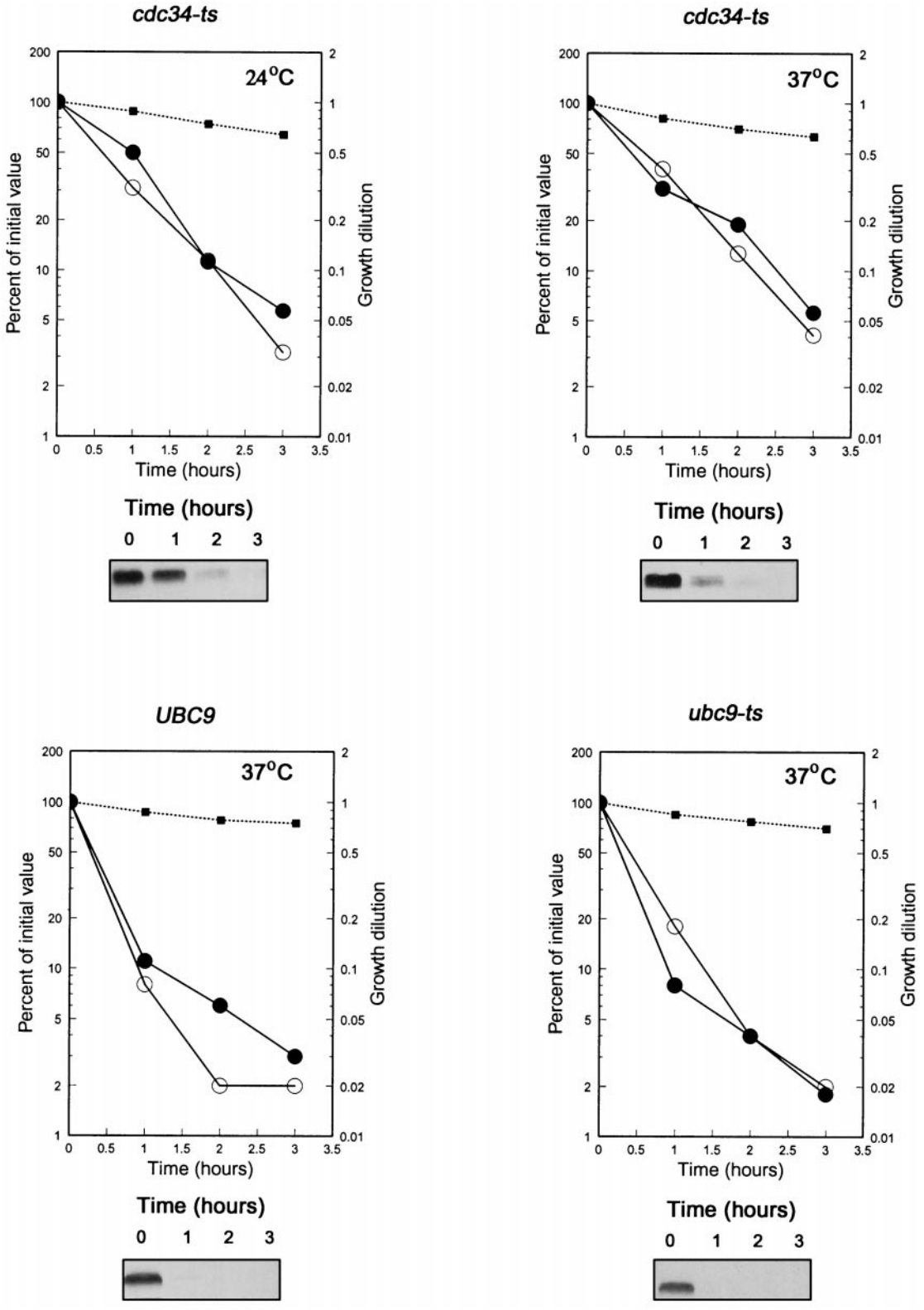

FIG. 6. **Glucose-induced inactivation of MAL61/HAp in *cdc34-ts*, and *ubc9-ts* strains.** Strain MGG15 containing the *cdc34–2ts* allele was transformed with plasmids containing the *MAL61/HA* gene and the *MAL* activator gene. Following growth to early log phase at room temperature, the standard inactivation assay protocol was carried out at the permissive temperature, 23 °C, and at the nonpermissive temperature 37 °C. Strains FM394, *UBC9*, and FM395, *ubc9-ts*, were transformed with plasmids containing the *MAL/61HA* allele and the *MAL63 MAL* activator genes. Strains were grown to early log phase in maltose media at 23 °C, equilibrated to 37 °C for 2 h, and then transferred to nitrogen starvation media plus 2% glucose at 37 °C. The standard inactivation assay was carried out at 37 °C, as described in the legend to Fig. 1 and under "Materials and Methods." The relative levels of Mal61/HA protein (●) and maltose transport activity (○) compared with the zero time sample are plotted along with the growth dilution (■).

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-ts* 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 suggest 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 by Galan and Haguenauer-Tsapis (47), who found that mutations at Lys-29 and Lys-48 had no effect on induced turnover but, when polyubiquitination was blocked at Lys-63, the rate of Fur4p endocytosis was reduced but not eliminated.

Experiments are under way to identify the site(s) of ubiquitination in Mal61p. Mutation of the lysine residue in the DAKISS sequence of the truncated Ste2p or on all seven lysine residues of the C-terminal cytoplasmic domain block endocytosis, clearly demonstrating a direct requirement for ubiquitin in Ste2p endocytosis (10, 46). Mutation of a "destruction box" sequence in Fur4p stabilized that permease against stress-induced degradation (48). Moreover, mutation of a single lysine to alanine (R294A) is resistant to stress-induced degradation (8).

The *UBC1*, *UBC4*, and *UBC5* genes have been implicated as the E2 enzymes required for substrate conjugation of certain yeast plasma membrane proteins including the α-factor receptor, the a-factor receptor, and the ABC transporter Ste6p (9–11). Double mutant *ubc4 ubc5* strains exhibit reduced rates of induced turnover of these proteins. Ubc1p is essential in *ubc4 ubc5* mutant strains, suggesting a possible role in endocytosis of membrane proteins (33). We explored the role of these ubiquitin-conjugating enzymes in maltose permease proteolysis. While we found that Ubc1p, Ubc4p, and Ubc5p appear to play an as yet unidentified post-transcriptional role in maltose permease expression, our results do not support the suggestion that these proteins are important for its glucose-induced proteolysis. This is in contrast to results for Ste2p (10, 46) but similar to findings regarding the inositol permease Itr1p (49) and uracil permease.[3]

Our results add the Mal61/HA maltose permease to the growing list of permeases, receptors, and other plasma membrane proteins that are marked by ubiquitination for rapid endocytosis and lysosomal (vacuolar) degradation. Moreover, for maltose permease and all of the other yeast membrane proteins for which this has been studied, Npi1p/Rsp5p appears to play an important role. Npi1p/Rsp5p contains a so-called C_2 domain also found in certain isoforms of protein kinase C and believed to mediate phospholipid interaction (6). This makes Npi1p an interesting candidate for a membrane-associated ubiquitin-protein ligase. Studies are currently under way to identify the target sites of this ubiquitination in maltose 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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[3] R. Haguenauer-Tsapis, unpublished results.

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CELL BIOLOGY AND METABOLISM:

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

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