Gly-46 and His-50 of Yeast Maltose Transporter Mal21p Are Essential for Its Resistance against Glucose-induced Degradation

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The maltose transporter gene is situated at the MAL locus, which consists of genes for a transporter, maltase, and transcriptional activator. Five unlinked MAL loci (MAL1, MAL2, MAL3, MAL4, and MAL6) constitute a gene family in Saccharomyces cerevisiae. The expression of the maltose transporter is also regulated post-translationally; Mal61p is rapidly internalized from the plasma membrane and degraded by ubiquitin-mediated proteolysis in the presence of glucose. We found that S. cerevisiae strain ATCC20598 harboring MAL21 could grow in maltose supplemented with a non-assimilable glucose analogue, 2-deoxyglucose, whereas strain ATCC96955 harboring MAL61 could not. These observations implied a MAL21p-specific resistance against glucose-induced degradation. Mal21p found in ATCC20598 has 10 amino acids, including Gly-46 and His-50, that are inconsistent with the corresponding residues in Mal61p. The half-life of Mal21p for glucose-induced degradation was 118 min when expressed using the constitutive TPI1 promoter, which was significantly longer than that of Mal61p (25 min). Studies with mutant cells that are defective in endocytosis or the ubiquitination process indicated that Mal21p was less ubiquitinated than Mal61p, suggesting that Mal21p remains on the plasma membrane because of poor susceptibility to ubiquitination. Mutational studies revealed that both residues Gly-46 and His-50 in Mal21p are essential for the full resistance of maltose transporters against glucose-induced degradation.

The utilization of maltose requires three gene products from the MAL locus, a maltose transporter encoded by the MALXI gene, a maltase encoded by MALX2, and a transcriptional activator by MALX3, where MALX stands for one of five unlinked MAL loci: MAL1, MAL2, MAL3, MAL4, and MAL6 located on chromosome VII, III, II, XI, and VIII, respectively (1). The allele type and copy number of the maltose transporter gene (MALXI) in S. cerevisiae vary depending on the strain (2). MALX1 and MALX2 genes are expressed under the control of a bidirectional common promoter located between their open reading frames (ORFs) (3). These genes are induced by maltose through the binding of the transcriptional activator MalX3p to the bidirectional upstream activating sequence (4) elements in the promoter region (4). Conversely, MalX3p functions negatively in the absence of maltose (5). Moreover, MALX1 and MALX2 are repressed by glucose, partly through the binding of non-phosphorylated Mig1p to the consensus sequence in the promoter region (6, 7).

This strict regulation is pivotal for avoiding excess sugar uptake. When sugar is taken up faster than it is metabolized, cell death may occur (8). This appears to be due to a rapid increase in intracellular glucose and glucose phosphate (8) and a rapid reduction of the internal pH (9). It has recently become clear that highly reactive substances generated during glycolysis (e.g., methylglyoxal) modify the Lys, Arg, and Cys residues of cellular proteins and consequently cause various problems in cellular stress responses and enzyme activities (10–12). The strict regulation of sugar uptake may be necessary to avoid generation of such toxic substances. Post-translational regulations of MAL gene products have been studied in depth (13). The addition of glucose to maltose-grown yeast cells results in a rapid inactivation of maltose transporters followed by their degradation. This degradation process requires endocytosis of the targeted proteins, their trafficking to the vacuole, and subsequent vacuolar proteolysis (13, 14). It has been reported that for various membrane transporters, phosphorylation of Ser, Thr, or Tyr residues and subsequent ubiquitination of the Lys residue are a prerequisite for their internalization on degradation. The ubiquitin ligase Npl1p/Rsp5p is necessary for ubiquitination of Mal61p (15, 16) as in the case of other membrane transporters (e.g., Ste2p (17, 18), Fur4p (19, 20), Gap1p (21), and Bap2p (22, 23)). With regard to glucose-induced inactivation and degrada-

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2 The abbreviations used are: ORF, open reading frame; 2-DOG, 2-deoxyglucose; HA, hemagglutinin; GST, glutathione S-transferase; BSA, bovine serum albumin.
Maltoose Transporter Resistant to Glucose-induced Degradation

TABLE 1

*S. cerevisiae* yeast strains used in this study

| Strain     | Genotype                          | Source               |
|------------|-----------------------------------|----------------------|
| JH1032     | MATa SUC2 mal mel gal2 CUT1 TP11::TRP1-MAL32-G418R ura3 | This study           |
| ATCC96955  | MALa MAL61 MAL62 MAL63 mal64 mal11 MAL12 mal13 ura3-52 leu2-3 leu2-112 trp1 his | ATCC                |
| ATCC20598  | MATa suc MAL2 MELI his4 leu2      | ATCC                |
| ATCC204508 | MATalpha SUC2 mal mel gal2 CUT1   | Berkeley Stock Center |
| CB1        | MALa ade1 MAL61 MAL62 MAL63 AGT1 MAL12 MAL31 MAL32 | This study           |
| D152       | identical to ATCC96955 except for mal61::TRP1 | This study           |
| RH1602     | MATa ura3 leu2 his4 bar1           | gift from H. Riezman |
| RH1597     | MATa ura3 leu2 his4 bar1 end4-1    | gift from H. Riezman |
| 23346c     | MATa ura3                           | gift from B. Andreé  |
| 27038a     | MATa ura3 npi1                      | This study           |
| HH108      | identical to JH1032 except for ura3::URA3::TRP1-MAL61 | This study           |
| HH206      | identical to JH1032 except for ura3::URA3::TRP1-MAL21 | This study           |
| HH207      | identical to JH1032 except for ura3::URA3::TRP1-MAL61 [D46G,L50H] | This study           |
| HH208      | identical to JH1032 except for ura3::URA3::TRP1-MAL61 [D46G] | This study           |
| HH209      | identical to JH1032 except for ura3::URA3::TRP1-MAL61 [L50H] | This study           |
| HH210      | identical to JH1032 except for ura3::URA3::TRP1-MAL61 [L50H] | This study           |
| HH227      | identical to JH1032 except for ura3::URA3::TRP1-MAL31 | This study           |
| HH229      | identical to JH1032 except for ura3::URA3::TRP1-MAL61 [S43A] | This study           |
| HH230      | identical to JH1032 except for ura3::URA3::TRP1-MAL61 [S43A] | This study           |
| HH231      | identical to JH1032 except for ura3::URA3::TRP1-MAL61 [S43A,S48A] | This study           |

tion, Mal61p has been extensively studied, whereas only a limited number of reports on Mal11p are available (24). The mutant Mal61p lacking in PEST-like sequences (residues 49–78 of the N-terminal cytoplasmic domain) has a significantly longer half-life and a decreased rate of glucose-induced degradation (25). This result correlates well with a decrease in the level of ubiquitination of the mutant Mal61p. Furthermore, it has been shown that three putative phosphorylation sites, Ser-295, Thr-363, and Ser-487, are determinants for glucose-induced inactivation and that Ser-295, a putative protein kinase C site, plays an important role in the degradation (24). On the other hand, it has been demonstrated that the fusion of Mal61p-green fluorescent protein is not internalized nor is it efficiently degraded in the presence of glucose in a yck mutant (mutant of the casein kinase I isoforms encoded by YCK1 and YCK2), although it is unclear whether this kinase directly phosphorylates Mal61p (26). As for MAL11, MAL12, and MAL61, very similar DNA sequences; the identities between these three genes are higher than 99%. Regardless of the high degree of identity, Mal11p has a noticeably longer half-life compared with Mal61p (24). As for MAL21 and MAL41, no information is available on their DNA sequences or the characteristics of their gene products.

In this study, the MAL21 gene was cloned, and its structure was compared with other maltose transporter genes. We report here the unusually long half-life of Mal21p in cells with the presence of glucose in the culture media. The amino acid residues underlying the resistance to glucose-induced degradation are shown, and possible mechanisms for the resistance are discussed.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Media, and Culture Conditions**—The *S. cerevisiae* strains used in this study are listed in Table 1. Strain JH1032 is a ura3 derivative of strain X2180-1A (27) created by the integration of a constitutive expression unit for the MAL32 gene. The strains initiating with “HH” are derivatives of JH1032 with disruption of transporter genes. D152 is the strain created by disruption of MAL61 with the TRP1 gene in strain ATCC96955 and was used for the expression of transporters. Yeast cells were cultured on YPD medium (1% yeast extract, 2% yeast peptone, 2% glucose) (28) or YPM medium (1% yeast extract, 2% yeast peptone, 0.5% maltose). YNB medium (6.7 g liter\(^{-1}\) yeast nitrogen base medium without amino acids (Difco), 0.5% maltose) was used to investigate maltose assimilation. Synthetic complete medium was prepared as described previously (28), except that 2% maltose was substituted for glucose. YNB medium containing 2% maltose (YNBHM) or synthetic complete medium supplemented with 0–2 mM 2-deoxyglucose (2-DG) was used to examine the resistance of yeast cells against 2-DG (Figs. 1, 4A, and 5A). YNBD medium (1.7 g liter\(^{-1}\) yeast nitrogen base medium without amino acids and ammonia sulfate (Difco), 2% glucose, and 25 μg ml\(^{-1}\) cycloheximide (24)) was used for the transporter degradation studies. The temperature-sensitive strain RH1597 was maintained at 25 °C and transferred to 37 °C for experiments under a restrictive temperature. The remaining strains were cultured at 30 °C.

**DNA Manipulations and Mutagenesis**—All of the primers used for PCR in this study are listed in supplemental Table S1. MAL61 and MAL31 were cloned from yeast strains ATCC96955 and S288C (ATCC204508), respectively, by PCR using chromosomal DNA as a template and oligonucleotides 1 and 2 as primers. The fragments obtained were sequenced, and their integrity was verified. The DNA sequences of the obtained MAL61 and MAL31 were identical to those deposited in GenBank\textsuperscript{TM} (MAL61, NC001134; MAL31, X17391). MAL21 was cloned from ATCC20598 chromosomal DNA by the homologous recombination method (29) using the upstream sequence (nucleotides 480 to −39 relative to the initiation codon) and the downstream sequence (nucleotides 1852–2307) of MAL31. The DNA fragments for homologous recombination were obtained by PCR using the chromosomal DNA of S288C as the template and sets of oligonucleotides (3 + 4 and 5 + 6) as primers. The fragment containing the MAL21 ORF (from −99 to 1871) was prepared as a SacI fragment from the homologously recombined plasmid and inserted into the Small site of pUC19 (L09137). The promoter sequence of MAL21 was cloned from yeast strains ATCC20598 by PCR using the chromosomal DNA as a template and oligonucleotides 7 and 8 as
primes. The centromeric expression vector, pHXSB (supplementary Fig. S1), was constructed with a fragment containing the replication origin (ori) and an ampicillin resistance gene (Amp R) from pGEM13Zf (Promega Corp.), the URA3 gene fragment from Ycp50 (30), and a fragment including CEN6 (CEN-ARS) from pRS317 (ATCC77157). The TPI1 promoter (TPI1p) and terminator (TPI1t) fragments were obtained from the chromosomal DNA of strain S288C by PCR using sets of oligonucleotides 9 + 10 and 11 + 12 as primers, respectively. The DNA fragments obtained for MAL21 and MAL61 were inserted into Sacl and BamHI sites downstream of the TPI1 promoter of plasmid pHXSB. The resultant plasmids, pHMAL21 and pHMAL61, were introduced into strains RH1602, RH1597 (31), 233346c, and 27038a (32). To create hemagglutinin (HA)-tagged transporter genes, an XhoI site was created right before the stop codons of MAL21 and MAL61 in pHMAL21 and pHMAL61, respectively, with oligonucleotides 13 + 14 for MAL61 and 15 + 16 for MAL21 by in vitro mutagenesis using an ExSite™ PCR-based site-directed mutagenesis kit (Stratagene, La Jolla, CA). The DNA fragment encoding the two tandem repeats of the HA tag was synthesized (oligonucleotides 17 + 18) and inserted at the XhoI site. The resultant plasmid, pJHML61-2HA, was introduced into strains JH1032 and RH1597 and pHJML61-2HA into JH1032. MAL21 and MAL61 were inserted into the centromeric expression vector, pYCWP4 (33), under the control of the PYK1 promoter, and the resultant plasmids were used for expression in D152. The integrative plasmid, pHJXSB, was constructed from pHXSB by removing CEN-ARS with digestion at the FseI sites. The DNA fragments obtained for pJHXSB by removing CEN-ARS with digestion at the FseI sites. The DNA fragments obtained for pJHXSB by removing CEN-ARS with digestion at the FseI sites. The DNA fragments obtained for pJHXSB by removing CEN-ARS with digestion at the FseI sites. The DNA fragments obtained for pJHXSB by removing CEN-ARS with digestion at the FseI sites. The DNA fragments obtained for pJHXSB by removing CEN-ARS with digestion at the FseI sites. The DNA fragments obtained for pJHXSB by removing CEN-ARS with digestion at the FseI sites.
Maltose Transporter Resistant to Glucose-induced Degradation

FIGURE 1. Yeast strains carrying MAL21 grows on medium containing 2-DOG. Control strains JH1032, ATCC96955 (carrying MAL61), ATCC20598 (MAL21), and CB11 (MAL31, MAL61, and AGT1) were grown in YPD. Cells were harvested, washed with water, and resuspended in water at OD600 = 2.0. The suspensions and their 10-fold dilutions were spotted onto synthetic complete medium with or without 3 mg liter⁻¹ antimycin A supplemented with 2-DOG (at 0, 0.5, and 1.0 mM). The plates were incubated at 30 °C for 3 days before being photographed.

Products, Inc.) or mouse anti-ubiquitin antibody (MMS-258R, Covance Research Products, Inc.).

Indirect Immunofluorescence Microscopy—Cells were grown and harvested as described above under “Transporter Degradation Studies.” Half of the collected cells were suspended for 1 h in YNBDS. Cells with and without exposure to glucose were processed for immunofluorescence microscopy as described by Omura et al. (23). Immunofluorescence directed to maltose transporters was performed using rabbit anti-Mal61p antibody at a dilution of 1:100 in PBS supplemented with 1% BSA and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Inc.) at a dilution of 1:500. Cells were examined using an epifluorescence microscope with a filter for blue excitation.

Nucleotide Sequence Accession Number—The nucleotide sequence data of MAL21 isolated from ATCC20598 have been deposited at DDBJ (getentry.ddbj.nig.ac.jp/) under accession number AB453253.

RESULTS

Growth of Yeast Strains Harboring Different Maltose Transporter Genes on the Maltose Medium Containing 2-Deoxyglucose—To estimate the inactivation or degradation rates of the different maltose transporters, we investigated the growth of the strains containing distinct MALXI genes on the maltose-based synthetic complete medium containing 2-DOG (Fig. 1). Although 2-DOG is not metabolized further than 2-DOG 6-phosphate and is not assimilated as a carbon source by yeast, it does induce inactivation and degradation of maltose transporters to the same extent as glucose (34). Although the host strain JH1032 used in this study is designated as a mal⁻ strain because of the nonfunctional MAL33, JH1032 could grow to a limited extent on maltose (Fig. 1). However, JH1032 could not grow at all on maltose when the medium was supplemented with antimycin A, an inhibitor of the electron transport chain. This may indicate that under a respiratory condition, a very slight expression of MAL31 or AGT1, or some distinct maltose uptake system other than a maltose transporter (e.g. hexose transporters), helped strain JH1032 to grow on maltose in combination with an overexpression of maltase. As shown in Fig. 1, strains ATCC96955 (in which MAL61 is expressed) and CB11 (MAL61, MAL31, and AGT1) could not grow in the presence of 1.0 mM 2-DOG, whereas ATCC20598 (MAL21) could, suggesting the possibility that Mal21p is more resistant to glucose-induced inactivation and degradation than are Mal61p, Mal31p, and Agt1p.

Cloning and Sequencing of the MAL21 Gene from Strain ATCC20598—A DNA fragment encompassing the MAL21 gene in strain ATCC20598 was cloned by homologous recombination (29) using upstream and downstream sequences of the MAL31 ORF. When compared with MAL61, the identity between the MAL21 and MAL61 genes was 98%. Of the 31 inconsistent bases, 21 were found within the region defined by nucleotides 483–541. This region was completely identical to the corresponding regions of MPH2 and MPH3, which are known as maltose transporter homologues (35). The rest of the inconsistent bases were scattered throughout the entire MAL21 ORF. The difference in DNA sequences leads to the replacement of 10 amino acid residues in Mal21p compared with Mal61p, as shown in Fig. 2. The MAL21 promoter sequence (5’-upstream sequence of the ORF, −1 to −876 relative to the initiation codon) appeared to have 12 distinct bases and one insertion compared with that of MAL61 but was completely identical to that of MAL31. Because the MAL upstream activating sequence was conserved in the MAL21 promoter region around position −515 to −582 (4, 36), it seems likely that MAL21 transcription is induced by maltose and repressed by glucose as in other MAL genes. For functional analysis, the isolated MAL21 gene and other transporter genes were placed under the control of the constitutive TPI1 promoter of pJHIXSB and were introduced into yeast strain JH1032. The resultant strain, HH206 (MAL21), and the control strain, HH108 (MAL61), were both found to grow on the maltose-based minimum medium, indicating that the isolated MAL21 gene conferred maltose uptake activity on the yeast cells (data not shown).

Maltose Uptake Activity and Steady-state Protein Expression Level of Mal21p—Strains HH206 (MAL21) and HH108 (MAL61) were grown in maltose-based rich medium, cells were harvested at early log phase, and their maltose uptake activities were measured using [14C]maltose (Table 2). The steady-state protein expression level of the transporters was determined by the signal intensities of the intact sized transporters observed in the Western blot analysis with the anti-Mal61p antibody of the transporter-expressing cells (Fig. 3). Considering the possibility that the anti-Mal61p antibody might recognize Mal21p less efficiently than Mal61p, we also examined the steady-state protein level of the transporters tagged with the HA sequence at their C termini using an anti-HA antibody. The ratio of the expression levels of the tagged transporter proteins was almost comparable with that of the native proteins measured with anti-
Maltose Transporter Resistant to Glucose-induced Degradation

The Mal61p amino acid sequence is from GenBank\textsuperscript{10} accession number X17391. The underlined and boxed regions are predicted transmembrane domains (46). Amino acid residues of Mal21p are shown where the corresponding residues of Mal61p are inconsistent. Basic amino acids are marked with a plus sign and acidic amino acids with a minus sign.

![FIGURE 2. Comparison of amino acid sequences in Mal21p and Mal61p. The Mal61p amino acid sequence is from GenBank\textsuperscript{10} accession number X17391. The underlined and boxed regions are predicted transmembrane domains (46). Amino acid residues of Mal21p are shown where the corresponding residues of Mal61p are inconsistent. Basic amino acids are marked with a plus sign and acidic amino acids with a minus sign.](Image)

### TABLE 2
Comparison of maltose uptake activity and steady-state protein expression level between Mal21p and Mal61p

The cells HH206 (expressing Mal21p) and HH108 (Mal61p) pregrown in YPD overnight at 30 °C were transferred to and grown in YPM for 2.5 h at 30 °C. Cells were harvested by centrifugation and separated into two portions. Maltose uptake activities were measured using 10 OD{}_{660} units of cells and [{}^{14}C]maltose substrate as described under "Experimental Procedures." Whole cell extracts were prepared from the cells in the logarithmic phase and were separated by SDS-PAGE. Maltose transporter proteins were analyzed by Western blot analysis with anti-Mal61p antibody. The amount of Mal21p in HH206 was taken as 100%. Assays were performed twice.

| Transporter | Maltose uptake rate | Relative protein expression | Relative activity |
|-------------|---------------------|-----------------------------|------------------|
| Mal21p      | 7.51 ± 0.28         | 100                         | 100              |
| Mal61p      | 3.74 ± 0.06         | 67.5 ± 3.15                 | 73.8             |

Mal61p antibody (data not shown). Mal21p with an intact size of 68 kDa in strain HH206 was more abundant than Mal61p in HH108. There seemed to be no modifications of maltose transporters judging from their electrophoretic mobilities. We observed several bands with lower molecular weight, which appeared to be degradation products originating from the transporter proteins. There were some differences in the pattern of degradation intermediates, possibly indicating differences in proteolysis efficiency at some cleavage sites of Mal61p and Mal21p. As HH206 showed a higher maltose uptake activity and a higher level of transporter expression, it appeared that the basal turnover rate of Mal21p in the absence of glucose was slower than that of Mal61p. Moreover, the relative specific activity of Mal61p was 74% of that of Mal21p. This could indicate that either the relative uptake activity of Mal21p was higher than that of Mal61p, because of the 10 amino acid substitutions, or that a higher ratio of Mal61p molecules with intact size (68 kDa) was in an inactive state in HH108 cells compared with the inactive ratio of Mal21p in HH206 cells.

### Determination of the Residues in Mal21p Responsible for Resistance against Glucose-induced Degradation
The growth of HH206 (MAL21) and HH108 (MAL61) was examined on maltose-based minimum medium containing 2-DOG. Only HH206 could grow in the presence of 2 mM 2-DOG (Fig. 4A), indicating a clear resistance of Mal21p against glucose-induced inactivation and degradation. On the other hand, Mal31p and Mal61p (99% identical in their amino acid sequences) were likely to have comparable inactivation and/or degradation rates judging from the resistance against 2-DOG (Fig. 4A). To confirm that Mal21p has a longer half-life than Mal61p, we examined their glucose-induced degradation rates (Fig. 4B). The half-lives of Mal61p and Mal21p were 25 ± 6 and 118 ± 5 min, respectively. The resistance of HH206 (MAL21) against 2-DOG suggested that Mal21p has a longer half-life than Mal61p and that Mal21p maintains its activity in the presence of glucose as well as 2-DOG. Thus, the degradation rates of Mal61p and Mal21p, expressed in strain D152, were determined in the presence of 2% glucose. The half-lives of Mal61p and Mal21p were 35 ± 2 and 113 ± 3 min, respectively. Using this yeast strain, the maltose uptake rates of Mal61p and Mal21p were measured before and after exposure to glucose for 1 h. The maltose uptake rate of Mal61p decreased to 12 ± 3% after treatment with glucose, whereas Mal21p remained at 57 ± 2% of the initial activity after treatment. It was verified that Mal21p had a longer half-life and also maintained a higher uptake activity than Mal61p in the presence of glucose. As there was no lysine residue (possible ubiquitination site) among the 10 residues that were inconsistent between Mal61p and Mal21p, we wished to know which amino acid substitution underlies the resistance of Mal21p. For this reason, we focused on the six residues of Mal21p (i.e. Gly-46, His-50, Leu-167, Leu-174, Val-175, and Thr-328) that were inconsistent in comparison between Mal21p and Mal31p as well as between Mal21p and Mal61p. Medintz et al. (25) have shown that some deletion mutants of Mal61p, with deletions in the cytoplasmic N-terminal region (Δ2–30 (i.e. deletion of amino acid residues 2–30), Δ31–60, Δ49–78, Δ49–60, and Δ61–78), have longer half-lives in the presence of glucose compared with the native Mal61p. We detected two inconsistent residues between Mal61p and Mal21p in the N-terminal cytoplasmic region; Asp-46 and Leu-50 in Mal61p are replaced by Gly-46 and His-50 in Mal21p. To explore the impact of these
two amino acid substitutions, the gene encoding a mutant Mal61p (Mal61p[Gly-46,His-50]) was constructed by replacement of the N-terminal part of Mal61p with the corresponding part of Mal21p and introduced into the test host strain, JH1032. The resultant strain, HH207 (in which MAL61[D46G, L50H] is expressed), could grow in the presence of 2 mM 2-DOG at the same level as HH206 (Mal21p) (Fig. 5A). It appeared that Gly-46 and/or His-50 were the key determinants for the resistance of Mal21p against glucose-induced degradation.

Both Gly-46 and His-50 Are Necessary for Resistance to Glucose-induced Degradation of Mal21p—To identify which of the two residues of Mal21p (Gly-46 or His-50) confers resistance against glucose-induced degradation, two mutant Mal61p transporters, one with amino acid substitution Asp-46 → Gly and another with Leu-50 → His, were constructed. The transformant strains HH207 (in which MAL61[D46G, L50H] is expressed), HH210 (MAL61[D46G]), and HH209 (MAL61[L50H]) were examined for their growth in the presence of 2 mM 2-DOG (Fig. 5A). The strains HH207 could grow in the presence of 2 mM 2-DOG as could HH206 (MAL21) (Fig. 4A). However, strains HH210 and HH209 could grow in the presence of 1 mM 2-DOG but not in 2 mM 2-DOG. Judging from these results, the coexistence of the two residues, Gly-46 and His-50, seemed necessary for the full resistance of Mal21p against glucose. To verify the effect of Gly-46 and His-50, a glucose-induced degradation test was conducted for these mutant proteins (Fig. 5B). The half-life of Mal61p[Gly-46, His-50] (134 ± 18 min) was close to that of Mal21p (118 ± 5 min). However, Mal61p[Gly-46] and Mal61p[His-50] were degraded faster than Mal21p. Therefore, it was confirmed that simultaneous substitutions to Gly-46 and His-50 are necessary to exert a Mal21p level of full resistance against glucose.

MAL61p Is Ubiquitinated to a Higher Degree than MAL21p in the Presence of Glucose—It is known that maltose transporters are not internalized until they are ubiquitinated (14). To examine differences in the efficiency of ubiquitination of Mal61p and Mal21p molecules in the presence of glucose, MAL61 and MAL21 were expressed in end4 and npi1 mutant cells, which are deficient in an early stage endocytosis process (37) and ubiquitin-conjugating activity (32, 38), respectively. After yeast cells were exposed to glucose for 1 h, each cell extract was prepared and separated by SDS-PAGE and analyzed by Western blotting with anti-Mal61p antibody (Fig. 6A). In END4 wild-type cells, the amount of Mal61p was lower than
that of Mal21p in the absence of glucose. Mal61p completely disappeared after a 1-h cultivation in the presence of glucose, whereas 70% of Mal21p remained intact after a 1-h glucose treatment. In end4 mutant cells, the degradation of Mal61p was largely circumvented by impairment of the endocytotic function. It is noteworthy that some additional bands with molecular weights higher than the intact Mal61p were detected after 1 h in the presence of glucose, and those bands were electrophoretically lined up with the gap of about 9 kDa in size, which is presumably equivalent to the size of the ubiquitin moiety. In npi1 mutant cells, the degradation of Mal61p and Mal21p was blocked again, however, unlike the case of the end4 mutant cells, in which no extra bands with higher molecular weight were detected. To make sure that the extra bands with a higher molecular weight indicated that the Mal61p molecules were modified with ubiquitin, the whole cell extract was prepared from the end4 mutant cells expressing HA-tagged Mal61p, and immunoprecipitation was conducted using anti-Mal61p antibody. Arrows indicate modified transporter proteins with slower mobilities. A, strain RH1597 (end4) cells carrying pJHMAL61-2HA were incubated in YPM at 37 °C for 30 min and then incubated in YNBDS medium at 30 °C for 1 h. Cells were collected and immediately frozen in ethanol-dry ice. Whole cell extracts were prepared, and immunoprecipitation was conducted with anti-Mal61p antibody. Extracts from the immunoprecipitate were separated with SDS-8% PAGE and analyzed by Western blot analyses using anti-HA antibody or anti-ubiquitin antibody. Arrows indicate modified transporter proteins with ubiquitin molecules. The arrowhead indicates a possible phosphorylated form of the transporter protein.

**FIGURE 5.** Simultaneous substitutions of Asp-46→Gly and Leu-50→His were necessary for resistance to glucose-induced degradation of Mal21p. A, yeast cells HH208 carrying vector DNA, HH108 (carrying MAL61), HH210 (MAL61-D46G), HH209 (MAL61-L50H), HH207 (MAL61-D46G, L50H), and HH206 (MAL21) were grown in YPD. Cells were collected, washed with water, and resuspended in water at OD650 = 2.0. Serial 10-fold dilutions of the suspension were spotted onto YNBHM plate containing 2-DOG (at 0, 0.5, 1.0, and 2 mM). The plates were incubated at 30 °C for 2 days before being photographed. B, the cells HH206 (expressing Mal21p), HH207 (Mal61p[Gly-46,His-50]), HH210 (Mal61p[Gly-46]), and HH209 (Mal61p[His-50]) pregrown in YPD overnight at 30 °C were transferred to and grown in YPM for 2.5 h at 30 °C. Cells were harvested and resuspended in YNBDS medium for incubation at 30 °C. The cells of 10 OD units were withdrawn at the times indicated. Whole cell extracts were prepared from the samples and analyzed by Western blot analysis using anti-Mal61p antibody.

**FIGURE 6.** Modification of Mal61p and Mal21p observed in endocytosis and ubiquitination mutant strains. A, yeast strains 23346c (NPI1) and 27038a (npi1) carrying pJHMAL21 or pJHMAL61 were grown in YPM for 2.5 h at 30 °C. Two fractions of cells corresponding to 10 OD units were collected by centrifugation. One was immediately frozen in ethanol-dry ice. The other was resuspended in YNBDS medium and incubated at 30 °C for 1 h. Strains RH1602 (END4) and RH1597 (end4) carrying pJHMAL21 or pJHMAL61 were treated in the same way, except that they were incubated at a restrictive 37 °C for 30 min prior to incubation in YNBDS medium. Cells were centrifuged and immediately frozen in ethanol-dry ice. Whole cell extracts were prepared and separated with SDS-8% PAGE and analyzed by Western blot analysis using anti-Mal61p antibody. Arrows indicate modified transporter proteins with slower mobilities. B, strain RH1597 (end4) cells carrying pJHMAL61-2HA were incubated in YPM at 37 °C for 30 min and then incubated in YNBDS medium at 30 °C for 1 h. Cells were collected and immediately frozen in ethanol-dry ice. Whole cell extracts were prepared, and immunoprecipitation was conducted with anti-Mal61p antibody. Extracts from the immunoprecipitate were separated with SDS-8% PAGE and analyzed by Western blot analyses using anti-HA antibody or anti-ubiquitin antibody. Arrows indicate modified transporter proteins with ubiquitin molecules. The arrowhead indicates a possible phosphorylated form of the transporter protein.

**FIGURE 6A.** The half-lives of Mal21p and Mal61p were determined by immunoprecipitation with anti-Mal61p antibody. Extracts from the immunoprecipitate were separated with SDS-8% PAGE and analyzed by Western blot analysis using anti-Mal61p antibody. Arrows indicate modified transporter proteins with slower mobilities. A, yeast strains 23346c (NPI1) and 27038a (npi1) carrying pJHMAL21 or pJHMAL61 were grown in YPM for 2.5 h at 30 °C. Two fractions of cells corresponding to 10 OD units were collected by centrifugation. One was immediately frozen in ethanol-dry ice. The other was resuspended in YNBDS medium and incubated at 30 °C for 1 h. Strains RH1602 (END4) and RH1597 (end4) carrying pJHMAL21 or pJHMAL61 were treated in the same way, except that they were incubated at a restrictive 37 °C for 30 min prior to incubation in YNBDS medium. Cells were centrifuged and immediately frozen in ethanol-dry ice. Whole cell extracts were prepared and separated with SDS-8% PAGE and analyzed by Western blot analysis using anti-Mal61p antibody. Arrows indicate modified transporter proteins with slower mobilities. B, strain RH1597 (end4) cells carrying pJHMAL61-2HA were incubated in YPM at 37 °C for 30 min and then incubated in YNBDS medium at 30 °C for 1 h. Cells were collected and immediately frozen in ethanol-dry ice. Whole cell extracts were prepared, and immunoprecipitation was conducted with anti-Mal61p antibody. Extracts from the immunoprecipitate were separated with SDS-8% PAGE and analyzed by Western blot analyses using anti-HA antibody or anti-ubiquitin antibody. Arrows indicate modified transporter proteins with ubiquitin molecules. The arrowhead indicates a possible phosphorylated form of the transporter protein.
Maltose Transporter Resistant to Glucose-induced Degradation

1-h exposure to glucose, the yeast strains expressing Mal21p exhibited a higher maltose uptake activity than those expressing Mal61p. To examine whether the internalization of these transporters was blocked in the presence of glucose, the localizations of Mal21p and Mal61p[Gly-46,His-50] along with other control transporters were observed by immunofluorescence microscopy using rabbit anti-Mal61p antibody as the first antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG as the second antibody. The cell margins are indicated by arrowheads.

DISCUSSION

Glucose-induced inactivation and degradation of yeast membrane proteins have been widely studied. It is known that phosphorylation and subsequent ubiquitination are essential for the internalization by endocytosis of membrane proteins such as Ste2p (17), Fur4p (19, 20), Gap1p (21), and Bap2p (22, 23). In this study, we found that Mal21p is more resistant to glucose-induced degradation than are Mal61p and Mal31p.

Gadura et al. (26) have shown that the regulation of the transport activity of Mal61p by YCK1 and YCK2 is exerted through an early step in glucose-induced internalization and also through a modification of the rate of Mal61p trafficking to the vacuole and/or its proteolysis. However, there has been no direct evidence indicating that casein kinase I directly phosphorylates Mal61p. We determined that Gly-46 and His-50 in the N-terminal cytoplasmic region of Mal21p are essential for its resistance to glucose-induced degradation (Fig. 5B). There are two serine residues, Ser-43 and Ser-48, located close to the Gly-46 and His-50. Those serine residues are found in the minimum consensus sequence, (S/T)X(D/E), for the phosphorylation sites by casein kinase II in Mal61p (39). Gly-46 in Mal21p disrupts the consensus sequence, and His-50 in Mal21p is poorly tolerated in the consensus sequence of casein kinase II (40). It is plausible that the breakage of the casein kinase II target sequence by the replacement of Gly-46 and His-50 could be the cause of the degradation resistance of Mal21p, although there have been no reports indicating that casein kinase II regulates transporter internalization in yeast. In mammalian cells, however, it has been reported that stress-induced casein kinase II phosphorylates the mouse predominant water channel, aquaporin 4 (41). This kinase is also involved in the regulation of the activity of the equilibrative nucleoside transporter hENT1 (42). Taking these findings into consideration, we explored the possibility that Ser-43 and Ser-48 in Mal61p could be the primary cause of the higher sensitivity to glucose-induced inactivation/degradation. To this end, Ser-43 and Ser-48 of Mal61p were replaced by alanine in order to examine whether their phosphorylation is associated with the ubiquitination and degradation efficiencies of Mal61p. The three mutant transporters in which one or both of these Ser residues were replaced (Mal61p[Ala-43], Mal61p[Ala-48], and Mal61p[Ala-43, Ala-48]) could not grow on maltose-based minimum medium containing 2 mM 2-DOG, and the degradation rates of the three above-mentioned mutant transporters were almost the same as that of Mal61p (data not shown). It is unlikely that the phosphorylation of Ser-43 and Ser-48 is an essential event that primarily defines the fate of the transporter molecule.

Experiments with a mutant strain defective in endocytosis (end4) allowed us to observe the modification status of maltose transporters when they are fated for degradation. In the presence of glucose, at least two distinct forms of Mal61p modified with ubiquitin molecules were observed in the end4 mutant cells, whereas Mal21p was not detected in the additional bands modified with ubiquitin molecules (Fig. 6). The poor modification of Mal21p with ubiquitin appears to be associated with its resistance to glucose-induced degradation (Fig. 5B). Our results suggest the possibility that more than one Lys residue of Mal61p can be ubiquitinated upon induction of the rapid internalization and proteolysis of this transporter protein. However, there is no difference between Mal21p and Mal61p as to the number and positions of Lys residues for the possible ubiquitination sites. The sole substitution of either Asp-46 → Gly or Leu-50 → His made Mal61p insufficiently resistant to glucose, whereas simultaneous substitutions of both amino acid residues were necessary to confer an equivalent resistance on Mal21p (Fig. 5B). Then how do those substitutions influence the ubiquitination efficiency of the maltose transporters? Because maltose transporters belong to the superfAMILY of sugar transporters with 12 transmembrane domains, amino acid residues Met-1 to Thr-95 in the N-terminal region of Mal61p are speculated to be cytoplasmic (43, 44). According to the estimation of a modified Chou-Fasman secondary structure prediction method (45), the region of Ser-17—Glu-51 is expected to form an α-helix structure. It is hypothesized that substitutions of both Asp-46 → Gly and Leu-50 → His shorten
the α-helix region starting with residue Ser-17. Loss of the α-helix Ser-40—Glu-51 might account for the blockage of phosphorylation and/or ubiquitination of Mal61p. Brondijk et al. (24) reported that Mal61p[ Ala-295 ], Mal61p[ Ala-363 ], and Mal61p[ Ala-487 ] exhibited a decrease in their inactivation rate. Ser-295 and Thr-363 are in a long cytoplasmic domain in the middle of Mal61p, which is rich in basic amino acids. Conversely, the N-terminal cytoplasmic domain is rich in acidic residues. It is tempting to believe that these two cytoplasmic domains of Mal61p possibly interact with each other and that their stereostructural relationship influences ubiquitination efficiency. It is possible that only when these two domains are maintained in a proper interactive conformation, the Lys residues are located in positions where they can be ubiquitinated. It is conceivable that mutations at both Ser-295 and/or Thr-363 in the mid-cytoplasmic region and at Asp-46 and/or Leu-50 in the N-terminal cytoplasmic region could impair the ubiquitination-mediated turnover of maltose transporter by disrupting the correct tertiary structure comprising those two cytoplasmic regions.

Five unlinked MAL loci, MAL1, MAL2, MAL3, MAL4, and MAL6, located on chromosomes VII, III, II, XI, and VIII, respectively, are found in various S. cerevisiae strains. The number of MAL loci depends on the yeast strain; some strains have no MAL locus, others have only one, and others have multiple loci. Generally speaking, lager yeast used in brewing has multiple MAL loci (46). Jespersen et al. (47) have investigated the number of genes present in ale and lager yeast strains in relation to maltose assimilation. Thirteen lager yeast strains have MAL21, whereas another 11 lager yeast strains do not. Lager yeast strains have been selected for industrial use by brewers because of their maltose assimilation ability. Having multiple MAL loci would be an advantage for efficient maltose assimilation. Weihenstephan 34/70, a representative lager brewing yeast, also appeared to have MAL21 on chromosome III judging from Southern hybridization in combination with a pulsed field gel electrophoresis (data not shown). However, the DNA sequence of MAL21 of this yeast was not identical to the sequence of MAL21 isolated in this study from strain ATCC20598. Mal21p originating from Weihenstephan 34/70 was found to have not only a degradation resistance determinant, His-50, but also a degradation-susceptible Asp residue at position 46 (data not shown), indicating that not all the MAL21 genes encode a glucose resistance-type maltose transporter. Under certain conditions, sugar uptake beyond the capacity of subsequent sugar metabolism causes cell death (8). In addition, it has been reported that methylglyoxal, generated from an intermediate in glycolysis, had some toxic effects on S. cerevisiae (10–12). From these perspectives, it would be interesting to understand whether having an ATCC20598-type Mal21p with full resistance to glucose-induced degradation could somehow be a disadvantage for industrial lager brewing yeast.

Acknowledgments—We are very grateful to M. Ishiguro for suggestions with regard to the three-dimensional structure of the maltose transporter, to H. Riezman and B. André for the gift of yeast strains, to S. Rainieri for critical reading of the manuscript, and to Y. Itokui for technical support.

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Maltose Transporter Resistant to Glucose-induced Degradation