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Catabolite Inactivation of the Yeast Maltose Transporter Occurs in the Vacuole after Internalization by Endocytosis

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The maltose transporter of *Saccharomyces cerevisiae* is rapidly degraded during fermentation in the absence of a nitrogen source. The location and mechanism of degradation of the transporter have been investigated. Using mutants defective in endocytosis, we have shown that degradation of this transporter requires internalization by endocytosis. In addition, studies of mutants defective in proteasome or vacuolar proteolysis revealed that degradation occurs in the vacuole and is independent of proteasome function. The results also revealed that degradation of the maltose transporter requires Sec18p and raised the question of whether in the absence of Sec18p activity the internalized maltose transporter is recycled back to the plasma membrane.

Sugar transporters are integral plasma membrane proteins that catalyze the first rate-limiting step of glycolysis in *Saccharomyces cerevisiae* (11). Several strategies are used by this organism to adjust the activities of these proteins to different environmental conditions (22). One of these strategies is the irreversible inactivation of the transporters, which occurs when protein synthesis is impaired upon exhaustion of a nitrogen source in the medium (3–5, 9, 23, 27) and which results in dramatic physiological effects (20, 21, 23). This inactivation, known as catabolite inactivation (17), affects mainly the V_{max} of the transporters, follows first-order kinetics, and is an energy-dependent process stimulated by fermentable substrates (3, 4, 9). By using polyclonal antibodies against a recombinant maltose transporter protein, it has been shown that catabolite inactivation is due to proteolysis (26). The experiments reported here attempt to establish the location and mechanism of the maltose transporter degradation. We investigated the inactivation of the maltose transporter by measuring the rate of maltose uptake with radioactive sugar as well as by determining the cellular content of the transporter with polyclonal antibodies. Possible loci investigated are the plasma membrane, which is the locus of transporter action, the cytoplasm, and the vacuole after internalization of the transporter by endocytosis. In this study, we used strains defective in the internalization step of endocytosis as well as strains that show a defect either in the "chymotrypsin-like" activity of the proteasome complex or in the two main vacuolar endopeptidases.

MATERIALS AND METHODS

Reagents. D-[U-^{14}C]maltose was from Amersham International (Amersham, United Kingdom). Zymolyase 20T was from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). 4′,6-Diamino-2-phenylindole (DAPI), *p*-phenylenediamine, and poly-L-lysine were from Sigma (St. Louis, Mo.). A mixture of goat anti-rabbit immunoglobulin G1 (IgG1), IgG2, IgA, IgM Fa, and Fab conjugated with rhodamine was from Nordic Immunological Lab (Tilburg, The Netherlands). Formaldehyde and 2-mercaptoethanol were from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

Yeast strains and growth conditions. The genotypes of the strains are described in Table 1. All of these strains were unable to grow on maltose and were transformed with the multicopy plasmid pRM1-1, which carries the *MAL1* locus (38). The transformed strains, which grew and transported maltose at normal rates (26), were grown aerobically at 24°C in a rotatory shaker (200 rpm) in a medium containing 2% peptone, 1% yeast extract, 2% maltose, and 3 ppm antimycin A. Cell growth was monitored by measuring optical densities at 640 nm.

Inactivation conditions. Unless otherwise indicated, cells were harvested during exponential growth (about 0.7 mg [dry weight] per ml), washed, and suspended in 3 volumes of an ammonium-free medium as described previously (4) in the presence of 2% glucose and 250 μg of tetracycline chlorhydrate per ml to avoid bacterial contamination. The suspension was incubated at 24 or 35°C (as indicated for each experiment) in a rotatory shaker (200 rpm).

Transport measurements. Cells were harvested by centrifugation, washed with 0.1 M tartaric acid adjusted to pH 4.2 with Tris, and suspended in the same buffer to a cellular density of about 70 mg (dry weight) per ml. Maltose uptake was measured as described previously (2), with 4 mM labeled maltose (0.5 μCi/μmol) and incubation for 15 s at 20°C. At this concentration only the high-affinity component of the maltose transport system is determined (5, 9). We have focused our attention exclusively on this component because the appearance of a low-affinity uptake seems to be due to an experimental artifact (2).

Cellular content of the transporter. The cellular content of the transporter was measured by using polyclonal antibodies as described previously (26). Immunoblots with increasing amounts of crude extracts and measurements of the intensity of the band corresponding to the maltose transporter were performed as described previously (26).

Detection of the transporter by immunofluorescence. Cells were harvested at the early exponential phase of growth (about 2×10^7 cells per ml), washed, and suspended in double the initial volume of inactivating medium. The suspension was divided into two aliquots. One aliquot was immediately used for immunofluorescence experiments, whereas the other one was incubated at 24 or 35°C, as indicated in Results and Discussion, for 4 h prior to immunofluorescence experiments. In either case the cells were fixed by adding 0.1 M KH_2PO_4 (pH 6.5) and 3.7% (vol/vol) formaldehyde (final concentrations). After incubation for 2 h at room temperature with gentle shaking, 100 ml of the cell suspension (about 10^9 cells) was centrifuged (3 min at 3,000 × *g*), and the cells were washed and resuspended in 1.5 ml of a buffer containing 1.2 M sorbitol, 50 mM KH_2PO_4 (pH 7.5), and 20 mM 2-mercaptoethanol. Zymolyase 20T (37 μg) was then added to 0.3 ml of this suspension, and after incubation at 37°C for 1 h with gentle shaking, the protoplasts obtained were collected by centrifugation (5 min at 2,000 × *g*). Thereafter they were washed and suspended in 0.3 ml of a buffer containing 1.2 M sorbitol and 50 mM KH_2PO_4 (pH 7.5). Ten microliters of this suspension was applied to polylysine-coated multiwell slides. After incubation for 10 s, the suspension was removed, and the slides were dried and immersed successively in precooled (−20°C) methanol and acetone for 5 min and 30 s, respectively, to permeabilize the cells. After the cells were blocked for 30 min with 25 μl of a buffer containing 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM NaH_2PO_4 (pH 7.4), and 1 mg of bovine serum albumin per ml (PBS-BSA), they were incubated for 2 h with 10 μl of 10-fold-diluted affinity-purified anti-maltose transporter antibodies (26). Affinity purification of the antibodies was done by incubation with the transporter bound to a nitrocellulose strip as described previously (25). The cells were then washed five times with 25 μl of PBS-BSA and incubated in the presence of 10 μl of the secondary antibody (affinity-purified goat anti-rabbit IgG conjugated to rhodamine, diluted 1:100 in PBS) for 1 h at room temperature in the dark. After being washed five times for 5 min with 25 μl of PBS, the cells were incubated for 5 min in the presence of 15 μl of DAPI diluted 1:100 in PBS, washed five times with 25 μl of PBS, and mounted with 10

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TABLE 1. Strains used in this work

Strain (reference)	Genotype
RH 144-3D (32)	*MAT***a** *ura3 leu2 his4 bar1-1*
RH 266-1D (32)	Isogenic with RH 144-3D, *end3 END4*
RH 268-1C (32)	Isogenic with RH 144-3D, *END3 end4*
WCG4a (14)	*MAT***a** *his3-11,15 leu2-3,112 ura3-Δ5 GAL*$^+$ *can*^{s}
WCG4-11a (14)	Isogenic with WCG4, *pre1-1 PRE2-1*
WCG4-21a (14)	Isogenic with WCG4, *PRE1-1 pre2-1*
WCG4-11/21a (14)	Isogenic with WCG4, *pre1-1 pre2-1*
YMTA (43)	Isogenic with WCG4, *pra1*::*HIS3 PRB1*
YMTAB (43)	*ura3 his3 leu2 pra1*::*HIS3 prb1*
X2180 (31)	*MAT***a** *SUC2 mel mal gal2 CUP1*
HMSF 176 (31)	Isogenic with X2180, *sec18*

μl of a medium containing 90% (vol/vol) glycerol, 0.1% (wt/vol) *p*-phenylenediamine, and 0.02 ppm DAPI. Visualization of the cells was performed by fluorescence microscopy with a Zeiss Axiophot microscope equipped with the following filters: excitation at 450 to 490 mm, FT510, LP520 for rhodamine, and BP546 FT580 LP590 for DAPI. The fluorescence was photographed with HP-50 film with an ASA of 400. Phase-contrast images of the same field were also photographed.

RESULTS AND DISCUSSION

Test for involvement of the proteasome in catabolite inactivation of the maltose transporter. The proteasome is a multicatalytic cytoplasmic and nuclear proteinase complex that has been identified in all eukaryotic cells tested so far. This proteinase complex is involved in ubiquitin-mediated proteolysis (7, 15, 37) and in the regulation of vital cellular proteins (34, 35, 39, 40, 46). In *S. cerevisiae*, as in all eukaryotes, the proteasome shows three different catalytic activities: a chymotrypsin-like activity, a trypsin-like activity, and a peptidylglutamyl-peptide-hydrolyzing activity (14, 15). Two mutants of this organism, the *pre1* and *pre2* mutants, that show a considerable reduction in the chymotrypsin-like activity of the complex have been isolated (14, 15). To investigate the possible involvement of the proteasome in the proteolysis of the maltose transporter occurring during catabolite inactivation (26), we used two single mutants carrying either the *pre1-1* allele or the *pre2-1* allele and a double mutant carrying both alleles, *pre1-1* and *pre2-1*. All of these mutants exhibit decreased protein degradation and accumulation of ubiquitin-protein conjugates under stress conditions (14, 15). We found that the activity (Fig. 1A) as well as the cellular content (Fig. 1B) of the maltose transporter decreased at similar rates in the double mutant and in the wild-type strain, and a half-life of about 1 h was calculated from the data shown in Fig. 1. Similar results were obtained with the two single mutants (results not shown). By contrast, fructose-1,6-bisphosphatase, whose degradation is dependent on the chymotrypsin-like activity, remains stable in these mutant strains (39, 40). All of these facts indicate that the chymotrypsin-like activity of the proteasome is not involved in catabolite inactivation of the maltose transporter. This catalytic activity seems to be most relevant in cytoplasmic proteolysis, since all known proteins which are degraded via the ubiquitin pathway accumulate in the *pre1-1* and *pre2-1* mutants (16). These experiments do not rule out a role for the two other catalytic activities of the proteasome in the inactivation of the maltose transporter.

Involvement of endocytosis in catabolite inactivation of the maltose transporter. The maltose transporter is an integral plasma membrane protein. One possible mechanism for its inactivation is the removal of the transporter from the plasma membrane by internalization. Internalization of plasma membrane proteins is the first step of endocytosis. With *S. cerevisiae*, two mutants, the *end3* and *end4* mutants, that are defective in the internalization step of endocytosis and are temperature sensitive for growth have been isolated (32). We used these two mutants to establish whether endocytosis is involved in catabolite inactivation of the maltose transporter. We found that inactivation of the maltose transporter, as monitored by measuring maltose uptake, was substantially reduced in the endocytosis mutants compared with the wild-type strain (Fig. 2A). While in wild-type cells the transport activity decreased by about 90% in 4 h, this decrease was lower than 20% in *end3* and *end4* mutant cells. In addition, while inactivation in the wild-type strain was accompanied by a parallel decrease in the cellular content of the transporter, the transporter content remained nearly constant in the endocytosis mutants (Fig. 2B and C). From the data in Fig. 2 it can be calculated that during catabolite inactivation at 35°C, the half-life of the transporter was increased six- to sevenfold in the endocytosis mutants compared with the wild-type strain (Table 2). Experiments carried out at 24°C revealed differences in transporter inactivation of two- to threefold between the mutants and the wild-type strain (Table 2). These findings strongly indicate that catabolite inactivation of the maltose transporter does not occur while the protein is located at the plasma membrane but that it occurs intracellularly after internalization of the transporter by endocytosis.

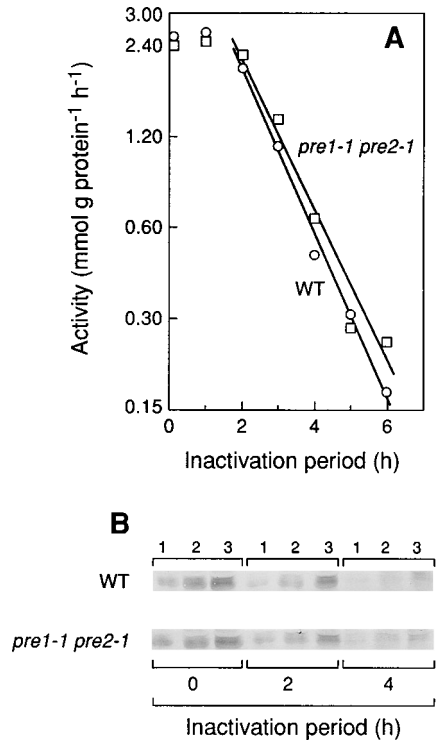

FIG. 1. Inactivation of the maltose transporter in mutants defective in proteasome function. Strains WCG4 (wild type [WT]) (○) and WCG4-11/21a (*pre1-1 pre2-1*) (□), transformed with the plasmid pRM1-1 carrying the *MAL1* locus, were harvested during exponential growth at 28°C, washed, and suspended in 3 volumes of the inactivating medium. (A) After incubation at 28°C for the indicated times, the cells were harvested and assayed for maltose transport activity. Data are means of results from two experiments. (B) Maltose transporter band detected by immunoblotting aliquots of cellular extracts (containing 10, 17, and 25 μg of protein) for lanes 1, 2, and 3, respectively) obtained at the indicated inactivation periods.

Involvement of the vacuolar function in the maltose transporter degradation. The plasma membrane proteins internal-

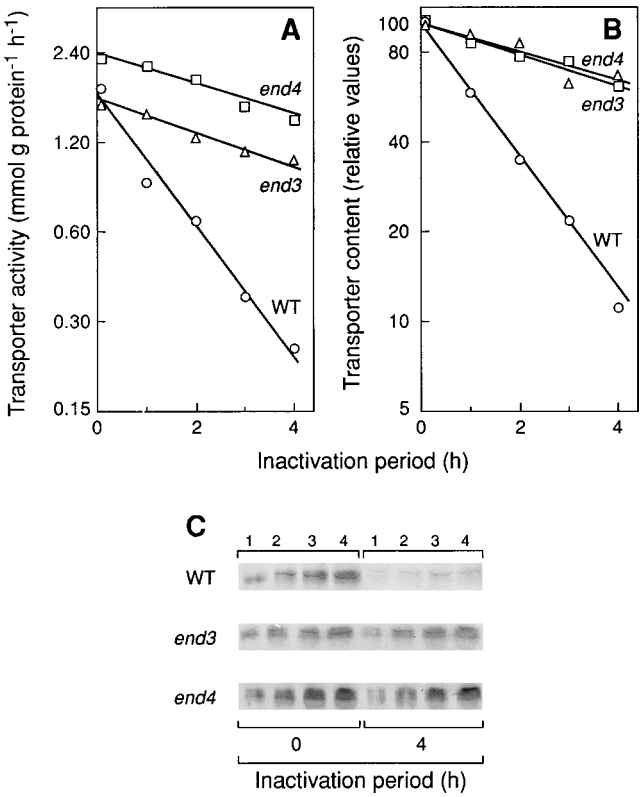

FIG. 2. Inactivation of the maltose transporter at 35°C in mutants defective in endocytosis. Strains RH 144-3D (wild type [WT]) (○), RH 266-1D (*end3*) (△), and RH 268-1C (*end4*) (□), transformed with the plasmid pRM1-1 carrying the *MAL1* locus, were harvested during exponential growth at 24°C, washed, and diluted in 3 volumes of the inactivating medium. After incubation at 35°C for the indicated times, the cells were harvested, washed, and assayed for maltose transport activity (A) and cellular content of the transporter (B) (see Materials and Methods). Data are means of results from two experiments. (C) Maltose transporter band detected by immunoblotting aliquots of cellular extracts (containing 15, 25, 40, and 50 μg of protein for lanes 1, 2, 3, and 4, respectively) obtained at the indicated inactivation periods.

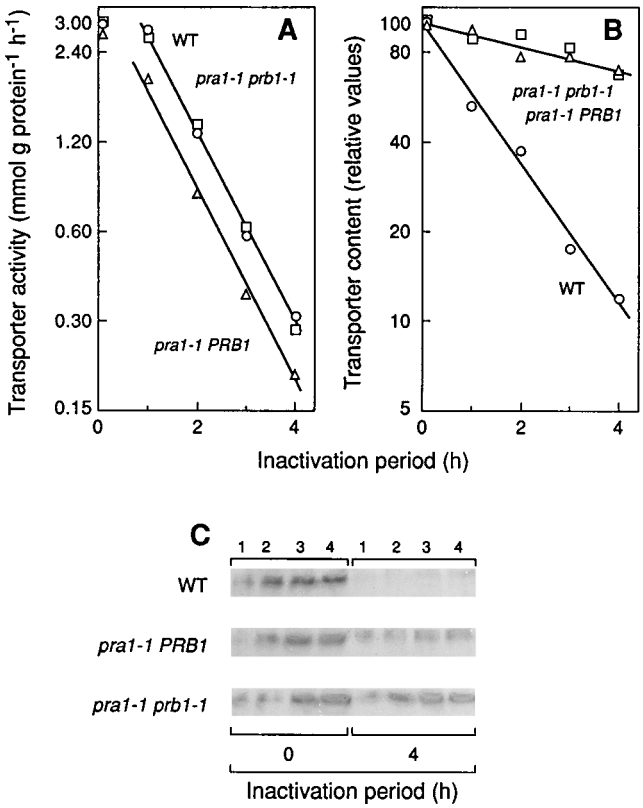

FIG. 3. Inactivation of the maltose transporter in mutants defective in vacuolar proteinases. Strains WCG4 (wild type [WT]) (○), YMTA (*pra1*) (△), and YMTAB (*pra1 prb1*) (□), transformed with the plasmid pRM1-1 carrying the *MAL1* locus, were harvested during exponential growth at 28°C, washed, and suspended in 3 volumes of the inactivating medium. After incubation at 28°C for the indicated times, the cells were harvested and assayed for maltose transport activity (A) and cellular content of the transporter (B) (see Materials and Methods). Data are means of results from two experiments. (C) Maltose transporter band detected by immunoblotting aliquots of cellular extracts (containing 15, 25, 40, and 50 μg of protein for lanes 1, 2, 3, and 4, respectively) obtained at the indicated inactivation periods.

ized by endocytosis pass through endosomes, from where they are either recycled back to the cell surface or delivered to the vacuole for proteolysis (12). The vacuole is a compartment where extensive protein degradation occurs in yeast cells (18, 19, 42, 43). It represents the equivalent of the lysosome in mammalian cells (28). The major proteolytic activity within the vacuole is provided by two endopeptidases encoded, respectively, by gene *PRB1* and gene *PRA1* (also known as *PEP4*) (19, 29, 30, 43, 45). To investigate the involvement of the vacuole in the proteolysis of the maltose transporter, we used a mutant carrying the *pra1-1* allele and a double mutant carrying the *pra1-1* and *prb1-1* alleles. These mutations reduced the vacuolar degradative capacity by 70 and 85%, respectively (43). We found that inactivation of the transporter, monitored by maltose uptake measurements, occurred at similar rates in the mutants and in the wild-type strain (Fig. 3A). This fact indicates that the disappearance of the transporter from the plasma membrane, i.e., endocytic internalization of the transporter, occurred at the same rate in all strains independent of their vacuolar function. By contrast, we found that the cellular content of the transporter remained almost constant in the mutants with defective vacuoles, while it decreased in parallel to maltose uptake in the wild-type strain (Fig. 3B and C). This fact indicates that degradation of the maltose transporter, once internalized, is dependent on vacuolar function and strongly suggests that proteolysis of this transporter during catabolite inactivation takes place in the vacuole. This conclusion is supported by the apparent accumulation of the maltose transporter in a compartment of the mutant cells, most likely the vacuole, as visualized by indirect immunofluorescence microscopy (Fig. 4). It was found that while in the wild-type cells immunofluorescence due to the transporter disappeared after 4 h under inactivating conditions, in the mutant cells immunofluorescence accumulated in a cellular compartment, different

TABLE 2. Half-life of the maltose transporter in mutants defective in endocytosis

Strain	Half-life (h) at[a]:	
	35°C	24°C
Wild type	1.0	1.4
end3 mutant	≃6.0	≃3.2
end4 mutant	≃7.0	≃4.4

[a] The values at 35°C were calculated from the data shown in Fig. 2. The values at 24°C were calculated from experiments similar to those described in Fig. 2 except that incubation in the inactivating medium was at 24°C.

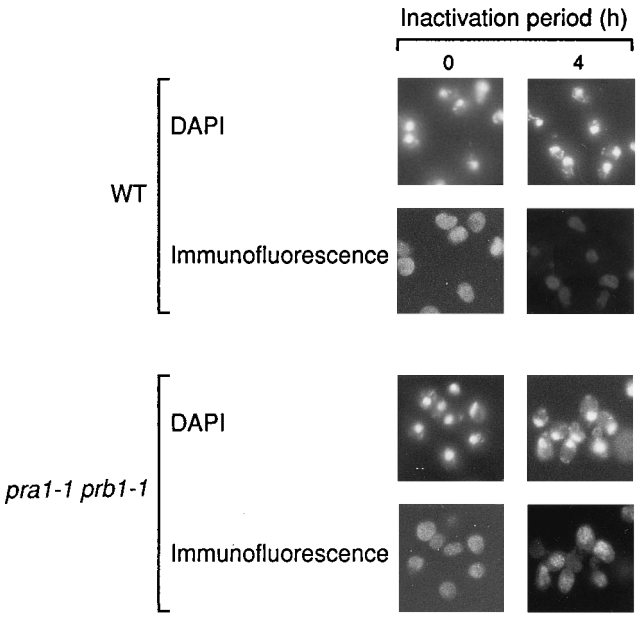

FIG. 4. Maltose transporter accumulation in mutants defective in vacuolar proteinases. Strains WCG4 (wild type [WT]) and YMBTAB (*pra1 prb1*), transformed with the plasmid pRM1-1 carrying the *MAL1* locus, were harvested during exponential growth at 28°C, washed, and suspended in 3 volumes of the inactivating medium. After incubation at 28°C for the indicated times, the cells were harvested and the transporter was detected by immunofluorescence (see Materials and Methods).

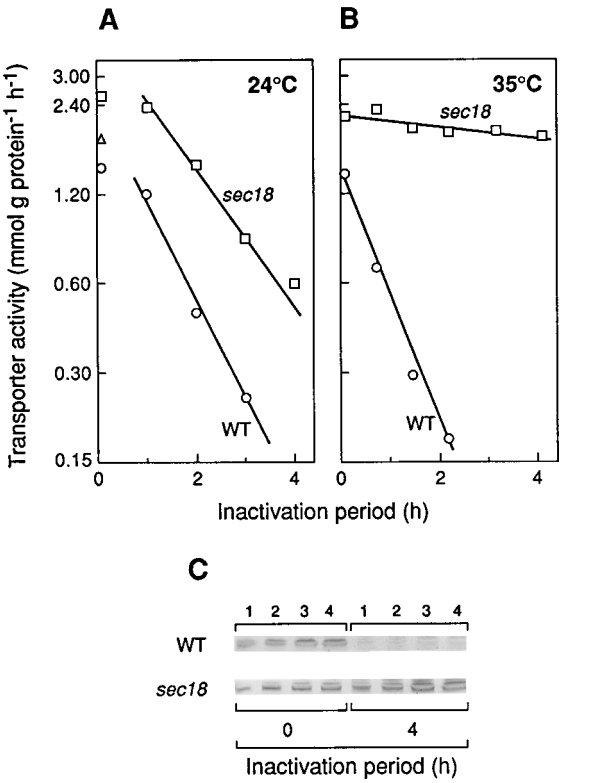

FIG. 5. Inactivation of the maltose transporter in Sec18 mutant cells. Strains X2180-1A (wild type [WT]) (○) HMSF 176 (*sec18*) (□), transformed with the plasmid pRM1-1 carrying the *MAL1* locus, were harvested during exponential growth at 24°C, washed, and diluted in 3 volumes of the inactivating medium. After incubation at 24°C (A) or 35°C (B) for the indicated times, the cells were harvested and assayed for maltose transport activity. Data are means of results from two experiments. (C) Maltose transporter band detected by immunoblotting aliquots of cellular extracts (containing 15, 25, 40, and 50 μg of protein (for lanes 1, 2, 3, and 4, respectively) obtained after incubation at 35°C for the indicated times.

from the nucleus as visualized with DAPI staining, that by its size and position seems to be the vacuole (Fig. 4).

In animal cells the main route of degradation of plasma membrane proteins is internalization by endocytosis and subsequent delivery to the lysosome (13). Most likely this is also the main route of degradation of plasma membrane proteins in *S. cerevisiae*. This is underlined by the observations that, in addition to that of the maltose transporter, degradation of **a**- and α-factor receptors (8, 41), uracil permease (44), and inositol permease (24) occurs in the vacuole after endocytosis and also that catabolite inactivation of the K^+ and glucose transporters is preceded by endocytosis (33). In addition to vacuolar proteolysis, other pathways may also contribute to degradation of plasma membrane proteins, but little is known about this possibility (13). Our results show that at least in the case of the maltose transporter, the proteasome is not involved in degradation to a noticeable extent. This is indicated by the fact that in vacuolar mutants, in which all catalytic activities of the proteasome are unaffected, degradation of the maltose transporter is not apparent (Fig. 3 and 4).

Involvement of Sec18p in catabolite inactivation of the maltose transporter. Endocytosis gives rise to early and late endosomes that are involved in the traffic of the plasma membrane proteins to the vacuole. In mammalian cells the *N*-ethylmaleimide-sensitive fusion protein is involved in the fusion of secretory vesicles and endosomes. Sec18p is the yeast homolog of the mammalian *N*-ethylmaleimide-sensitive fusion protein (1, 10) and seems to be required for the delivery of proteins internalized by endocytosis to the vacuole (36). This idea is based on the fact that *sec18* mutant cells are able to internalize the peptide pheromone α-factor when bound to its receptor, but subsequent delivery to the vacuole is blocked. In these mutant cells α-factor accumulates in an early endocytic compartment, indicating that Sec18p is necessary for progress of the endocytic pathway (36).

To investigate whether Sec18p is involved in the degradation pathway of the maltose transporter, we used a temperature-sensitive *sec18* mutant strain as well as its isogenic wild-type strain (6, 31). As expected, at the permissive temperature of 24°C, a rapid decrease in the maltose transport activity was observed in the two strains upon application of the inactivating conditions (Fig. 5A). However, at the nonpermissive temperature of 35°C, the rate of maltose uptake remained almost constant in *sec18* mutant cells, while this rate rapidly decreased in the wild-type strain. These results can be explained in at least two ways. (i) *sec18* mutant cells cannot internalize the maltose transporter at the nonpermissive temperature, and, as a consequence, the transporter stays in the plasma membrane. According to this possibility, internalization of the maltose transporter and of the α-factor bound to its receptor would show different requirements. (ii) The mutant cells can internalize the transporter, but it gets stuck in early endosomes. However, in contrast to the pheromone that stays in the endosomes (36), the maltose transporter is recycled back to the plasma membrane. The two possibilities predict that degradation of the maltose transporter would be impaired in *sec18* mutant cells at the nonpermissive temperature. Indeed, the results obtained are consistent with this prediction. Immunoblotting experiments show that at 35°C the cellular content of the transporter remains almost constant in *sec18* mutant cells

after 4 h under inactivating conditions, whereas the protein nearly disappears in the control strain (Fig. 5C).

In animal cells many internalized plasma membrane proteins, most of them receptors, do not readily pass through early and late endosomes to be degraded in the lysosome but are rapidly recycled back to the cell surface from early endosomes after dissociation of their ligands. This enables the recovery of plasma membrane proteins and lipids of the cell (for a review, see reference 12). In *S. cerevisiae* a similar recycling has not been demonstrated, but the results obtained with the maltose transporter in *sec18* mutant cells, as well as other results obtained with the α-factor receptor (36), are consistent with this possibility. As mentioned above, in wild-type cells α-factor bound to its receptor is internalized via endocytosis and degraded in the vacuole. However, when Sec18p is defective because of mutation, the pheromone is not delivered to the vacuole but accumulates in early endosomes (36). A similar accumulation of the receptor of α-factor in endosomes has not been demonstrated, and an interesting possibility is that the receptor is recycled back to the plasma membrane after dissociation of the pheromone.

In summary, the results reported here show that catabolite inactivation of the maltose transporter in *S. cerevisiae* is independent of the function of the proteasome and that degradation of the transport protein takes place in the vacuole after internalization by endocytosis. The results also show that catabolite inactivation of the maltose transporter requires Sec18p and raise the question of whether in the presence of a mutational defect of this protein the internalized maltose transporter is recycled back to the plasma membrane.

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