INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

INVESTIGATION OF THE GLUCOSE–INDUCED INACTIVATION OF MALTOSE PERMEASE IN *SACCHAROMYCES*

by

Igor Medintz

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1999

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

September 25, 1998
Date

Chair of Examining Committee
Dr. Corinne Michels, Queens College

September 29, 1998
Date

Executive Officer
Dr. Richard L. Chappell

Dr. Harold Magazine, Queens College

Dr. Timothy Short, Queens College

Dr. Dan Eshel, Brooklyn College

Dr. Lorraine Marsh, Albert Einstein College of Medicine

Supervising Committee

The City University of New York

ABSTRACT

CHARACTERIZATION OF THE GLUCOSE-INDUCED INACTIVATION OF MALTOSE PERMEASE IN *SACCHAROMYCES*

by

Igor Medintz

Advisor: Professor Corinne A. Michels

The addition of glucose to maltose fermenting *Saccharomyces cerevisiae* cells results in the rapid loss of maltose transport activity. This results both from the repression of the maltose permease gene transcription and from the post-translational inactivation of maltose permease through a process termed glucose-induced inactivation of maltose permease. The inactivation consists of two separable processes, a rapid inhibition of maltose transport activity and by a slower degradation of maltose permease protein. Degradation is dependent on endocytosis, vesicular sorting and vacuolar proteolysis, and is independent of the proteasome. Furthermore, maltose permease exists in differentially phosphorylated forms.

Ubiquitin and the ubiquitin-conjugating system are necessary for glucose-induced proteolysis of maltose permease. The rate of glucose-induced proteolysis of maltose permease is slowed by deletion of *DOA4* encoding a ubiquitin recycling enzyme and this is suppressed by overexpression of ubiquitin. Moreover, maltose permease exists in a ubiquitinated state whose abundance increases upon the addition of glucose. A mutation that reduces the expression of *NPI1/RSP5*, encoding a putative

ubiquitin-protein ligase, also dramatically decreases the rate of glucose-induced proteolysis of maltose permease. Double null mutations in *ubc1 ubc4* and *ubc4 ubc5* had no significant impact on the rate of glucose-induced proteolysis of maltose permease.

Mutational analysis was carried out on the predicted cytoplasmic N- and C-terminal domains of maltose permease. The N-terminal cytoplasmic domain of maltose permease contains a putative PEST sequence and a di-leucine sequence. The C-terminal cytoplasmic domain contains a putative endocytosis signal, NPF, and numerous lysine, serine, and threonine residues. The PEST sequence is required for rapid glucose-induced inhibition and degradation of maltose permease while alterations in the C-terminal domain cause only a modest decrease.

In summary, our results indicate that glucose stimulates the ubiquitination of maltose permease and that this marks this protein for endocytosis and vacuolar proteolysis. The PEST sequence found in residues 49-78 of the N-terminal cytoplasmic domain plays an essential role in this process and appears to be required for recognition by the ubiquitin conjugating enzymes.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 2

Chapter 3

INTRODUCTION

In *Saccharomyces*, the level of maltose permease expression is regulated at both the transcriptional and post-translational level by glucose. Strains of *Saccharomyces* that ferment maltose utilize maltose permease for maltose uptake or transport. Transcription of the gene encoding the permease is induced by maltose in the growth medium and repressed by the presence of glucose. This is referred to as glucose repression. Glucose also triggers an irreversible inactivation of the permease, a process referred to as glucose-induced inactivation. The goal of my project is to characterize the glucose-induced inactivation of maltose permease and to identify regions in the permease critical to this process.

Maltose permease and maltose fermentation in *Saccharomyces*

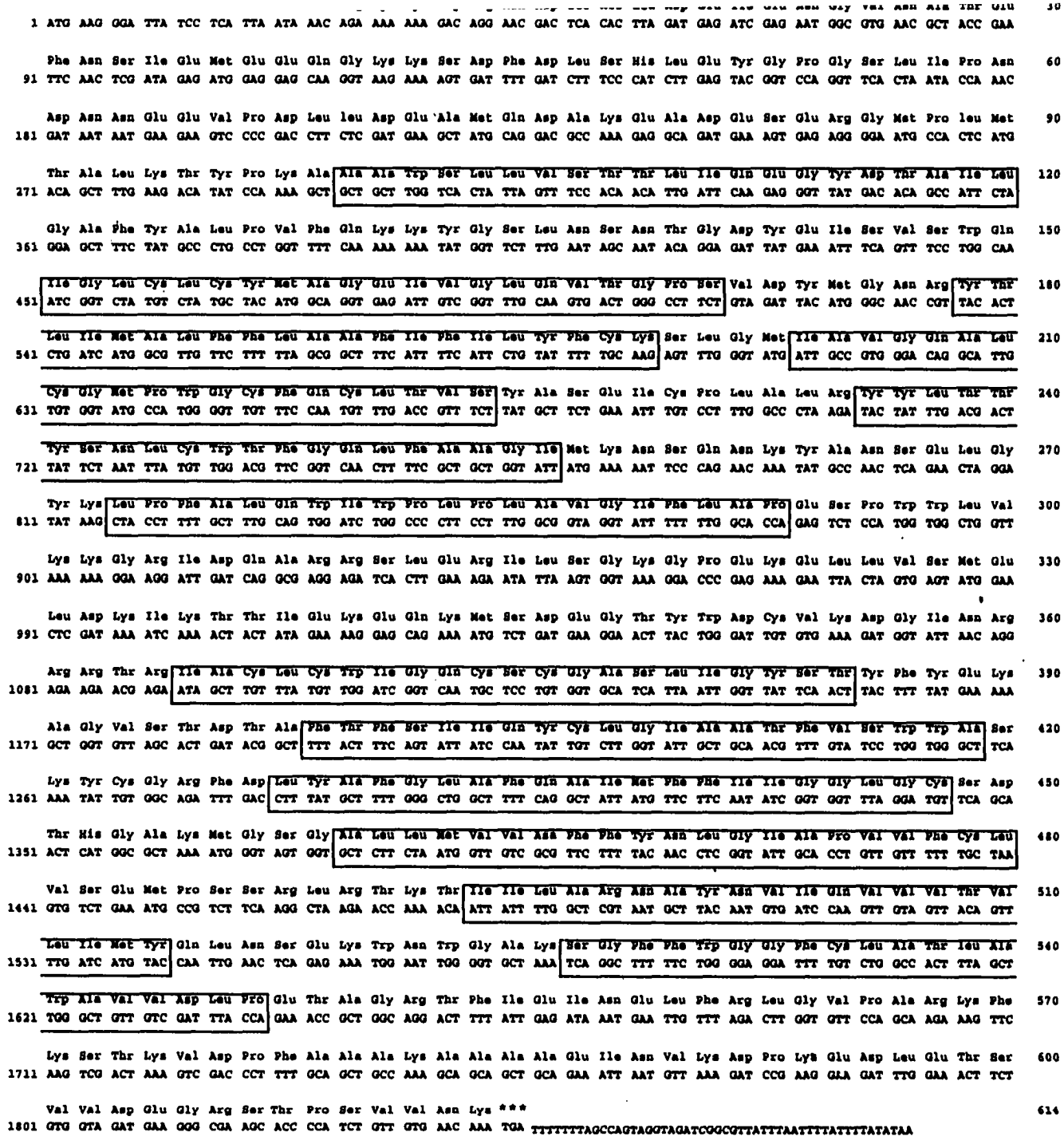
Maltose fermenting yeast strains require one of the five *MAL* loci, *MAL1,2,3,4,6*, which map near the telomeres of different *Saccharomyces* chromosomes. Each locus is a cluster of three genes: Gene 1 encodes the maltose permease; Gene 2 encodes the maltase enzyme and Gene 3 encodes the *trans*-acting *MAL* gene transcription activator, the Mal-activator (Charron, *et al.*, 1989). The genetic nomenclature designates both the locus position and specific gene. The maltose permease gene, Gene 1, found at the *MAL6* locus is designated *MAL61*. Gene 1 homologues at each of the *MAL* loci encode maltose permease (Cheng and Michels, 1989). Expression of both the maltose permease and maltase genes requires the *Mal*-activator protein to induce above

a very low basal rate. Expression is maltose inducible and glucose repressible and regulation is at the transcriptional level (Lagunas, 1993; Needleman and Michels, 1983; Needleman, *et al.*, 1984, Hu *et al.*, 1995).

MAL61, encodes a proposed 614 residue protein [see Figure 1] with 12 putative hydrophobic transmembrane domains (designated by the boxed areas) which exhibits significant homology to members of the superfamily of sugar transporters including the human facilitative glucose transporter GLUT-5 (Cheng and Michels, 1989). Transport by maltose permease is an active process that is coupled to the electrochemical proton gradient and is independent of ATP, that is, by proton symport (Serrano, 1977). Kinetic studies of maltose transport demonstrated the presence of two transporters in maltose induced cells. The high affinity transport has an apparent K_m of 4mM versus a K_m of 70mM for the low affinity transport (Cheng and Michels, 1991). High affinity transport is dependent on the maltose permease protein encoded by *MAL61* or its homologues at the other *MAL* loci (*MAL11, MAL21, MAL31, MAL41*). Deletion of *MAL61* eliminates the high affinity component but does not affect the low affinity component. Evidence exists that the low-affinity maltose transport is an artifact caused by the trapping of maltose in the yeast periplasmic space (Benito *et al.*, 1992). Another high-affinity α-glucoside transporter, called *AGT1*, has been identified and has 57% amino acid identity to the Mal61p. (Han *et al.*, 1995).

Figure 1. Nucleotide and predicted amino acid sequence of the Mal61 gene. (Adapted from Cheng and Michels, 1989)

```
                AGTACTCAGCATATAAAGAGACACAATATACTCCATACTTGTTGTGAGTGGTTTTAGCGTATTCAGTATAACAATAAGAATTACATCCAAGACTATTAATTAACT

     Met Lys Gly Leu Ser Ser Leu Ile Asn Arg Lys Lys Asp Arg Asn Asp Ser His Leu Asp Glu Ile Glu Asn Gly Val Asn Ala Thr Glu    30
   1 ATG AAG GGA TTA TCC TCA TTA ATA AAC AGA AAA AAA GAC AGG AAC GAC TCA CAC TTA GAT GAG ATC GAG AAT GGC GTG AAC GCT ACC GAA

     Phe Asn Ser Ile Glu Met Glu Glu Gln Gly Lys Lys Ser Asp Phe Asp Leu Ser His Leu Glu Tyr Gly Pro Gly Ser Leu Ile Pro Asn    60
  91 TTC AAC TCG ATA GAG ATG GAG GAG CAA GGT AAG AAA AGT GAT TTT GAT CTT TCC CAT CTT GAG TAC GGT CCA GGT TCA CTA ATA CCA AAC

     Asp Asu Asn Glu Glu Val Pro Asp Leu leu Asp Glu Ala Met Gln Asp Ala Lys Glu Ala Asp Glu Ser Glu Arg Gly Met Pro leu Met    90
 181 GAT AAT AAT GAA GAA GTC CCC GAC CTT CTC GAT GAA GCT ATG CAG GAC GCC AAA GAG GCA GAT GAA AGT GAG AGG GGA ATG CCA CTC ATG

     Thr Ala Leu Lys Thr Tyr Pro Lys Ala Ala Ala Trp Ser Leu Leu Val Ser Thr Thr Leu Ile Gln Glu Gly Tyr Asp Thr Ala Ile Leu   120
 271 ACA GCT TTG AAG ACA TAT CCA AAA GCT GCT GCT TGG TCA CTA TTA GTT TCC ACA ACA TTG ATT CAA GAG GGT TAT GAC ACA GCC ATT CTA

     Gly Ala Phe Tyr Ala Leu Pro Val Phe Gln Lys Lys Tyr Gly Ser Leu Asn Ser Asn Thr Gly Asp Tyr Glu Ile Ser Val Ser Trp Gln   150
 361 GGA GCT TTC TAT GCC CTG CCT GGT TTT CAA AAA AAA TAT GGT TCT TTG AAT AGC AAT ACA GGA GAT TAT GAA ATT TCA GTT TCC TGG CAA

     Ile Gly Leu Cys Leu Cys Tyr Met Ala Gly Glu Ile Val Gly Leu Gln Val Thr Gly Pro Ser Val Asp Tyr Met Gly Asn Arg Tyr Thr   180
 451 ATC GGT CTA TGT CTA TGC TAC ATG GCA GGT GAG ATT GTC GGT TTG CAA GTG ACT GGG CCT TCT GTA GAT TAC ATG GGC AAC CGT TAC ACT

     Leu Ile Met Ala Leu Phe Phe Leu Ala Ala Phe Ile Phe Ile Leu Tyr Phe Cys Lys Ser Leu Gly Met Ile Ala Val Gly Gln Ala Leu   210
 541 CTG ATC ATG GCG TTG TTC TTT TTA GCG GCT TTC ATT TTC ATT CTG TAT TTT TGC AAG AGT TTG GGT ATG ATT GCC GTG GGA CAG GCA TTG

     Cys Gly Met Pro Trp Gly Cys Phe Gln Cys Leu Thr Val Ser Tyr Ala Ser Glu Ile Cys Pro Leu Ala Leu Arg Tyr Tyr Leu Thr Thr   240
 631 TGT GGT ATG CCA TGG GGT TGT TTC CAA TGT TTG ACC GTT TCT TAT GCT TCT GAA ATT TGT CCT TTG GCC CTA AGA TAC TAT TTG ACG ACT

     Tyr Ser Asn Leu Cys Trp Thr Phe Gly Gln Leu Phe Ala Ala Gly Ile Met Lys Asn Ser Gln Asn Lys Tyr Ala Asn Ser Glu Leu Gly   270
 721 TAT TCT AAT TTA TGT TGG ACG TTC GGT CAA CTT TTC GCT GCT GGT ATT ATG AAA AAT TCC CAG AAC AAA TAT GCC AAC TCA GAA CTA GGA

     Tyr Lys Leu Pro Phe Ala Leu Gln Trp Ile Trp Pro Leu Pro Leu Ala Val Gly Ile Phe Leu Ala Pro Glu Ser Pro Trp Trp Leu Val   300
 811 TAT AAG CTA CCT TTT GCT TTG CAG TGG ATC TGG CCC CTT CCT TTG GCG GTA GGT ATT TTT TTG GCA CCA GAG TCT CCA TGG TGG CTG GTT

     Lys Lys Gly Arg Ile Asp Gln Ala Arg Arg Ser Leu Glu Arg Ile Leu Ser Gly Lys Gly Pro Glu Lys Glu Leu Leu Val Ser Met Glu   330
 901 AAA AAA GGA AGG ATT GAT CAG GCG AGG AGA TCA CTT GAA AGA ATA TTA AGT GGT AAA GGA CCC GAG AAA GAA TTA CTA GTG AGT ATG GAA

     Leu Asp Lys Ile Lys Thr Thr Ile Glu Lys Glu Gln Lys Met Ser Asp Glu Gly Thr Tyr Trp Asp Cys Val Lys Asp Gly Ile Asn Arg   360
 991 CTC GAT AAA ATC AAA ACT ACT ATA GAA AAG GAG CAG AAA ATG TCT GAT GAA GGA ACT TAC TGG GAT TGT GTG AAA GAT GGT ATT AAC AGG

     Arg Arg Thr Arg Ile Ala Cys Leu Cys Trp Ile Gly Gln Cys Ser Cys Gly Ala Ser Leu Ile Gly Tyr Ser Thr Tyr Phe Tyr Glu Lys   390
1081 AGA AGA ACG AGA ATA GCT TGT TTA TGT TGG ATC GGT CAA TGC TCC TGT GGT GCA TCA TTA ATT GGT TAT TCA ACT TAC TTT TAT GAA AAA

     Ala Gly Val Ser Thr Asp Thr Ala Phe Thr Phe Ser Ile Ile Gln Tyr Cys Leu Gly Ile Ala Ala Thr Phe Val Ser Trp Trp Ala Ser   420
1171 GCT GGT GTT AGC ACT GAT ACG GCT TTT ACT TTC AGT ATT ATC CAA TAT TGT CTT GGT ATT GCT GCA ACG TTT GTA TCC TGG TGG GCT TCA

     Lys Tyr Cys Gly Arg Phe Asp Leu Tyr Ala Phe Gly Leu Ala Phe Gln Ala Ile Met Phe Phe Ile Ile Gly Gly Leu Gly Cys Ser Asp   450
1261 AAA TAT TGT GGC AGA TTT GAC CTT TAT GCT TTT GGG CTG GCT TTT CAG GCT ATT ATG TTC TTC AAT ATC GGT GGT TTA GGA TGT TCA GCA

     Thr His Gly Ala Lys Met Gly Ser Gly Ala Leu Leu Met Val Val Asn Phe Phe Tyr Asn Leu Gly Ile Ala Pro Val Val Phe Cys Leu   480
1351 ACT CAT GGC GCT AAA ATG GGT AGT GGT GCT CTT CTA ATG GTT GTC GCG TTC TTT TAC AAC CTC GGT ATT GCA CCT GTT GTT TTT TGC TAA
```



However the Mal61 maltose permease transports only maltose and turanose, while the Agt1p has a much broader substrate specificity (Cheng and Michels, 1991; Han *et al.*, 1995).

Glucose-induced inactivation of maltose permease.

Glucose is a global metabolic regulator in *Saccharomyces* that controls the expression of many genes at both the transcriptional and post-translational levels (Thevelein and Hohmann, 1995). Glucose-induced inactivation (sometimes referred to as catabolite inactivation) was first identified in *Saccharomyces* more than 50 years ago (Gorts, 1969, reviewed in Holzer, 1984). The most extensively studied enzyme subject to glucose-induced inactivation is fructose-1,6-bisphosphatase. In response to the addition of glucose to the growth media, this enzyme is reversibly inactivated by phosphorylation on a single serine residue resulting in a loss of catalytic activity. The enzyme is then irreversibly inactivated by proteolytic degradation (Chiang and Schekman, 1991, Schork, *et al.*, 1994).

The addition of glucose to maltose grown cells results in a complete inactivation of maltose uptake within 60 minutes (Gorts, 1969). Ethanol and acetate, carbon-starvation conditions, do not stimulate this rapid inactivation (Gorts, 1969; Lucero *et al.*, 1993). The inactivation is irreversible since *de novo* protein synthesis is required to recover the ability to transport maltose suggesting that it could result from the proteolytic degradation of the permease (Gorts, 1969). In contrast, Peinado and Loureiro-Dias (1986) reported that glucose-

induced inactivation of the maltose permease is a result of a change in substrate affinity due to some reversible modification process.

Chapter 1 describes work carried out by myself and Hua Jiang characterizing the glucose-induced inactivation of maltose permease in a genetically defined strain that expresses only a hemagglutinin epitope-tagged allele of *MAL61* referred to as *MAL61/HA* (Medintz *et al.*, 1996). The results show that glucose, but not ethanol, inactivated maltose transport activity in two steps: glucose causes an initial very rapid loss of maltose transport activity with very little loss in protein levels and then a slower loss of maltose transport activity that is associated with a decrease in maltose permease protein levels. We further demonstrated that this glucose-induced proteolysis of Mal61/HAp is dependent on endocytosis, vesicular sorting, and vacuolar degradation, and is independent of proteasomal degradation. We also show that Mal61/HAp exists in differentially phosphorylated states depending upon carbon source.

Two glucose sensing/signaling pathways for glucose-induced inactivation of maltose permease.

Since glucose not only causes the inactivation of maltose permease, but also many aspects of cellular metabolism associated with carbohydrate utilization at both the transcriptional and the post-translational level, an understanding of the glucose sensing/signaling pathways becomes important. This became the main focus of the thesis project of Hua Jiang. Many of the genes involved in glucose regulation of transcription have been identified and include *SNF3, RGT2, GRR1,* and *RGT1* (Ozcan *et al.*, 1996a,b). By testing the roles of these genes in

the glucose-induced inactivation of maltose permease, at least two signaling pathways were identified that are used to monitor glucose levels and to stimulate glucose-induced inactivation of maltose permease (Jiang *et al.*, 1997).

Pathway 1, which is independent of glucose transport, appears to be modulated by Rgt2p, a glucose sensor present in the plasma membrane. Rgt2p and the Snf3p are homologous membrane localized proteins which have been identified as extracellular glucose sensors (Ozcan and Johnston, 1996). The *RGT2-1* allele, which was identified as a dominant mutation causing constitutive expression of *HXT1* encoding a low-affinity glucose transporter, also causes the constitutive proteolysis of maltose permease in the absence of glucose. The proposed model of glucose-induced inactivation of maltose permease proposes that a high concentration of extracellular glucose is sensed by Rgt2p and transduction is mediated by Grr1p and Glc7p/Reg1p protein phosphatase type-1 (Jiang *et al.*, 1997; Jiang *et al* , submitted). Pathway 2 uses the high rates of glucose transport and phosphorylation (or other fermentable sugars) to generate a signal (Jiang *et al.*, 1997; Jiang *et al* , submitted[b]).

Proteolytic pathways in ***Saccharomyces***.

There are two major proteolytic sites in *Saccharomyces cerevisiae* and other eucaryotes that are utilized for the degradation of intracellular proteins: the cytosolic proteasome and the vacuole (the *Saccharomyces* lysosome)(Jones, 1991). The vacuole is the site of proteolysis of proteins that enter the cell from the extracellular space, via endocytosis, and of the degradation of long-lived proteins. Proteosomal degradation is responsible for the highly selective turnover

of intracellular proteins. Under stress conditions, the vacuole will also be utilized for intracellular protein degradation by a process callled autophagy (Ciechanover, 1994).

The ubiquitin-proteasome pathway.

Ubiquitin, a 76 residue polypeptide, plays a major role in non-lysosomal proteolysis. In an ATP-dependent manner, ubiquitin is covalently bound to proteins that are to be degraded in the proteasome. Strains carrying mutations in the protein components of the proteasome accumulate proteins which are normally degraded via this pathway (Simoen, *et al.*, 1992; Hochstrasser,1996).

Conjugation of ubiquitin to proteins destined to be degraded by the proteasome follows a general 3-step mechanism. First, the C-terminal Gly of ubiquitin is activated to a high energy intermediate, using ATP, by the ubiquitin-activating enzyme, E1. The substrate protein to be ubiquitinated is bound to the ubiquitin-protein ligase, E3. Ubiquitin conjugating enzyme (Ubc proteins), E2, transfers ubiquitin from E1 to the substrate bound to E3. A bond is formed between the activated C-terminal Gly of ubiquitin and the side chain of a Lys residue of the substrate protein. Successive reactions synthesize a polyubiquitin chain to various lysines of the previous ubiquitin. E2 and E3 complexes may also be involved in substrate recognition. Monoubiquitin modification, by E2, does not target substrates for degradation (Hochstrasser, 1996).

Following formation of the full ubiquitin conjugate, the protein substrate is delivered to and degraded by the proteasome. The proteasome is a 26S multi-subunit complex of proteases that contains a 'catalytic core' known as the 20S

protease complex. The proteasome specifically degrades proteins conjugated to ubiquitin in an ATP-dependent manner (Ciechanover, 1994). Free, reutilizable ubiquitin is released during the degradation of ubiquitin conjugates (Ciechanover, 1994). In yeast this is the function of the *DOA4* gene product and several other ubiquitin hydrolases. Doa4p is integral to the degradation of ubiquinated proteins and functions late in the proteolytic pathway by cleaving ubiquitin from substrates still bound to protease (Papa and Hochstrasser, 1993).

Many of the genes that code for homologues to these proteins have been identified in yeast. These include 13 genes that encode distinct E2 enzymes (*UBC* genes) and other enzymes which function in the ubiquitin-dependant degradation of proteins in several subcellular compartments including the nucleus and peroxisome. *UBA1* codes for the ubiquitin-activating enzyme and is essential for viability (Jentsch, 1992, Hochstrasser, 1996). *UBC1* mediates vital functions along with other Ubc proteins including sporulation/germination and cell cycle induction. Most notable in this group are *UBC4* and *UBC5,* involved in the degradation of most short-lived and abnormal cytoplasmic proteins (Seufert and Jentsch, 1990; Ciechanover, 1994). *UBC2* is involved in a variety of cellular processes such as DNA repair, sporulation and repression of retrotransposition (Ciechanover, 1994). *UBC6* and *UBC7* are involved in the degradation of the *MATα2* regulator (Hochstrasser, *et al.*, 1991). *UBC3* (*CDC34*) and *UBC9* are essential for viability and function in cell cycle progression (Jentsch, 1992). *UBC10* is essential for peroxisome biogenesis (Ciechanover, 1994). E3 proteins have also been isolated and characterized in yeast. These include the

NPI1/RSP5 gene, an essential gene involved in induced degradation of the general amino acid permease and the uracil permease (Hein *et al.*, 1995)

ac olar roteolysis.

The yeast vacuole (lysosome) functions as the central organelle for non-specific proteolysis. The bulk of proteins to be degraded in the vacuole enter via some sort of endocytotic process, including receptor mediated endocytosis and autophagocytosis (Knop, *et al.*, 1993). These are complicated processes involving recognition of coated pits by adaptor proteins, sorting into early and late endosomes, recycling of reusable proteins, followed by delivery to the vacuole as well as activation of proteolysis (Robinson, 1992).

The vacuole is the site of degradation of long-lived proteins. Mutants defective in the vacuolar proteases, such as PrA (encoded by *PEP4*), accumulate long-lived proteins in this organelle (Egner, *et al.*, 1993). During active growth, the vacuole accounts for 40% of overall protein degradation, and, under starvation conditions, this increases to 85% (Teichert, *et al.*, 1989). In animal cells the main route of degradation of plasma membrane proteins is endocytotic internalization followed by lysosomal proteolysis (Hare, 1990). The same may be true of yeast plasma membrane proteins. Examples of yeast membrane proteins demonstrated to be degraded in the vacuole include the maltose permease (Medintz *et al.*, 1996), the inositol permease (Lai, *et al.*, 1995), the uracil permease (Volland, *et al.*, 1994), the *PDR5* multidrug transporter (Egner and Kuchler, 1996), the ABC-transporter *STE6* (Kolling and Hollenberg, 1994), the galactose transporter *GAL2* (Horak and Wolf, 1997) as

well as the a-factor and α-factor receptors (Davis, *et al.*, 1993; Singer, *et al.*, 1990; Hicke *et al.*, 1995).

The role of ubiquitination in signaling endocytosis.

Recent evidence has implicated ubiquitin conjugation in more than just cytosolic and denatured protein degradation. Ubiquitination allows the timed destruction of key proteins in the cell cycle including cyclins, chromosomal tethers and kinase inhibitors (Barinaga, 1995). Ubiquitination-dependent activation of IKBα protein kinase activity has been demonstrated (Hochstrasser, 1996). Several recent studies have implicated ubiquitin conjugation as a signal for endoytosis in yeast and higher eucaryotic cells. These include the yeast a- and α-factor receptors, galactose transporter (Gal2p), the yeast uracil permease (Fur4p), the ABC-transporter Ste6, and the Gap1 general amino acid permease (Hicke and Riezman, 1996; Roth and Davis, 1996; Horak and Wolf, 1997; Kolling and Hollenberg, 1994; Galan *et al.*, 1996; Marchal *et al.*, 1998; Springael and Andre, 1998). The exact mechanism by which ubiquitination stimulates endocytosis of these proteins remains to be elucidated. Current models suggest that covalent modification of Ub to a cell surface protein might constitute a sufficient signal for endocytosis or it might allow the protein to be recognized by endocytosis machinery or even promote movement of ubiquitinated proteins into membrane regions undergoing active endocytosis (Hicke and Riezman, 1996).

Ubiquitination signals the ligand-stimulated endocytosis of the α-factor receptor. Upon binding of α-factor, the Ste2p receptor activates a signal

transduction pathway that stimulates endocytosis of the receptor-ligand complex and also induces the ubiquitination of the Ste2p C-terminal cytoplasmic tail. This ubiquitination is required for stimulated endocytosis of Ste2p since endocytosis is 5 to 15 fold slower in strains with multiple ubiquitin conjugation enzyme (*ubc1 ubc4 ubc5*) mutants. A C-terminal truncation of Ste2p did not affect the rapid ubiquitination and endocytosis, but both could be eliminated by a single lysine to arginine substitution in a SINNDAKISS sequence of this C-terminal truncated tail (Hicke and Reizman, 1996).

Similar results have also been demonstrated for the yeast a-factor receptor, Ste3p (Roth and Davis, 1996). Phosphorylation of the Ste3p increases upon binding by the a-factor followed by ubiquitination. In strains mutated for ubiquitin recycling, the level of receptor ubiquitination is substantially decreased. This correlates with an impairment in receptor turnover of up to 6-fold. Mutations in ubiquitination conjugating enzymes resulted in substantially less receptor ubiquitination. Ligand binding to the receptor stimulated not only ubiquitination but subsequent endocytosis, which could be impaired by depleting available ubiquitin. This suggested that the covalent attachment of ubiquitin to this membrane receptor triggered its endocytosis (Roth and Davis, 1996).

The yeast plasma membrane ABC-transporter *STE6* undergoes ubiquitination. Degradation of Ste6p occurs in the vacuole since the strongest stabilizing effect, half-life increase to > 2 h from 13 min, is seen in strains carrying mutations in the vacuolar protease, Pep4p. The half-life of Ste6p is also increased 3-fold in the *ubc4 ubc5* double mutant. Additionally Ste6 protein

accumulates in a ubiquitinated form in the plasma membrane of endocytosis mutants, suggesting that ubiquitination may serve as the internalization signal for endocytosis of this protein (Kolling, *et al.*, 1994).

The yeast uracil permease, *FUR4*, also undergoes ubiquitination as part of the signal for endocytosis. This process appears to be mediated by the *NPI1/RSP5* ubiquitin protein ligase, E3, since ubiquitinated uracil permease is barely detectable in *npi1* loss-of-function mutants (Galan *et al.*, 1996). A PEST-like sequence located within the cytoplasmic N-terminus of the protein is essential for the efficient phosphorylation and subsequent ubiquitination of this protein (Marchal *et al.*, 1998). Degradation of this endocytosed protein also occurs in the vacuole since turnover is greatly reduced in *pep4* vacuolar degradation mutants and is unaffected in proteasomal mutants (Galan *et al.*, 1996).

Several other examples of this process also exist. The yeast multidrug transporter, *PDR5*, is ubiquitinated in the plasma membrane prior to endocytosis and degradation in the vacuole (Egner and Kuchler, 1996). Evidence also exists for ubiquitin dependent signaling of endocytosis and vacuolar degradation of the *STE6* gene product, the a–factor transporter (Loayza and Michaelis, 1998). Glucose- induced proteolysis of the Gal2 galactose permease is dependent on endocytosis and vacuolar degradation and is independent of proteasomal degradation. Moreover this protein has been shown to be ubiquitinated under conditions of glucose-induced inactivation suggesting a similar process (Horak and Wolf, 1997). The rich nitrogen-regulated ubiquitination of the Gap1

permease of yeast has been shown to be associated with internalization of this protein (Springael and Andre, 1998). Ubiquitination and subsequent vacuolar degradation of Gap1p are impaired in *npi1* strains that contain a loss-of-function mutation in that ubiquitin-protein ligase (Springael and Andre, 1998).

A close analog of this endocytosis signalling pathway also may occur in higher eucaryotic cells. Studies have shown that the ubiquitin conjugation system is required for ubiquitination of the growth factor receptor following binding of the ligand. Endocytosis of ligand-bound growth hormone receptor requires this ubiquitin conjugation and degradation of the receptor occurrs within the endosomal/lysosomal compartment (Strous *et al.*, 1996).

Endocytosis and internalization signals.

Many integral plasma membrane proteins, including receptors, transporters and ATPases, utilize the endocytotic process for internalization and down-regulation. During endocytosis, the plasma membrane pinches off after invaginating and internalizes membrane lipids, proteins and extracellular solutes. This may occur as a result of ligand binding to a specific receptor, in a constitutive manner, or as a result of some other signal (Hubbard,1989). Ligand-driven, receptor-mediated endocytosis involves clathrin coated pit formation while the internalization of other proteins may be more non-selective (Hubbard, 1989). After entering the cell, both the membrane and its contents, now in coated vesicles, are delivered to the first subcompartment of a complex membrane system, collectively referred to as endosomes. Once in these 'early' endosomes the contents are sorted into one of several pathways, including the recycling back

to the plasma membrane of uncoupled receptors. A second pathway delivers, via 'late' endosomes, dissociated ligands, solutes and other proteins to the lysosome (Hubbard,1989; Mellman, 1996; Schmid, 1996). The following short discussion will focus on the characteristics of internalization signals found on various membrane proteins.

Short amino acid sequences often serve as signals for endocytosis

Analysis of constitutively recycled receptors in higher eukaryotes has revealed specific short peptide internalization signals that are necessary for endocytosis (Trowbridge, 1991; Mellman, 1996). These peptides may be important to the concentration of receptors into coated pits, either bound to ligand or unbound in a constitutive manner. Through the use of mutational analysis many of the internalization signals on receptors have been identified and characterized.

Rapid internalization of the low-density lipoprotein receptor (LDLR) requires the first 22 amino acids (residues 790-811) of the carboxy-terminal cytoplasmic domain. Tyr807 and Phe802 are considered critical for rapid internalization. The peptide Phe-X-Asn-Pro-X-Tyr is believed to be the complete LDLR internalization signal and is highly conserved across at least six species whose genome encodes the LDLR protein or similar genes (Trowbridge,1991; Davis, *et al.*, 1987; Chen, *et al.*, 1990; Lehrman, *et al.*, 1985).

Mutational analysis of the transferrin receptor (TR) indicated that residues 19-28 of the cytoplasmic domain were sufficient for rapid internalization and that Tyr20, the only tyrosine residue in the cytoplasmic tail, is critical (the number, 20,

indicates the position in the C-terminal cytoplasmic tail after the last transmembrane domain). The tetrapeptide Tyr-X-Arg-Phe has been identified as the internalization signal and it has been shown to be independent of position, in the carboxy-terminus, provided that it is separated from the transmembrane region by at least 7 residues. A second copy of the signal can even enhance endocytosis (Collawan, *et al.,* 1993; Girones, *et al.*, 1991; Jing, *et al.,* 1990; Dargemont, *et al.*, 1993).

Other examples of membrane protein internalization signals have been defined. The mannose-6-phosphate receptor signal, defined by deletion and truncation mutations, is Tyr-X-Tyr-X-Lys-Val at positions 24 thru 29 of the carboxy-terminal cytoplasmic tail (numbers indicate position in the C-terminal cytoplasmic tail) (Canfield, *et al.*, 1991). The erythropoietin receptor contains the carboxy-terminal signal WSAWSE, which is believed to cause internalization after ligand binding in erythroid cells. Specific mutation of the tryptophan residues in this signal results in failure to internalize receptor after ligand binding (Quelle, *et al,* 1992). Although a lysosomal membrane protein, the internalization signal found in the cytoplasmic tail of lysosomal acid phosphatase consists of the peptide PGYRHV. This functions in a similar manner to other membrane proteins internalization signals in that it mediates the endocytosis of this protein into the lysosome (Lehmann, *et al.*, 1992). The carboxy-terminal domain of the adipocyte/skeletal muscle glucose transporter (GLUT4) contains a double leucine rapid endocytosis signal, although other research indicates that the amino

terminus of GLUT4 may also function as an internalization motif (Garippa, *et al.*, 1994; Corvera,*et al.*, 1994)).

The internalization capability of some of these proteins can also be restored by using transplanted endocytosis signals from other proteins. High efficiency endocytosis of the transferrin receptor can be restored by transplanted LDL and mannose-6-phosphate receptor internalization signals (Collawn, *et al.*, 1991). The amino terminus of GLUT4 also can function as an internalization signal when substituted by the transferrin receptor cytoplasmic domain (Garippa, *et al.*, 1994). Tyrosine residues as part of the internalization signal.

Analysis of peptide internalization signals found in several membrane proteins strongly suggests a role for tyrosine residues and phosphorylation as part of the internalization signal. By using the internalization signal of the influenza virus hemagglutin protein competent for internalization in coated pits, HA-Y543, a consensus signal of a short amino acid sequence with polar or basic residues preferred at certain positions on either side of an essential tyrosine residue, was defined by mutational analysis (Ktistakis, *et al.*, 1990). Other consensus sequences containing tyrosine residues, that are highly variable, may also substitute and function as the signal in a degenerate manner (Naim, *et al.*,1994). The LDLR, TR, mannose-6-phosphate receptor and lysosomal acid phosphatase all contain essential tyrosine residues in the internalizaton signal (Trowbridge, 1991; Peters, *et al.*, 1990). Internalization of the polymeric immunoglobulin receptor requires two internalization signals, each of which contains a tyrosine residue, in the C-terminal cytoplasmic domain (Okamoto, *et*

al., 1992). Replacement of the key tyrosine residue on the bovine mannose-6-phosphate receptor with other aromatic residues gave rise to mutant proteins with only 10% of the turnover seen in wild-type proteins (Jadot, *et al.*, 1992).

Phosphorylation plays a role in internalization.

In many instances, phosphorylation may be a key signal. It appears to be involved in either the clustering of receptors in coated pits or in the subsequent events of endocytosis. Phosphorylation is essential for insulin receptor internalization and interaction with its substrate(s) (Kublaoui, *et al.*, 1995). Inhibitors of tyrosine kinases affect early receptor internalization from the plasma membrane of the asialoglycoprotein receptor. This correlates with their inhibition of tyrosine phosphorylation of this receptor *in vitro.* Mutation of the asialoglycoprotein tyrosine at position 5 of the H1 subunit relieved this inhibitory effect when subjected to the same treatment (Fallon, *et al.*, 1994). These same inhibitors affected constitutive internalization of the transferrin receptor.

Hyperphosphorylation is required for endocytosis of the a-factor receptor in yeast (Zanolari, *et al.*, 1992). These results suggest phosphorylation, especially of the tyrosine residues in the internalization signal, as an early event in the endocytosis of some integral membrane proteins.

Conformation of the region surrounding the signal.

A tight turn is implicated as a conformational determinant for high efficiency endocytosis (Trowbridge, 1991). Collawn *et al* (1990) reasoned that the TR signal Tyr-X-Arg-Phe might have a preferred local structure. A search of the Brookhaven Protein Data Bank of crystallographic structures revealed that

the most closely analogous structures had a strong intrinsic propensity to be surface-exposed tight turns. The conformation of the tetrapeptide is not influenced by adjacent residues. Analogues to the LDLR signal were also predominantly tight turns but in the form of a reverse-turn. By assuming a tight conformation independent from the surrounding structures, these signal residues may interact with other proteins. This may also explain why transplanting signals from one protein, LDLR, to another, an internalization signal deletion mutant transferrin receptor, allows that transferrin receptor to still retain rapid internalization capabilities. This may also explain why the GLUT4 amino terminus may function as an internalization signal, assuming the right conformational turn is available. Four to six residue signals may thus adopt conformations that facilitate the endocytotic process (Trowbridge, 1991; Collawn, *et al.*, 1990; Ktistakis, *et al.*,1990; Bansal, *et al.*, 1991).

Endocytosis signals in yeast membrane proteins.

The carboxy-terminal domain of the yeast a-factor receptor (*STE2*), a G-protein-coupled a-pheromone receptor, is the putative regulatory domain (Reneke, *et al.*, 1988). Receptor endocytosis is independent of G-protein mediated signal transduction (Zanolari, *et al.*, 1992). The first 39 residues of the cytoplasmic tail contain sufficient information for internalization, and a specific point mutation in this region (K^{337} to R^{337}) renders the receptor resistant to endocytosis. The putative endocytosis signal appears to be SINNDAKSS (Rohrer, *et al.*, 1993). Additionally, adding the sequence DAKSS onto a C-terminally truncated receptor restores endocytosis. A mutant, in which part of the

cytoplasmic tail of the Ste2p receptor is deleted, is defective in constitutive, but not pheromone-induced internalization suggesting that different mechanisms regulate constitutive and pheromone-mediated internalization (Davis, *et al.*, 1993). In fact, later studies have shown this peptide signal is actually critical for endocytosis, and is the site for a-factor-stimulated ubiquitination that results in rapid receptor-ligand internalization (Hicke and Riezman, 1996).

Other examples of short amino acid sequences in yeast that contribute to catabolite inactivation, ubiquitination and endocytosis are also being revealed. It has been shown that a di-leucine motiff and nearby sequences are required for the ammonia-induced inactivation and degradation of the general amino acid permease encoded by *GAP1* (Hein and Andre, 1997). An *in vivo* isolated mutant that contained a single Glu→Lys substitution in an amino acid context, similar to the DXKSS context mentioned above, EEKAI, protected this permease against ammonia-induced inactivation. By substituting two alanines for a di-leucine motif, a similar result was obtained. Mutation of a putative phosphorylation site upstream from this di-leucine motif had no effect on the regulation or degradation of activity of Gap1p. In contrast, deleting the last eleven amino acids of Gap1p, a region conserved in other amino acid permeases, conferred resistance to ammonia-induced inactivation (Hein and Andre, 1997). It is likely that these changes in the Gap1 permease rendered it resistant to ammonia-triggered inactivation by impairing the internalization of the permease from the membrane. The process of ammonia-triggered inactivation seems to be a progressive removal of the permease from the plasma membrane, since cells deficient in

endocytosis also become deficient in ammonia-triggered inactivation (Hein and Andre, 1997). As previously mentioned, ubiquitination is likely to be involved since depletion of ubiquitin or loss of Npi1p/Rsp5p ubiquitin protein ligase function results in loss of ammonia-triggered inactivation (Hein *et al,* 1995). Further studies on the ubiquitination and endocytosis of Gap1p have shown that the area around the di-leucine motif may adopt a predicted stable α-helical turn and that a nearby glutamate residue may also be important to this structure. Interestingly, Gap1p mutants could be constructed that were insensitive to ammonia-inactivation but still were ubiquitinated (Springael and Andre, 1998). Taken together, these results argue for a complex system whereby different sequences within the Gap1p may interact and also separately contribute to ubiquitination, endocytosis and degradation.

The di-leucine motif, which is upstream from the EEAKI sequence in Gap1p, has been shown to be part of a peptide signal required for internalization of several-surface proteins of higher cells including the T-cell surface antigen CD4, the γ and δ chains of the T-cell receptor, the insulin receptor, the adipocyte/skeletal muscle glucose transporter Glut4p, the interleukin-6-receptor and the β_2-adrenergic receptor (Letourneur and Klausner, 1992; Shin *et al.*, 1994; Garippa *et al.*, 1994; Haft *et al.*, 1994; Dittrich *et al.*, 1996; Gabilondo *et al.*, 1997). A role of di-leucine peptides in targeting membrane proteins to endosomes and lysosomes has also been reported (Sandoval and Bakke, 1994). It has been proposed that these di-leucine sequences act as binding sites for

adaptor proteins required for intracellular protein trafficking (Gabilondo *et al.*, 1997).

Another recently discovered endocytosis signal in yeast is the short peptide sequence NPFXD (Tan *et al.*, 1996). The trans-golgi network membrane Kex2 protein is mislocalized to the plasma membrane in a *vps1* mutant and then undergoes subsequent endocytosis that is dependent on clatharin and the *END3* gene product. By constructing a chimeric protein consisting of the Kex2p cytoplasmic domain fused to an endocytosis-defective form of the α-factor receptor the critical residues to this process were investigated (Tan *et al.*, 1996). Internalization of this chimeric protein, Stex22p, did not require the Kex2p Golgi localization motif. Instead the sequence NPFSD located 37 amino acids from the COOH terminus was shown to be critical to Stex22p endocytosis. Converting the N, P, or F residues to alanine abolished internalization and changing the D to A resulted in severely impaired internalization. This peptide signal restored uptake to an endocytosis-defective Ste2p chimera lacking its own intrinsic lysine based endocytosis signal when added to the C-terminus.

A similar NPF sequence is present in the cytoplasmic domain of the a-factor receptor, Ste3p. A truncated form of this protein, Ste3-Δ365p, is efficiently transported to the membrane but is not internalized constitutively, rather it is only internalized in response to a-factor (Davis *et al.*, 1993). Mutation of this NPF sequence prevents pheromone-stimulated endocytosis in this truncated form of Ste3p (Tan *et al.*, 1996). This suggests that NPFSD is a clathrin-dependent

endocytosis signal distinct from the Golgi localization motif and from the lysine-based, ubiquitin-dependent endocytosis signals (Tan *et al.*, 1996).

Functional domains in selected yeast membrane proteins.

Through the use of deletion/mutational analysis, many recent studies have begun to elucidate the function of domains or regions within several eukaryotic membrane proteins. An understanding of the context from which these domains work within has also begun to emerge. Several recent examples exist and will be briefly discussed here.

Two glucose transporter homologs exist in *Saccharomyces cerevisiae* that are encoded by the *SNF3* and *RGT2* genes. These two membrane proteins contain unusually long C-terminal tails which are predicted to be cytoplasmic. It is believed that these proteins act as glucose sensors that can detect both high and low glucose concentrations extracellularly (Ozcan *et al.*, 1998). By transplanting and attaching the Snf3 and Rgt2 C-terminal tail regions onto the Hxt1 and Hxt2 glucose transporters, respectively, the authors were able to show that the two Hxt transporters were converted into glucose sensors that were able to generate a signal for glucose-induced *HXT* gene expression. These results suggest that perhaps, among other functions, the C-terminal tail regions of these two proteins are responsible for generating a glucose-induced signal and are thus signaling domains (Ozcan *e al.*, 1998).

Another yeast integral membrane protein that has undergone a significant functional domain analysis is the ABC-transporter Ste6. This protein contains two homologous halves, each with six membrane spanning segments and

predicted ATP nucleotide binding domains (Berkower and Michaelis, 1991). Both of these halves are critical for function. So called 'half molecules' are unable to provide a-factor transport when individually expressed, however co-expression of both half-molecules in the same cell leads to a functional reconstitution of STE6-mediated a-factor transport (Berkower and Michaelis, 1991).

Further analysis of the Ste6p showed that it can actually be sub-divided into 4 modular units consisting of the 2 membrane spanning domains (MSDs) and the 2 nucleotide-binding domains (NBDs). Through the dissection and analysis of the interaction between these modules, physical interaction between 'half-molecules' was demonstrated as well as interaction between 'half-molecules and full-length Ste6p (Berkower *et al.*, 1996). Defective activity in a full-length STE6 mutated in one of its NBDs can be corrected by co-expression of the corresponding 'wild-type' half molecule. Furthermore, a 'quarter molecule' consisting of the N-terminal MSD interacts physically and functionally with a C-terminal 'three quarter' molecule which suggests that information regarding the assembly of Ste6p from partial molecules is partially contained within its membrane spans (Berkower *et al.*, 1996).

The linker region which connects the two homologous halves of Ste6p has been shown to contain a signal that mediates ubiquitination and fast-turnover of Ste6p (Kolling and Losko, 1997). This signal is also functional in the context of another plasma membrane protein, the plasma membrane Atpase Pma1p, and results in the rapid turnover of this usually stable membrane protein when fused

to its C-terminal region. The linker region contains an acidic stretch called an A-box. Deletion of this A-box strongly stabilizes Ste6p turnover. The A-box also contains the sequence DAKTI which resembles the putative endocytosis signal of the α-factor receptor, Ste2p. Deletion of DAKTI also stabilized Ste6p turnover, but not as strongly as entire A-box deletion. A correlation between ubiquitination and half-life of the protein could also be established with loss of ubiquitination stabilizing the protein at the membrane suggesting that ubiquitination is required for efficient endocytosis from the plasma membrane (Kolling and Losko, 1997).

Another sequence/domain that may contribute to the rapid degradation of yeast proteins is that of the PEST sequence. PEST sequences are rich in proline [P], glutamic acid [E], serine [S] and threonine [T] (Rechsteiner and Rogers, 1996). PEST sequences known to function as proteolytic signals can be found on proteins such as Gcn4, Cln2, Cln3 and $I_K\beta_\alpha$ (Rechsteiner and Rogers, 1996). Evidence also exists for the function of these signals as conditional proteolytic signals.

Recently it has been shown that a PEST-like sequence mediates phosphorylation and efficient ubiquitination of the yeast uracil permease encoded by the *FUR4* gene (Marchal *et al.*, 1998). This sequence is located within the cytoplasmic N-terminus of the protein. Internalization of the transporter was reduced when serines within this region were converted to alanines and internalization was severely impaired when all five serines were mutated or the entire region was deleted. Phosphorylation and ubiquitination was inversely correlated to the number of serines replaced by alanine. A serine free version of

this sequence resulted in the protein being very poorly phosphorylated and elimination of the sequence prevented ubiquitination. Replacement of serines in the PEST-like sequence with glutamic acids allowed efficient permease turnover, suggesting that PEST serines are phosphoacceptors (Marchal *et al.*, 1998).

Summary of thesis goals

The major goal of my thesis project is to characterize the glucose-induced inactivation of maltose permease and to identify sequences within the maltose permease that are key to its function. I have shown that the maltose permease undergoes a bi-phasic inactivation characterized by a rapid loss of transport capability and a slower degradation. Degradation occurs in the vacuole, independent of the proteosome, and requires endocytosis. Maltose permease is ubiquitinated in response to glucose. Ubiquitination of maltose permease depends on the *RSP5/NPI1* ubiquitin protein ligase. A direct/indirect role for ubiquitin in induction of *MAL* genes is also suggested. Mutational/deletional analysis demonstrates that the C-terminus of the protein effects stable expression and may also be involved in induction. The N-terminus is involved in determining substrate affinity and glucose-induced inhibition of maltose permease. The role of ubiquitin in glucose-induced inactivation of maltose permease is described in chapter 2. Chapter 3 describes the mutational/deletional analysis of the maltose permease.

OVERVIEW

This thesis is organized into 3 main chapters. Chapter 1 describes the characterization of the glucose-induced inactivation of maltose permease, with emphasis especially placed on the mechanism of maltose permease proteolysis. Chapter 2 describes the role of ubiquitin and the ubiquitin conjugation system on glucose-induced proteolysis of maltose permease. Chapter 3 describes the mutational/deletion analysis of the N-terminal and C-terminal cytoplasmic domains of the Mal61/HAp maltose permease and characterization of the resultant mutants.

Chapter 1 is entitled "Characterization of the glucose-induced inactivation of maltose permease in *Saccharomyces*". This was published as a full paper which was co-authored by H. Jiang, myself, E.-K. Han, W. Cui, and C.A. Michels, and appeared in the Journal of Bacteriology (1996) 178:2245-2254. In this study, we characterized the glucose-induced inactivation of maltose permease in a genetically defined maltose fermenting strain that carried only a single *MAL* locus and 1 copy of each of the required *MAL* genes, including the *MAL61/HA* allele of maltose permease. Using strains carrying mutations in *END3*, *REN1*(*VPS2*), *PEP4*, and *PRE1 PRE2*, we demonstrated that the proteolysis of Mal61/HAp is dependent on endocytosis, vesicular sorting, vacuolar degradation, and is independent of the proteosome. Additionally, we demonstrate that Mal61/HAp exists in differentially phosphorylated forms in response to different carbon sources. Hua Jiang and myself were equally contributing first authors to this

study. Hua Jiang's contribution includes the construction of the parental strain, CMY 1001, and its isogenic *pep4Δ*, *end3-ts*, and *hxk2Δ* mutant strains as well as performing all of the sugar transport assays. My contribution to this study included the construction of the *ren1Δ* mutant strain and carrying out most of the Western analysis. In addition, E.-K. Han constructed the HA-tagged allele of Mal61 maltose permease and W. Cui demonstrated the differentially phosphorylated forms of maltose permease.

Chapter 2 is entitled "The role of ubiquitin conjugation in the glucose-induced proteolysis of *Saccharomyces* maltose permease". This manuscript is co-authored by myself, H. Jiang and C.A. Michels and has been accepted for publication to the Journal of Biological Chemistry. This paper addresses the role of ubiquitin, ubiquitin conjugation and the ubiquitin conjugation system on glucose-induced degradation of maltose permease. My contribution to this work includes the construction of all the mutant strains utilized, and carrying out most of the sugar transport assays, maltase assays and Western Analysis. Hua Jiang assisted in the construction of some of the strains and assisted in carrying out some of the sugar transport assays presented in Figure 1 and Figure 4.

Chapter 3 is entitled "Mutational analysis of the N-terminal and C-terminal cytoplasmic domains of *Saccharomyces* maltose permease. It is currently being prepared for submission as a manuscript and will be co-authored by myself and C.A. Michels. In this report we undertook the determination of portions of Mal61/HAp critical to not only function but also to glucose-induced inactivation of maltose permease. We found that the C-terminal cytoplasmic domain is

important for transport activity and signaling maltose induction of the *MAL* genes but does not appear to play a role in glucose-induced turnover. The N-terminal cytoplasmic domain is important for glucose-induced inactivation of maltose permease and a putative PEST sequence is identified as a potential targeting sequence for degradation. In addition we found that mutation of potential endocytosis internalization signals to alanine did not significantly alter kinetics of glucose-induced inactivation and proteolysis of maltose permease.

CHAPTER 1

Characterization of the Glucose-induced Inactivation of Maltose Permease in *Saccharomyces*

This chapter has been reproduced with the permission of the publisher of the **Journal of Bacteriology.** It appeared in the **Journal of Bacteriology** 178:2245-2254.

ABSTRACT

The addition of glucose to maltose fermenting *Saccharomyces* cells causes a rapid and irreversible loss in the ability to transport maltose resulting both from the repression of transcription of the maltose permease gene and from the inactivation of maltose permease. The latter is referred to as glucose-induced inactivation or catabolite inactivation. We describe an analysis of this process in a maltose fermenting strain expressing a hemagglutinin-tagged allele of *MAL61*, encoding maltose permease. The transfer of maltose-induced cells expressing the Mal61/HA protein to rich medium containing glucose produces a decrease in maltose transport rates which is paralleled by a decrease in Mal61/HA maltose permease protein levels. In nitrogen-starvation medium, glucose produces a biphasic inactivation, that is, an initial, rapid loss in transport activity (inhibition) followed by a slower decrease in transport activity which correlates with a decrease in the amount of maltose permease protein (proteolysis). The inactivation in both rich and nitrogen-starved media results from a decrease in V_{max} with no apparent change in K_m. Using strains carrying mutations in *END3*, *REN1(VPS2)*, *PEP4*, and *PRE1 PRE2*, we demonstrate that the proteolysis of Mal61/HAp is dependent on endocytosis and vacuolar proteolysis, and is independent of the proteosome. Moreover, we show that the Mal61/HA maltose permease is present in at least two differentially phosphorylated forms.

INTRODUCTION

The addition of glucose to maltose fermenting *Saccharomyces* cells causes an irreversible loss in the ability to transport maltose which is more rapid than can be explained by the combined effects of glucose repression of maltose permease gene transcription and cell growth (Bustaria and Lagunas, 1985; Cheng and Michels, 1989; Gort, 1969). Similar irreversible inactivation of the high-affinity galactose and glucose transporters and of the gluconeogenic enzymes has been described, and the phenomenon is generally referred to as glucose-induced inactivation or catabolite inactivation (Bustaria and Lagunas, 1986; Dejuan and Lagunas, 1986; Holzer, 1976; Matern and Holzer, 1989; Ramos and Cirillo, 1989; Ramos *et al.*, 1989).

Early studies of the mechanism of glucose-induced inactivation of the gluconeogenic enzymes demonstrated glucose-stimulated proteolysis of these cytoplasmic enzymes (Muller and Holzer, 1981; Needleman, 1991; Tortora *et al.*, 1981). The mechanism of fructose-1,6-bisphosphatase inactivation has been investigated most extensively. Here, two distinct processes are stimulated in response to glucose: an initial, rapid, reversible phosphorylation which is correlated with a decrease in enzyme activity but not protein levels; and a slower, irreversible proteolysis (Chiang and Schekman, 1991; Mazon *et al.*, 1982; Tortora *et al.*, 1981). The pathway of the proteolysis is as yet unresolved with conflicting published reports of both vacuolar-dependent and proteosome-dependent proteolysis (Chiang and Schekman, 1991, 1994, Schork *et al.*, 1994).

MAL61 of the *MAL6* locus and its nearly identical homologues at the other *MAL* loci (*MAL11*, *MAL21*, *MAL31*, and *MAL41*) encode *Saccharomyces* maltose permease. Mal61p is a high-affinity (2-4 mM) proton/maltose symporter, and is a member of the twelve transmembrane domain family of sugar transporters (Cheng and Michels, 1989). Expression of *MAL61* is maltose-induced and glucose-repressed (Cheng and Michels, 1989, 1991; Needleman, 1991). Maltose induction is mediated at the transcriptional level by the Mal-activator, a sequence-specific DNA-binding transcription activator encoded by *MAL63* of the *MAL6* locus and its nearly identical homologues at the other *MAL* loci (*MAL13*, *MAL23*, *MAL33*, *MAL43*, and *mal64*). Glucose regulates maltose transport at two levels. First, glucose represses maltose permease gene transcription by mechanisms involving several gene products including Mig1p (the downstream target of the Snf1 protein kinase signal transduction pathway), Hxk2p (hexokinase2), and *grr1* (unknown function) reviewed in Johnston and Carlson. Second, glucose induces post-transcriptional events resulting in the rapid, irreversible loss in maltose transport activity (Gort, 1969).

In this report we use molecular genetic analysis to explore the mechanism of glucose-induced inactivation of maltose permease. Our results show that, inactivation in both rich and nitrogen-starvation medium results from a decrease of apparent V_{max} with no change in apparent K_m; in rich medium, loss of maltose transport activity is paralleled by a loss in maltose permease protein; in nitrogen-starvation medium, maltose transport is inactivated by two independent

mechanisms, a very rapid inhibition of transport activity, and a slower proteolysis of maltose permease protein; and that this proteolysis is dependent on endocytosis and vacuolar proteases, and is independent of a functional proteosome. In this last regard, our results are consistent with those in a recent report of the glucose-induced inactivation of maltose permease which employed some of the same mutations used in the present study (Riballo *et al.*, 1995). Additionally, our results suggest that differentially phosphorylated species of the Mal61p maltose permease are present in maltose-induced cells, and that the distribution of the phosphorylated species varies with carbon-source.

MATERIALS AND METHODS

Strains and growth conditions. The *Saccharomyces* strains used in this study and their relevant genotypes are listed in Table 1. Cells were grown either in rich medium (1% yeast extract, 2% peptone) plus the indicated carbon source or selection medium (0.67% yeast nitrogen base with ammonium) plus the appropriate supplements and the indicated carbon source.

Strain construction. An epitope-tagged allele of the cloned *MAL61* gene containing the sequence encoding the HA-epitope at the 5' end of the ORF was constructed by oligonucleotide-directed site-specific mutagenesis using the BioRad Mutagene kit (Kolodziej and Young, 1991). The added sequence encodes 15 residues: a Met residue, the 12 residue epitope derived from the influenza virus hemagglutinin protein, Pro and Gly residues to act as a hinge separating the epitope from the remainder of the protein, all followed by the Met residue at the start of the *MAL61* coding region. This cloned *MAL61/HA* gene was subcloned into a yeast *LEU2* integrating vector and targeted to the *MAL1* locus of strain 100-1A using homology between the plasmid copy of *MAL61/HA* and the genomic *mal11Δ::URA3* gene, containing an internal deletion of *MAL11*. A single copy integration strain was chosen by Southern analysis of the transformants, and uracil$^-$ recombinants were selected using 5-fluoro-orotic acid (Boeke *et al.*, 1984). Maltose fermentation was confirmed as in reference Charron *et al.* (1986). These were then screened using Southern analysis to confirm the loop-out, and Western analysis was done to confirm the presence of the epitope-tag. This procedure replaced at least part if not

all of the *mal11Δ::URA3* gene in strain 100-1A with *MAL61/HA* to produce strain 100-1A::MAL61/HA.

To facilitate the construction of isogenic mutant strains, we introduced a series of yeast selectable markers by crossing strain 100-1A::MAL61/HA strain to YPH500 (see Table 1), a maltose nonfermenting strain lacking a Mal-activator gene but carrying *MAL1*-linked and *MAL3*-linked structural genes (*AGT1 MAL12* and *MAL31 MAL32*) (unpublished results, Sikorski and Hieter, 1989). Strain CMY1001 is a maltose fermenting random segregant containing solely the *MAL1* locus derived from 100-1A::MAL61/HA, and was used in the following constructions (Table 1).

Strain CMY1002 carries a *pep4Δ* deletion/disruption and is otherwise isogenic to CMY1001. It was constructed by two-step gene replacement using plasmid pPLO2010 (obtained from Steven Nothwehr) which contains a *pep4Δ* allele lacking a *Hin*dIII fragment of the wild-type sequence carried on an *URA3* integrating vector. Plasmid DNA was linearized at a unique *Eco*RI site, which targets integration at the genomic *PEP4*, used to transform CMY1001, and uracil$^+$ transformants selected. Uracil$^-$, potential loop-out of the *URA3* gene and the adjacent *PEP4*, were selected using 5-fluoroorotic acid, and loss of vacuolar protease function was tested by the APNE plate assay (Boeke *et al.*, 1984; Wolf and Fink, 1975). Deletion of *PEP4* was confirmed by Southern analysis.

Strain CMY1003 is isogenic to CMY1001 but contains a *ren1Δ*. Plasmid pSL1572 (obtained from George Sprague) contains a *ren1Δ::LEU2* deletion/disruption. The plasmid was digested with *Bam*HI and *Sac*I, and used to

transform CMY1001 to leucine$^+$. Replacement of the genomic *REN1* was confirmed by Southern analysis.

Strain CMY1004 carries a temperature sensitive allele of *END3* constructed by integrative disruption of the genomic copy with plasmid pLC2 (obtained from Howard Reizman). Plasmid pLC2 carries the *end3-ts* gene sequence with a small deletion of the 5' end of the gene in a *LEU2* integrating vector. Plasmid DNA was digested at a unique *XhoI* site within the ORF, which targets integration to the genomic *END3* so as to create a deletion of the genomic copy plus integrate an *end3-ts* gene copy. Southern analysis was used to confirm that the event had occurred as expected (Raths *et al.*, 1993).

Strains WCG4a, WCG4-11/21a, and WCG11/22a are an isogenic strain series containing *PRE1 PRE2*, *pre1-1 pre2-1*, and *pre1-1 pre2-2* alleles, respectively (18). *PRE1* and *PRE2* encode components of the proteasome. None of these strains ferment maltose because they lack a Mal-activator gene, despite the fact that they carry the *MAL* structural genes. Thus, in order to carry out our analyses, we introduced into these strains the *MAL63* Mal-activator gene carried on a *URA3* CEN plasmid, pRS316MAL63 (Needleman,1991; Sikorski and Hieter, 1989). In order to be able to follow maltose permease protein levels, we also introduced into these strains the *MAL61/HA* allele constructed for this study on a *LEU2* CEN plasmid, pRS315MAL61/HA (Sikorski and Hieter, 1989).

Table 1. Saccharomyces strains used in this study

Strain	Genotype	Reference
CMY1001	*MATa MAL61/HA MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200*	This study
CMY1002	*pep4Δ* (isogenic to CMY1001)	This study
CMY1003	*ren1Δ::LEU2* (isogenic to CMY1001)	This study
CMY1004	*end3-ts* (isogenic to CMY1001)	This study
CMY1006	*hxk2Δ::URA3* (isogenic to CMY1001)	This study
WCG4a	*MATa his3-11,15 leu2-3,112 ura3-Δ5 GAL*	Heinemeyer et al., 1993
WCG4-11/21a	*pre1-1 pre2-1* (isogenic to WCG4a)	Heinemeyer et al., 1993
WCG4-11/22a	*pre1-1 pre2-2* (isogenic to WCG4a)	Heinemeyer et al., 1993
YPH500	*MATα leu2-Δ1 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 ura3-52 AGT1 MAL12 MAL31 MAL32*	Sikorski and Hieter, 1989
600-1B	*MATa MAL11 MAL12 MAL13 leu2-3,112 ura3-52*	Charron et al., 1986
100-1A	*MATa mal11Δ::URA3 MAL12 MAL13 leu2-3,112 ura3-52*	Charron et al., 1986

The *Saccharomyces* strains used in this study are listed. It should be noted that the *MAL61/HA* gene in CMY1001 is located at the *MAL1* locus position and replaces the *MAL11* sequence of the wild-type *MAL1* locus (See Materials and Methods)

Strain CMY1006 is isogenic to CMY1001 but contains a *hxk2Δ* null allele. Plasmid pRB528 (from David Botstein) carries a *hxk2::URA3* deletion/disruption. It was digested with *Eco*RI and used to transform CMY1001 to uracil$^+$. Replacement of the genomic copy of *HXK2* was confirmed by Southern analysis. CMY1006 was transformed with the constitutive Mal-activator gene *MAL64-R10* carried on the episomal *HIS3* vector pRS413 (Dubin *et al.*, 1989).

Inactivation protocol. Cells were grown at 30°C to early log phase (OD_{600} 0.1-0.3) in rich medium containing 2% maltose, harvested by filtration using cellulose filters, and resuspended in either rich medium or nitrogen-starvation medium (1.74 gm/liter yeast nitrogen base without amino acids and without ammonium sulfate) plus either 2% ethanol (vol/vol) or 2% glucose (wt/vol). At selected time intervals cells were harvested for Western analysis and maltose transport assays using cellulose filters. Growth dilution was calculated as the OD_{600} at time 0 divided by the OD_{600} at time X.

Maltose transport assay. Maltose transport was measured by the uptake of ^{14}C-maltose as described in Cheng and Michels (1991). Cells were harvested by filtration, washed and resuspended in 0.1M tartaric acid (pH 4.2) to $O.D._{600}$ 30. Eighty microliters of cells were taken and incubated with ^{14}C-maltose for 10 sec. at room temp. then washed 4 times with ice cold water, followed by scintillation counting. With the exception of the results shown in Figure 3, maltose transport rates were determined using 1mM maltose. Assays were done in duplicate on at least duplicate cultures.

Western analysis and quantitation of relative protein levels. Cells were harvested, quick frozen in a dry ice-ethanol bath, and stored frozen at -70°C until the preparation of protein extracts. The preparation of total cell protein extracts is according to the method described by Davis *et al.* (1993) with the addition of a protease inhibitor cocktail (Ausubel, *et al.*, 1995). Where noted, protein kinase and phosphatase inhibitors were also included (James, *et al.*, 1989). After assaying, equal amounts of protein (usually about 80-100 μg) were loaded into each lane of a standard 10% or 7.5% (where noted) acrylamide gel for SDS-PAGE analysis using standard methods (Laemmli, 1970). Duplicate gels were stained with Commassie Blue to insure even loading and membranes were stained with amido black to insure even transfer. Membranes were probed with mouse anti-hemagglutinin anti-body (12CA5 Boehringer Mannheim) as the primary antibody and sheep anti-mouse Ig, HRP-linked secondary antibody. Detection was visualized using the ECL Western Blotting kit (Amersham) on ECL-Hyperfilm.

The intensity of the signal was quantitated by scanning with a Beckman DU640 spectrophotometer and relative Mal61/HA protein levels determined by comparison of the area under the curve. Westerns were done in duplicate on all samples for duplicate experimental cultures and relative protein levels were determined twice for each film.

Phosphatase treatment of Mal61/HA. CMY1001 was grown in rich medium under maltose-induced conditions to early log phase as described above. About 15 OD_{600} units of cells were harvested by filtration, quick frozen at -70°C. For the

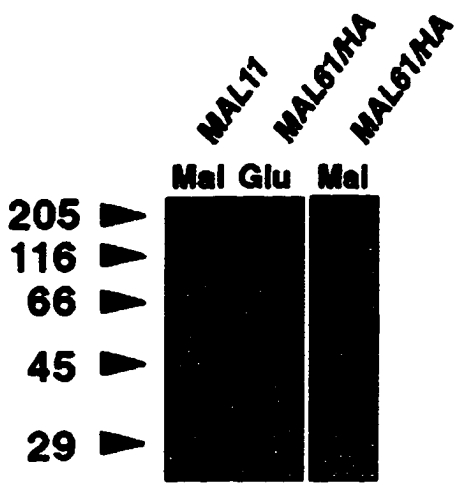
phosphatase treated samples, the cells were thawed by resuspending in 200 µl of 40 mM PIPES buffer, pH 6.0, 1 mM DTT, plus protease inhibitors and an equal volume of glass beads was added. This was vortexed for 10 minutes, centifuged to recover the membrane fraction in the pellet. The pellet was resuspended in the PIPES buffer described above for the acid phosphatase treatment, or in 0.1 M HEPES buffer, pH 7.2, plus the protease inhibitors for the alkaline phosphatase treatment. The indicated number of units of acid phosphatase (Sigma) or alkaline phosphatase (Boehringer Mannheim) were added to the membrane preparations, and treatment was carried out at 37°C for 1 hour. Following this, protein was extracted from the membranes using the same procedure as described above for whole cells, and size separated by SDS-PAGE in 7.5% acrylamide gels run at constant amperage. Western analysis was done as described above. The sample receiving no treatment, labeled "None" in Figure 7B, was extracted directly from whole cells as described above.

RESULTS

Construction, expression and characterization of *MAL61/HA*, a hemagglutinin-tagged allele of *MAL61*. In order to follow the level of maltose permease protein, we constructed a hemagglutinin epitope-tagged allele of *MAL61*, referred to as *MAL61/HA* (described in Materials and Methods). This allele was used to replace the disrupted *mal11::URA3* gene in strain 100-1A, a disruption of the maltose permease gene of *MAL1*, using two-step gene replacement thereby creating a *MAL1* locus containing the *MAL61/HA* sequence. This strain was crossed to YPH500 (see Table 1), and strain CMY1001 (see Table 1) is a maltose fermenting segregant carrying only this reconstructed *MAL1* locus (*MAL61/HA MAL12 MAL13* encoding maltose permease, maltase and the Mal-activator respectively) and no other *MAL* genes. Thus, *MAL61/HA* is the sole maltose permease gene present in the strain.

Two isogenic *MAL1* strains, one containing the wild-type *MAL11* gene (strain 600-1B) and the other containing *mal11Δ::URA3* but transformed with a plasmid carrying *MAL61/HA* (strain 100-1A[pMAL61/HA]), were grown in selection medium on either 2% maltose or 2% glucose as the carbon source. As can be seen in Figure 1, no cross-reacting species of protein is detected in the strain lacking the HA-tagged allele under these growth conditions. Additionally, the expression of *MAL61/HA* is maltose-induced. The induced level of maltose transport activity is comparable to that in strains carrying the wild-type allele (data not shown).

Figure 1. Characterization and regulation of the Mal61/HA protein. Strains 600-1B and 100-1A[pMAL61/I-HA] were grown in rich medium plus 2% maltose or 2% glucose. Protein extracts were prepared and analyzed by Western blotting, as described in Materials and Methods.

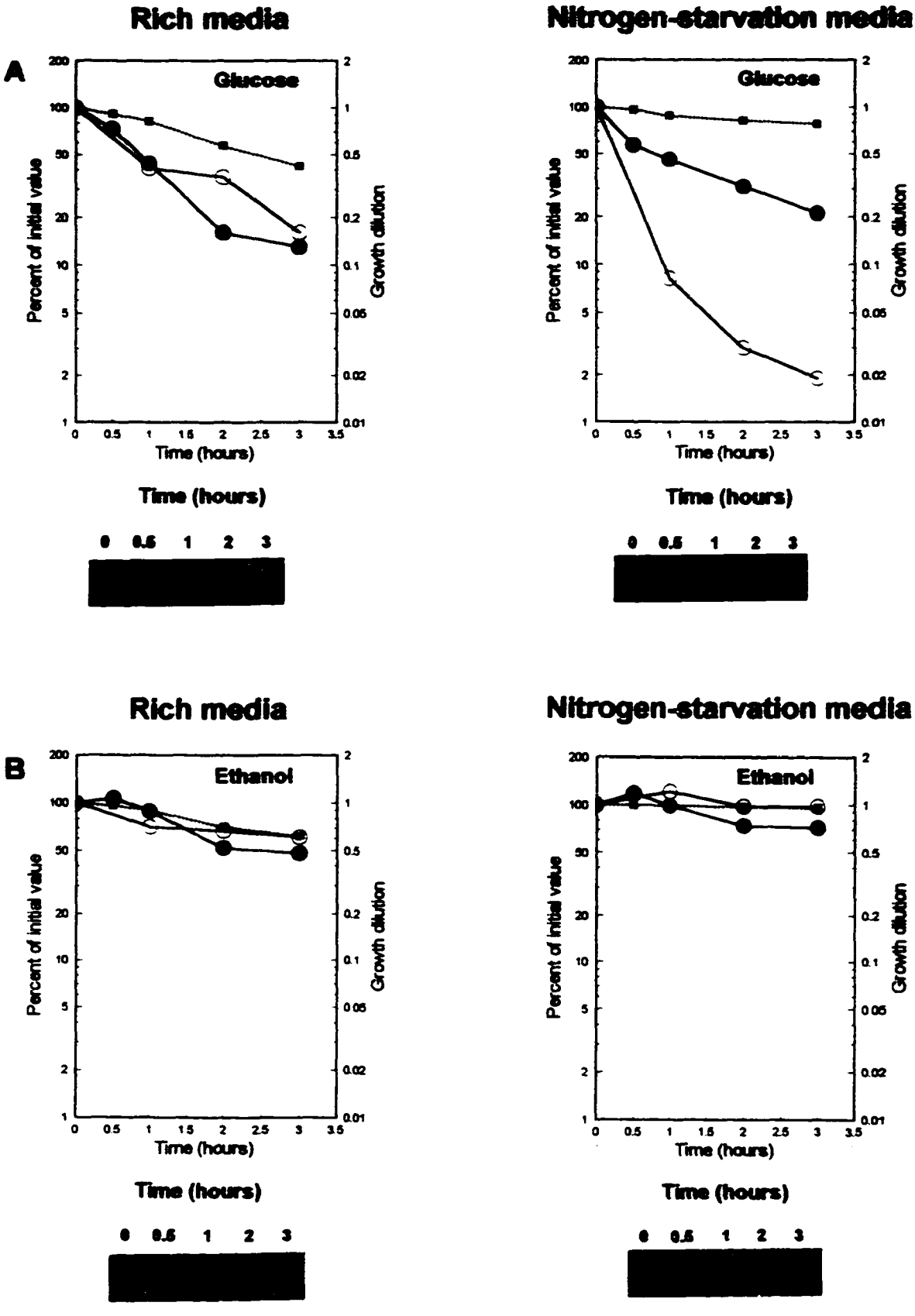


Moreover, the K_m of the Mal61/HA maltose permease (approximately 1-2 mM) is not significantly different from that reported for wild-type Mal61p (Cheng and Michels, 1991).

Glucose-induced inactivation of maltose transport exhibits two components in nitrogen-starvation, but only one in rich media. CMY1001 cells were grown to early log phase in rich medium with 2% maltose, harvested, and transfered to either rich medium (YEP) or nitrogen-starvation medium (YNB without amino acids or ammonium) with the indicated carbon source. At the times shown following the transfer, maltose transport activity was assayed, and total cell protein extracts were prepared. The relative amount of Mal61/HA protein in the total cell extracts was determined by Western analysis of size-separated proteins using the anti-HA antibody as described in Materials and Methods. Each of these measures (growth dilution, maltose transport rate, and maltose permease protein levels) were then plotted in a single graph relative to the value at the time of glucose addition. Semi-log plots are used because they demonstrate the kinetics of the glucose-induced responses more clearly than linear plots.

Figure 2 compares the effects of glucose and ethanol on maltose transport and maltose permease protein levels in rich media versus nitrogen-starved media. Several conclusions can be drawn. Glucose, but not ethanol, stimulates the inactivation of maltose transport and a decrease in level of maltose permease protein. In rich medium, the glucose-induced loss in transport activity parallels the loss in maltose permease protein in what appears to be a single process with simple

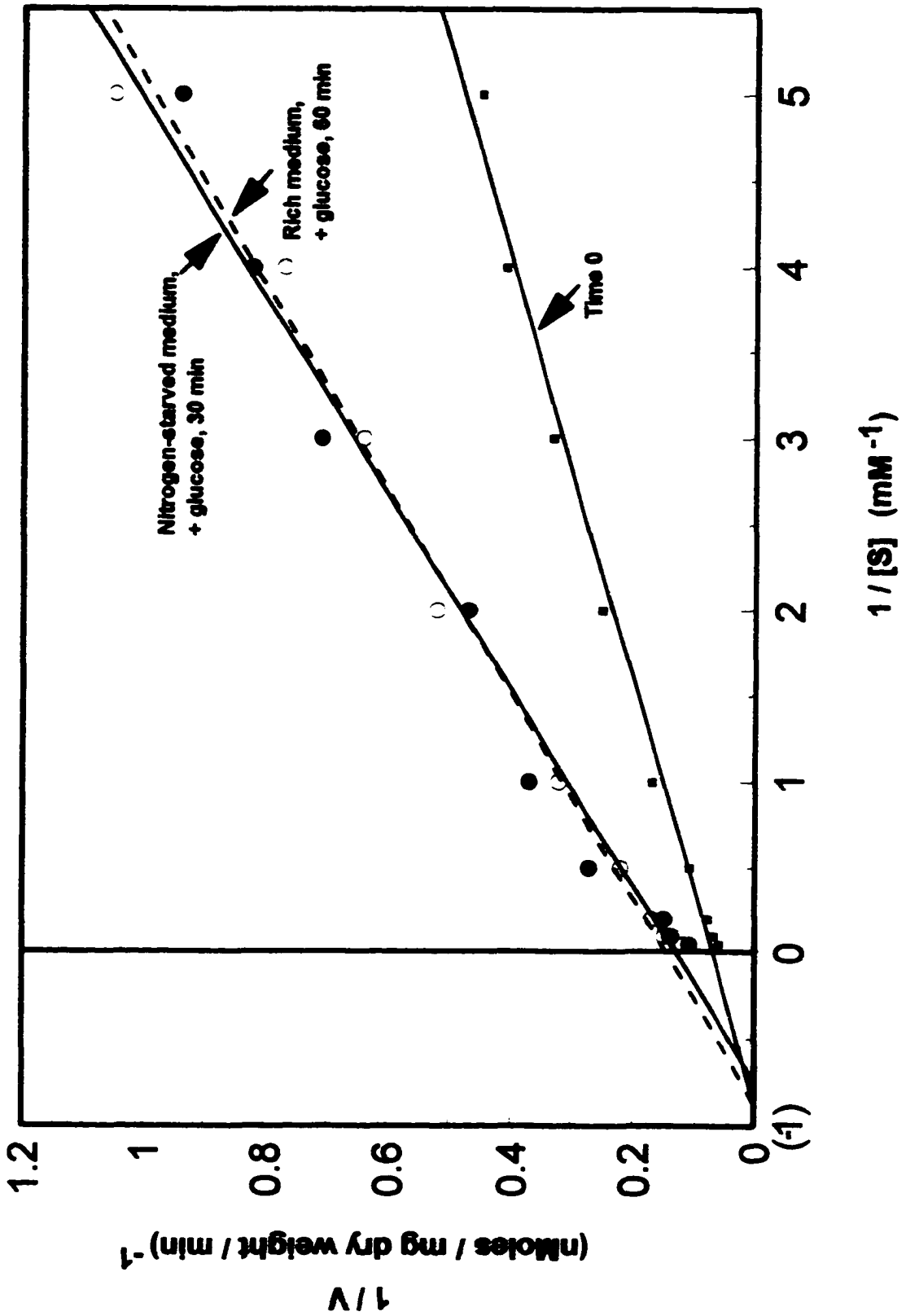
Figure 2. Glucose-induced inactivation of maltose permease in rich versus nitrogen-starvation medium. Strain CMY1001 was grown in rich medium plus 2% maltose, harvested, transferred to either rich medium or nitrogen-starvation medium plus either 2% glucose (Panel A) or 2% ethanol (Panel B). At the indicated times, the OD_{600} was determined and aliquots of the culture were removed for maltose transport assay and the preparation of total protein extracts for Western analysis of Mal61/HA protein levels, as described in 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 experiments, each run on duplicate gels, and each scanned twice. The relative levels of Mal61/HA protein (•) and maltose transport activity (O) compared to the zero time sample are plotted along with the Growth dilution (■, dotted line). Growth dilution represents the growth of the culture during the course of the experiment and is calculated as the OD_{600} at time zero divided by the OD_{600} at time X.



kinetics. In contrast, in nitrogen-starvation medium, the glucose-induced decrease in transport activity is more rapid than the decrease in protein levels at early time points. Ninety percent of the activity is lost within the first 60 minutes. On the other hand, loss of maltose permease protein occurs at the same rate as is seen in rich medium (half-life of approximately 45 minutes). These results indicate that, when strain CMY1001 is transferred to nitrogen-starvation medium plus glucose, two kinetically distinct processes contribute to the inactivation of maltose permease. The first is a rapid loss of the transport activity which we refer to as the inhibition of transport activity, and the second is a slower loss in transport activity which correlates with the proteolytic degradation of the maltose permease protein. The rapid inhibition of transport activity is not seen in rich medium, nor did Riballo *et al.* (1995) see evidence of this inhibition in their studies which utilized different strains. Moreover, in our analysis of strains carrying *PRE* mutations (described below), which utilized the same strains as did Riballo *et al.* (1995), we also did not detect the rapid inhibition component.

Glucose affects the V_{max} but not the K_m of maltose transport. We carried out a kinetic analysis of the maltose transport activity remaining after 50% inactivation to determine whether the rapid inactivation seen in nitrogen-starvation medium represents an increase in K_m of the maltose transporter or a decrease in V_{max}. CMY1001 cells were grown in rich medium plus maltose, harvested, and subjected to glucose inactivation in rich or nitrogen starved media. Samples were taken at time zero and at the indicated time where 50% loss in transport activity was

Figure 3. Lineweaver-Burk plot of maltose transport in maltose-induced and glucose-inactivated cells. Strain CMY1001 was grown in rich medium, and allowed to undergo a 50% glucose-induced inactivation in either rich (○) or nitrogen-starvation medium (●), as described for Figure 2. At the indicated time, maltose transport was determined at a range of substrate concentrations. These were compared to maltose transport in cells at time zero before inactivation (■).



achieved in each medium. Lineweaver-Burk analysis of the results is shown in Figure 3. As expected for the inactivation in rich medium, a 50% decrease in V_{max} was observed with no significant change in the K_m (approximately 1.25 mM). In nitrogen-starvation medium, the V_{max} also is reduced approximately 50% with no apparent change in K_m. Thus, in both media the amount of functional maltose permease is reduced with no change in kinetic characteristics, as had been suggested in a previous report (Peinado and Loureiro-Dias, 1986).

Mechanism of proteolysis of maltose permease. We explored the pathway of proteolysis of the maltose permease protein using mutations in genes known to control early steps in endocytosis (*END3*), vesicle targeting to the vacuole (*REN1*), vacuolar proteolysis (*PEP4*), and proteosome activity (*PRE1*, *PRE2*).

A temperature sensitive mutation of *END3* was introduced into strain CMY1001 by targeted integrative disruption of the genomic copy of the gene using a cloned copy of an *end3-ts* allele (34). Glucose-induced inactivation of maltose permease was followed in the parental (CMY1001) and *end3-ts* (CMY1004) strains with the following exception. The strains were grown in induced conditions at the permissive temperature of 25°C and transferred to nitrogen-starvation medium equilbrated to the nonpermissive temperature of 35°C. Clearly, *END3* is required for the glucose-induced proteolysis of maltose permease protein (see Figure 4). The level of maltose permease protein in the *end3-ts* strain grown induced at the permissive temperature is approximately 2-3 fold higher than that found in the isogenic parent strain (data not shown), and this increased protein level is reflected

Figure 4. Glucose-induced inactivation of maltose permease in an *end3-ts* strain defective in early endocytosis. Strains CMY1001 (Panel A) and CMY1004 (*end3-ts*) (Panel B) were grown as described for Figure 1 except at 25°C, harvested, and transferred to nitrogen-starvation medium containing 2% glucose or 2% ethanol at 35°C. Samples were taken at the indicated times, and the growth dilution (■), maltose transport rate (○), and relative Mal61/HA protein levels (●)were determined, as described in Materials and Methods.

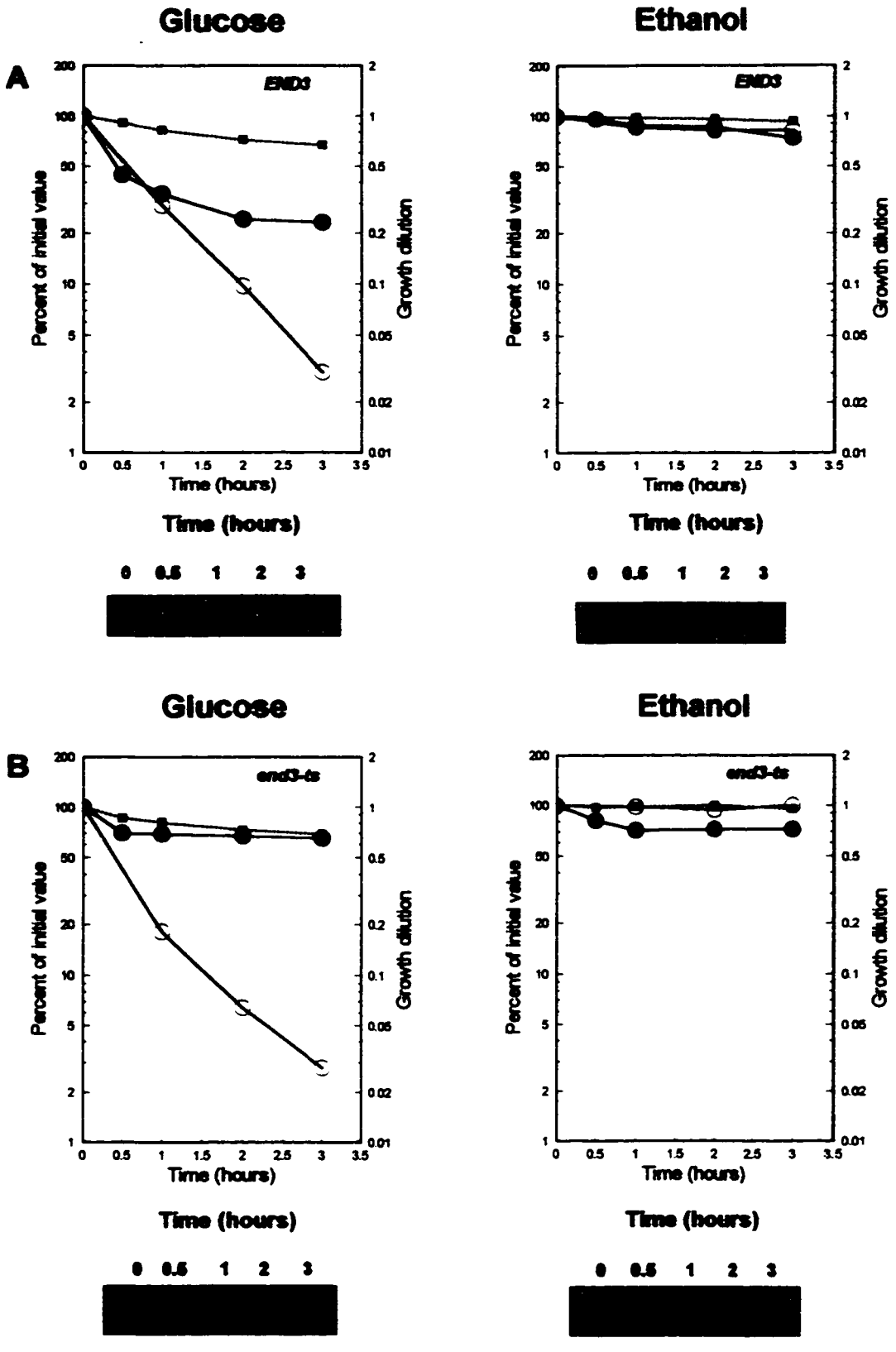


Table 2. Maltose transport rates of strains mutated in genes required for endocytosis, vesicle trafficking and proteolysis.

Strain	Relevant genotype	Transport rate (nmol/mg dry wt/min)
CMY1001	*END3 REN1 PEP4*	2.40, 2.47 (25°C)
CMY1002	*pep4Δ*	2.91
CMY1003	*ren1Δ::URA3*	1.49
CMY1004	*end3-ts*	4.84 (25°C)
WCG4a[pMAL61/HA] [pMAL63]	*PRE1 PRE2*	3.23
WCG4-11/21a[pMAL61/HA] [pMAL63]	*pre1-1 pre2-1*	2.63
WCG4-11/22a[pMAL61/HA] [pMAL63]	*pre1-1 pre2-2*	3.77

The strains were grown in rich medium with 2% maltose to early log phase at 30°C, unless otherwise indicated. Maltose transport rates were determined as described in Materials and Methods.

in an increased rate of maltose transport (Table 2). Interestingly, the rapid inhibition of maltose transport is still seen immediately after glucose addition at the nonpermissive temperature, suggesting that the inhibition of transport activity is independent of the proteolysis process or is upstream of the End3p function.

The *ren1Δ* and *pep4Δ*,mutations were created in CMY1001 by one-step gene replacement. *PEP4* encodes vacuolar proteinase A which is required for vacuolar proteolysis and for the enzymatic activation of all of the vacuolar proteases including itself (Ammerer *et al.*, 1986; Hemmings *et al;* 1981). Ren1p (Vps2p) functions late in endocytosis, and appears to be involved in vesicle transport from the endosome to the vacuole (Davis *et al.*, 1993). Inactivation of maltose permease was followed in these strains, and the results are shown in Figure 5. No glucose-induced proteolysis of maltose permease is seen in either mutant strain. Quantitation of the relative amounts of maltose permease protein in these mutant strains compared to the isogenic wild-type shows approximately 2-3 fold higher levels in the *pep4Δ* strain but no comparable increase in maltose transport (Table 2), and 2-3 fold lower levels of maltose permease protein in the *ren1Δ* strain with a coordinate decrease in transport rate (Table 2). The rapid inhibition of maltose transport is unaffected in both the *ren1Δ* and *pep4*Δstrains.

Finally, we tested the role of the proteosome in the glucose-induced proteolysis of maltose permease by using strains containing mutations in *PRE1* and *PRE2* encoding components of the proteosome (Heinemeyer *et al.*, 1993). An

Figure 5. Glucose-induced inactivation of maltose permease in a *pep4Δ* and a *ren1Δ* strain defective in vacuolar proteolysis and vesicle trafficking to the vacuole, respectively. Strains CMY1002 (*ren1Δ*) (Panel A) and CMY1003 (*pep4Δ*) (Panel B) were grown as described in Figure 1, transferred to nitrogen-starvation medium containing 2% glucose or 2% ethanol, and, at the indicated times, the growth dilution (■), maltose transport rate (○), and relative Mal61/HA protein levels (●) determined, as described in Materials and Methods.

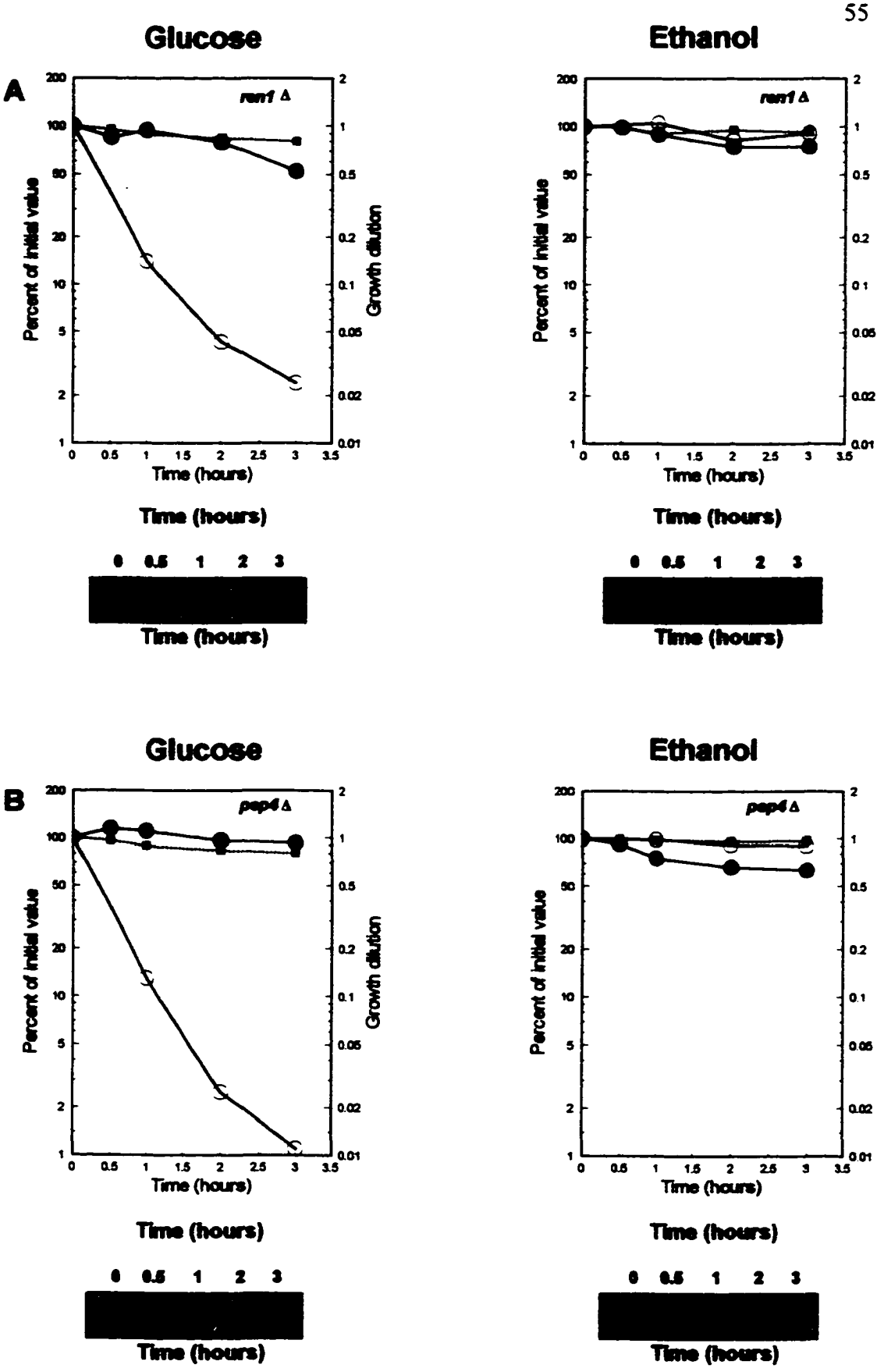


Figure 6. Glucose-induced inactivation of maltose permease in *pre1 pre2* mutant strains with defects in proteosome function. Strains WCG4a (*PRE1 PRE2*) (Panel A), WCG4-11/21a (*pre1-1 pre2-1*) (Panel B), and WCG4-11/22a (*pre1-1 pre2-2*) (Panel C) were all transformed with plasmids pMAL61/HA and pMAL63 to allow these host strains to express maltose-inducible *MAL61/HA*. The transformed strains were grown in selection medium plus 2% maltose, transferred to nitrogen-starvation medium containing 2% glucose or 2% ethanol, and, at the indicated times, the growth dilution (■), maltose transport rate (○), and relative Mal61/HA protein levels (●) were determined, as described in Materials and Methods.

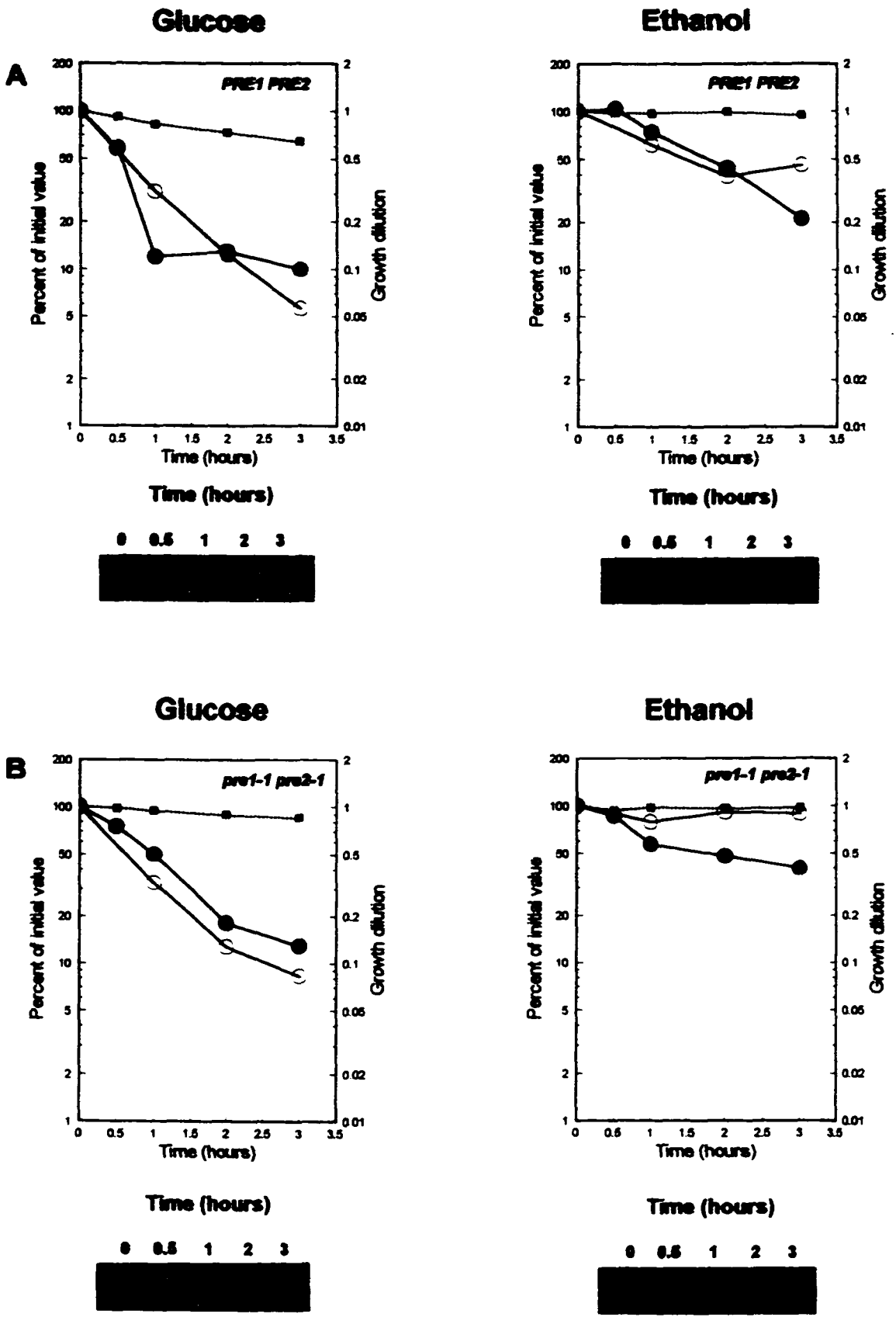
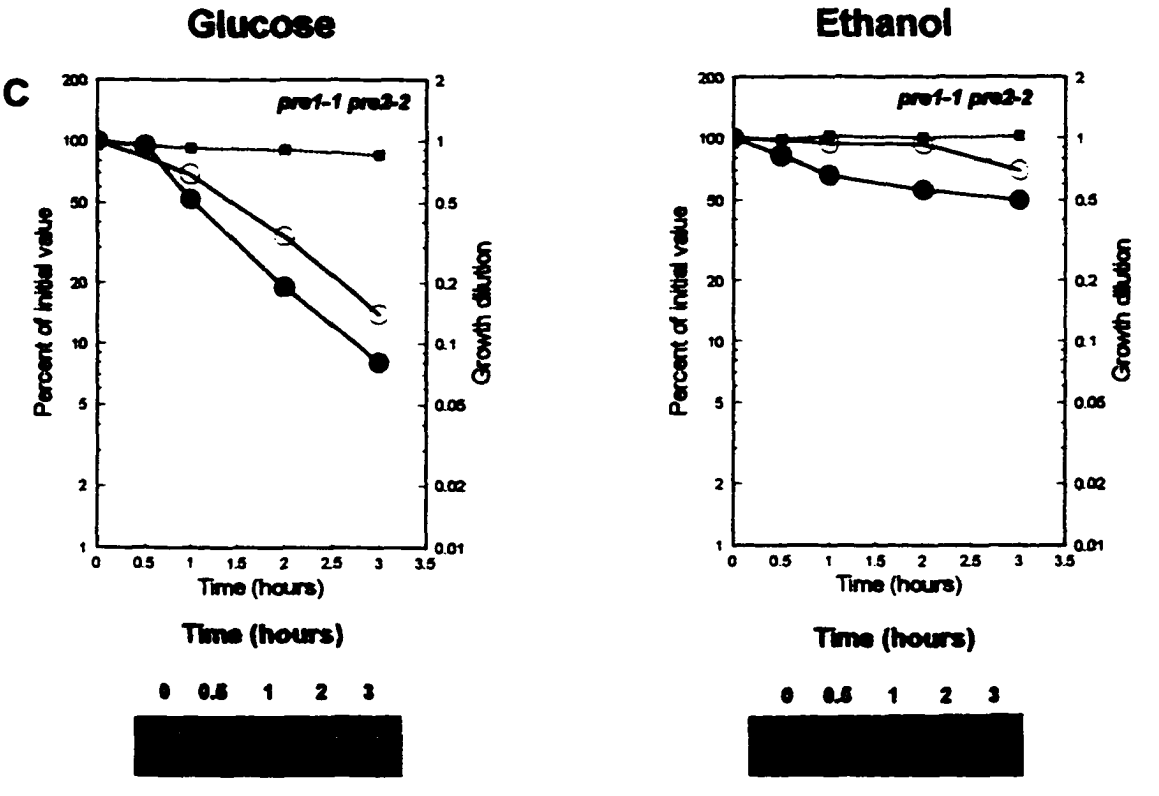


Figure 6 continued



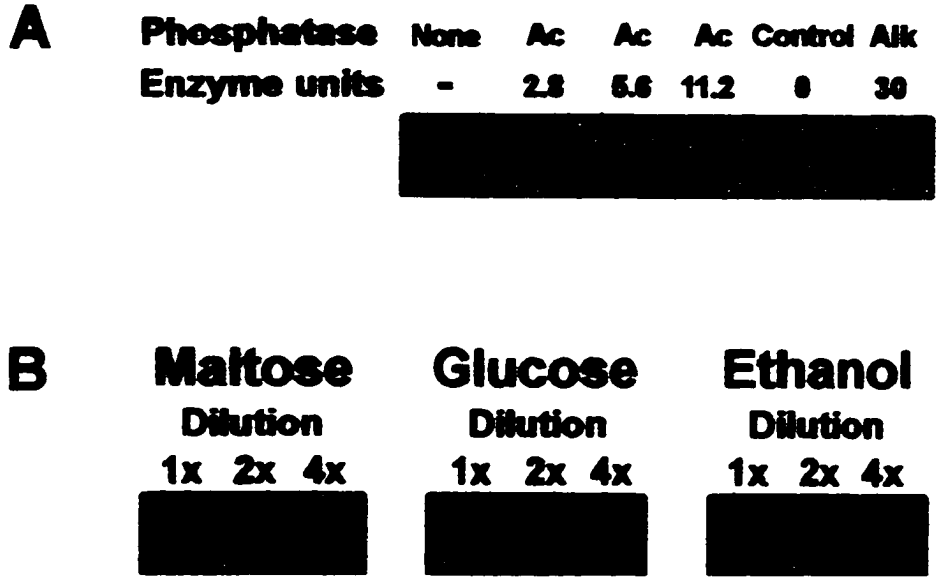
isogenic series of strains of the genotype *PRE1 PRE2* (WCG4a), *pre1-1 pre2-1* (WCG4-11/21a), and *pre1-1 pre2-2* (WCG4-11/22a) were transformed with a CEN plasmids carrying the HA-tagged *MAL61/HA* allele and the inducible Mal-activator gene *MAL63* (required because these strains lacked a Mal-activator gene and an appropriately tagged maltose permease). Glucose-induced inactivation of maltose permease was followed using the standard inactivation protocol, and the results are shown in Figure 6.

No significant difference can be seen between the *pre1 pre2* mutant strains and the isogenic wild type in the rate of glucose-induced proteolysis of maltose permease protein. Interestingly, the rapid glucose-induced inhibition of maltose transport seen in the CMY1001 strains is not evident in these strains. Instead the loss in maltose transport activity correlates with the loss in maltose permease protein even in the nitrogen-starvation conditions, as was reported by Riballo *et al.* (1995).

Differentially phosphorylated forms of Mal61/HA protein. The anti-HA antibody detects at least two species of Mal61/HA protein which exhibit slightly different mobilities in the gel conditions used in Figure 7A. Treatment of total protein extracts with increasing amounts of acid phosphatase decreases the relative amount of the slower migrating form(s). At the maximal treatment, only the faster migrating form is evident. Alkaline phosphatase treatment had no effect. This result suggests that the Mal61/HA maltose permease is differentially phosphorylated in maltose grown cells.

Preliminary studies were carried out to explore the possibility that the extent of phosphorylation of Mal61/HA protein varied in cells grown on different carbon sources. For this, we needed to express Mal61/HAp at detectable levels even under uninduced and glucose repressed conditions. This was achieved by disrupting the *HXK2* gene of CMY1001 and introducing a copy of the *MAL64-R10* constitutive Mal-activator gene to produce strain CMY1006[pMAL64-R10] (see Materials and Methods and Table 1). In work to be reported elsewhere, we showed that glucose repression of *MAL61/HA* transcription is almost fully relieved by disruption of *HXK2,* but that the kinetics of both the glucose-induced inhibition of maltose transport and the proteolysis of maltose permease are unaffected in the *hxk2Δ* mutation (Jiang *et al.*, 1997). Strain CMY1006[pMAL64-R10] was grown in selective medium with either 2% maltose, 2% glucose, or 2% ethanol as the carbon source. Total protein extracts were prepared using extraction buffer containing protease inhibitors, and protein kinase and protein phosphatase inhibitors. Western analysis was carried out on different dilutions of protein extract to be sure that the intensity of the band was linearly related to protein levels. As can be seen in Figure 7B, carbon sources affect the pattern of distribution of the phosphorylated species of Mal61/HAp, and cells grown on glucose appear to accumulate the fully phosphorylated form.

Figure 7. Phosphorylation of Mal61/HAp. Panel A. Strain CMY1001 was grown in rich medium plus 2% maltose. A crude membrane preparation was made, and treated with the indicated number of units of either alkaline or acid phosphatase for 1 hour at 37°C. The lane labeled "None" is a sample extracted from whole cells using Sample Buffer and given no treatment at all and the lane labeled "Control" was treated as were the acid-phosphatase-treated samples but without added phosphatase enzyme. Differences in the total amounts of Mal61/HAp in the different lanes are the result of differences in the treatment procedures used for the acid versus alkaline phosphatase assays, and that approximately 3-times less membrane preparation was used for the acid phosphatase treatment. Protein extracts were prepared and analyzed by Western blotting, as described in Materials and Methods. The SDS-PAGE was done using 7.5% acrylamide gels at constant amperage to achieve the separation shown. Panel B. Strain CMY1006:pMal64-R10 was grown in rich media in either 2% maltose, 2% glucose, or 2% ethanol. Total protein extracts were prepared using the extraction buffer containing a cocktail of protein kinase and phosphatase inhibitors (James *et al.*, 1989). Three different dilutions of each extract were used for Western analysis.



DISCUSSION

Gorts (1969) first described the glucose-induced inactivation of maltose transport. He noted that the inactivation took about 90 minutes, and was irreversible in the absence of *de novo* protein synthesis in maltose-induced conditions. Kinetic analysis of maltose transport in *Saccharomyces* strains identified both high-affinity (K_m 2-4 mM) and low-affinity (K_m about 70 mM) components (Bustaria and Lagunas, 1985; Cheng and Michels, 1991). Using genetically defined strains carrying a single maltose permease gene (*MAL61* or *MAL11)*, Cheng and Michels (1991) demonstrated that only the high-affinity component is the product of that gene. This report focuses on the glucose-induced inactivation of the high-affinity maltose permease encoded by *MAL61*. By using a strain expressing only a hemagglutinin epitope-tagged allele of *MAL61*, we were able to follow both maltose transport rates and levels of maltose permease protein, and directly compare the two.

We show that glucose but not ethanol stimulates the proteolysis of maltose permease in both rich and nitrogen-starvation medium, a finding that is consistent with previous reports (Lucero *et al.*, 1993; Riballo *et al.*, 1995). The half-life of Mal61/HAp measured in nitrogen-starvation conditions, where little or no cell growth is occurring, is 8 hours or greater in ethanol and 30-60 minutes in glucose. The half-life of several *Saccharomyces* membrane proteins has been determined and shown to vary from 13 minutes to 30 hours depending on the protein, and, for most, the half-life is regulated by the physiological state of the cell and/or the presence or

absence of ligand (Beino *et al.*, 1991; Davis *et al.*, 1993; Kolling and Hollenberg, 1994; Lai *et al.*, 1995; Ramos *et al.*, 1988; Volland *et al.*, 1994). The rate of degradation of the uracil permease is increased in a variety of stress conditions including starvation for carbon, phosphate and nitrogen (Volland *et al.*,1994). On the other hand, degradation of the otherwise stable inositol permease is induced by the addition of inositol to the growth medium which appears to stimulate a ligand-induced change in the protein targeting it to the degradation pathway (Lai *et al.*, 1995). Recently reported studies of the α-factor receptor Ste2p showed that α-factor stimulated the already high constitutive rate of turnover (Hicke and Riezman, 1996).

Endocytosis and vacuolar proteolysis has been demonstrated as the pathway of degradation for all of the *Saccharomyces* membrane proteins studied to date, and maltose permease is no exception (Benito *et al.*,1991; Davis *et al.*, 1993; Hicke and Riezman, 1996; Kolling and Hollenberg, 1994; Lai *et al.*, 1995; Raths *et al.*, 1993; Riballo *et al.*, 1995; Stanbrough and Magasanik, 1995; Volland *et al.*, 1994). Isogenic strains carrying mutations in *END3*, *REN1(VPS2)*, or *PEP4* all lack glucose-induced proteolysis of the Mal61/HA maltose permease protein. Both the *end3-ts* (at the permissive temperature) and the *pep4Δ* strains accumulate 2-3 fold higher levels of Mal61/HA protein but only the *end3-ts* strain exhibits comparably higher rates of maltose transport. *END3* encodes a very early function in the endocytosis pathway, and this result suggests that the accumulated permease in the *end3-ts* strain is present at the plasma membrane in a functional

form. Only a modest increase in maltose transport is seen in the *pep4Δ* strain despite the abundant increase in levels of Mal61/HAp, and this suggests that the permease is not at the plasma membrane but instead is in an internal compartment(s) such as the vacuole. The finding that the *ren1Δ* strain exhibits reduced levels of Mal61/HAp is somewhat surprising considering that the *ren1-1* allele was selected for its ability to accumulate **a**-factor receptor at the cell surface as a result of a reduced rate of internalization of ligand-bound Ste3p (Davis *et al.*, 1993). We had expected that the *ren1Δ* strain used in this study would accumulate Mal61/HAp and would exhibit higher levels of maltose transport if the maltose permease were able to recycle to the plasma membrane from the endosome as suggested by Riballo *et al.* (1995). Perhaps these unexpectedly low levels of maltose permease can be attributed to our use of a *ren1Δ* as opposed to the *ren1-1* aliele. If so, this suggests a role for Ren1p in secretion as well as targeting vesicles to the vacuole, at least for the maltose permease.

We found that in nitrogen-starvation medium the CMY1001 isogenic strains used in this study exhibited a loss of maltose transport activity that was more rapid than the loss of maltose permease protein suggesting that inhibition of maltose permease specific activity is occurring in addition to its proteolysis. This inhibition was not reported by Riballo *et al.* (1995), and we believe that this reflects differences in the strains used in the two studies. Consistent with Riballo *et al.* (1995), we also did not detect the inhibition component when we followed the glucose-induced inactivation of Mal61/HA maltose permease in the WCG4a

isogenic series of strains (Figure 6).

Among the mechanisms one could propose for the initial glucose-induced inhibition of maltose transport activity are post-translational modification, alteration in subcellular compartment or structural conformation, and interaction with a negative regulatory factor. Phosphorylation of fructose 1,6 bisphosphatase at a site near the N-terminal end following glucose addition to the medium is responsible for the rapid inhibition of its enzyme activity, but the glucose-induced proteolysis of FBPase does not appear to require phosphorylation at this site (Marcus *et al.*, 1988; Mazon *et al.*, 1982; Rittenhouse *et al.*, 1986; Tortora *et al.*, 1981). In contrast, the general amino acid permease, Gap1p, is phosphorylated in the active state, and dephosphorylation of Gap1p is correlated with its rapid inhibition in response to rich nitrogen sources (Stanbrough and Magasanik, 1995). The finding that the inhibition of maltose permease occurs at wild-type rates in the *end3-ts* mutant strain where endocytosis is severely depressed suggests that a change in compartment is not involved. The mechanism of this inhibition and the basis of these apparent strain differences are under investigation.

The rapid inhibition of maltose permease transport activity does not appear to be a prerequisite for the proteolysis of maltose permease since the proteolysis occurs in strains and/or growth conditions where inhibition is absent. Inhibition appears to occur early in inactivation prior to the End3p-dependent step and this is unlikely to represent a change in compartment. Moreover, both the inhibition of maltose transport and the proteolysis of maltose permease result in a decrease of

V_{max} and not a change in K_m, which is consistent with studies reported by Busturia and Lagunas (1986) but not with those of Peinado and Loureiro-Dias (1986) who found that glucose induced an increase in K_m but no change in V_{max}. Taken together, these results suggest that the inhibition process occurs to maltose permease in the plasma membrane and could be a modification of the protein that fully inhibits function. Whatever, the process, there appears to be strain-dependent variation of an as yet unidentified gene(s) and/or physiological conditions required for inhibition.

Two species of maltose permease with different mobility in SDS-PAGE analysis were reported in studies of a maltose fermenting strain of undefined *MAL* genotype (Lucero *et al.*, 1993; Van den Broek *et al.*, 1994). Lucero *et al.* (1993) detected maltose permease protein using a polyclonal antibody, and thus it was not clear whether the two species represented different modified forms of the permease or if the antibody also detected Agt1p, a second maltose permease present in many laboratory strains (Han *et al.*, 1995). Analysis of the endopeptidase Lys-C peptides carried out by Van den Broek *et al.* (1994) suggested that the two forms were highly sequence homologous indicating that Agt1p is an unlikey candidate for either species (Han *et al.*, 1995). We report here that the Mal61/HA maltose permease is present in differentially phosphorylated forms in maltose-grown cells, and that the hyperphosphorylated species accumulate in glucose-grown cells. Phosphorylation has been implicated in the receptor-mediated endocytosis of the *Saccharomyces* α-factor receptor and in several mammalian membrane receptors, including the

insulin receptor and the asialoglycoprotein receptor (Fallon *et al.*, 1994; Kublaoui *et al.*, 1995; Zanolari *et al.*, 1992). The transport activity of the *Saccharomyces* general amino acid permease Gap1p is activated by phosphorylation, and the level of Gap1p phosphorylation is nitrogen source regulated (Peinado and Loureiro-Dias, 1986). Similarly, the human insulin-responsive glucose transporter *GLUT4* is functionally activated in the phosphorylated state (James *et al.*, 1989). The *Saccharomyces* uracil permease is also differentially phosphorylated, but the functional significance of this phosphorylation is unknown (Volland *et al.*, 1994). Thus, phosphorylation of maltose permease might impact on its transport activity and/or turnover. We are currently investigating Mal61/HAp phosphorylation in detail with particular interest in its role, if any, in transport activity and glucose-induced inhibition and/or proteolysis.

CHAPTER 2

The role of ubiquitin conjugation in glucose-induced proteolysis of *Saccharomyces* maltose permease

This chapter has been accepted for publication in the **Journal of Biological Chemistry**.

ABSTRACT

In *Saccharomyces*, the addition of glucose induces a rapid degradation of maltose permease that is dependent on endocytosis and vacuolar proteolysis (Medintz *et al.*, 1996). 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 mono-ubiquitination. A promoter mutation that reduces expression of *RSP5/NPI1*, a postulated ubiquitin protein-ligase (E3), dramatically reduces the rate of glucose-induced proteolysis of maltose permease. The role of various ubiquitin-conjugating enzymes (E2) was investigated using strains carrying mutant alleles *ubc1Δ ubc4Δ*, *ubc4Δ ubc5Δ*, *cdc34-ts2/ubc3*, and *ubc9-ts*. Loss of these functions was not shown to effect glucose-induced proteolysis of maltose permease, but loss of Ubc1,4, and 5 was found to inhibit maltose permease expression at the post-transcriptional level.

INTRODUCTION

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 (Hu *al.*, 1995), and it inactivates maltose permease by a process referred to as glucose-induced inactivation or catabolite inactivation (Medintz *al.*, 1996; Lucero and Lagunas, 1997). 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 (Medintz *al.*, 1996). 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. Similar results have been reported describing the glucose-induced proteolysis of the galactose permease encoded by *GAL2* (Horak and Wolf, 1997).

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 addition of ammonium ions to yeast cells growing on proline as the sole nitrogen source (Stanbrough and

Magasanik, 1995; Hein *et al.*, 1995). Inactivation occurs as a 2-fold process with enzymatic inactivation by phosphorylation proceeding degradation of the permease (Stanbrough and Magasanik, 1995; Hein *et al.*, 1995). The high affinity Pho84 phosphate transporter undergoes rapid degradation once the supply of phosphate and/or carbon source is exhausted (Martinez *et al.*, 1998). Uracil permease (Fur4p) is phosphorylated on serine residues at the plasma membrane and is rapidly degraded under adverse growth conditions (Galan *et al.*, 1996). 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 (Horak and Wolf, 1997; Hein *et al.*, 1995; Galan *et al.*, 1996; Roth and Davis, 1996; Hicke and Riezman, 1996; Kolling and Hollenberg, 1994). 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 (Hicke and Riezman, 1996). *END4* mutations inhibit endocytosis of α-factor and stimulate the appearance of multi-ubiquitinated species. A sequence in the C-terminal cytoplasmic domain of Ste2p, SINNDAKSS (Rohrer *et al.*, 1993), 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. It also has been shown that ubiquitination is required for endocytosis of yeast uracil permease (Galan *et al.*, 1996). Galactose transporter, Gal2 protein, is ubiquitinated in response to the addition of glucose to the growth media and this ubiquitinated species has a relatively short half-life (Horak and Wolf, 1997).

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 Hicke, L, 1997). The ABC-transporter Ste6 accumulates in a ubiquitinated form in the plasma membrane of strains that are deficient in endocytosis (Kolling and Hollenberg, 1994). 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 multi-drug 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 (Egner and Kuchler, 1996). Similar results have been reported for the human fibroblast growth factor receptor (Strous *et al.*, 1996). Moreover many other plasma membrane receptor proteins are found as ubiquitin conjugates including the lymphocyte homing receptor, the platelet-derived growth factor receptor, the c-Kit receptor, and the mammalian immunoglobulin E receptor (Siegelman *et al.*, 1986; Mori *et al.*, 1992; Miyazawa *et al.*, 1994; Paolini and Kinet, 1993).

In this study, we used molecular genetic analysis to explore the role of ubiquitin in the glucose-induced inactivation of the maltose permease. Our

results indicate that loss of free ubiquitin, via a *DOA4* null mutation, impairs the glucose-induced proteolysis of maltose permease and that the effects of the *doa4Δ* null mutation can be suppressed by the overexpression of ubiquitin. We demonstrate that the maltose permease exists as a ubiquitinated species and that the amount of this ubiquitinated species increases dramatically upon the addition of glucose to maltose fermenting cells. Rsp5/Npi1 ubiquitin protein-ligase is implicated in the proteolysis of maltose permease. Mutations in *UBC1*, *UBC4* and *UBC5* encoding ubiquitin conjugation enzymes, UBC (E2), in combination were found to dramatically decrease the level of maltose permease expressed, apparently by affecting a post-transcriptional process. However, loss of these functions or of Cdc34p/ubc3p or Ubc9p had no significant effect on the rate of glucose-induced proteolysis of maltose permease.

Materials and Methods

Strains and plasmids. The *Saccharomyces cerevisiae* strains used in this study and their relevant genotypes are listed in Table 1. Plasmid pDOA4-8 carries the wild-type allele of *DOA4*. Plasmid YEp96, contains *UBI4* encoding ubiquitin expressed from the copper-inducible *CUP1* promoter, and YEp105, contains a *c-myc* tagged ubiquitin allele also expressed from the *CUP1* promoter (Ecker *et al.*, 1987; Hochstrasser *et al.*, 1991). These plasmids were obtained from Mark Hochstrasser, University of Chicago. Plasmid pUN70 does not contain any inserted gene sequence and serves as a control (Elledge and Davis, 1988) as does plasmid yATAG200 which contains a *CUP1* promoter also without any inserted 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 (Elledge and Davis, 1988; Sikorski and Heiter, 1989). 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 basepairs upstream of the start codon of the permease gene *MAL61/HA*. The promoter sequence of this gene was removed by a digestion with *Xho*I and *Sac*I and

replaced with the 400 basepair *ADH1* promoter, amplified from plasmid pGAD424 (Clontech Inc., Palo Alto, CA.) by PCR.

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 (Papa and Hochstrasser, 1993). 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)(Charron *et al.*, 1986).

Inactivation assay. The standard inactivation assay protocol was used as described previously (Medintz *et al.*, 1996). Unless otherwise indicated cells were grown at 30°C to early log phase (OD_{600} 0.1 to 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% (wt/vol) 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 2 experiments and were carried out in duplicate. Variation was less than 15%. Growth dilution was calculated as the OD_{600} at time zero divided by the OD_{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 (Medintz *et al.*,

1996, Cheng and Michels, 1989). Transport assays were done in duplicate on at least duplicate cultures. Maltase activity was determined as described previously (Dubin *et al.*, 1985). Maltase activity describes the nmoles of *p*-nitrophenol α-D-glucopyranoside (PNPG) cleaved per mg protein per minute as measured spectrophotochemically.

Western analysis and quantitation of relative protein levels. Cells were harvested and total protein extracts prepared by the methods described previously (Medintz *et al.*, 1996, Davis *et al.*, 1993). Equal amounts of total protein are loaded per well for comparison of time courses or relative protein levels. SDS-PAGE analysis and detection were carried out for the HA-tagged Mal61 maltose permease (Medintz *et al.*, 1996). 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 (Medintz *et al.*, 1996).

Table 1. *Saccharomyces. cerevisiae* strains used in this study.

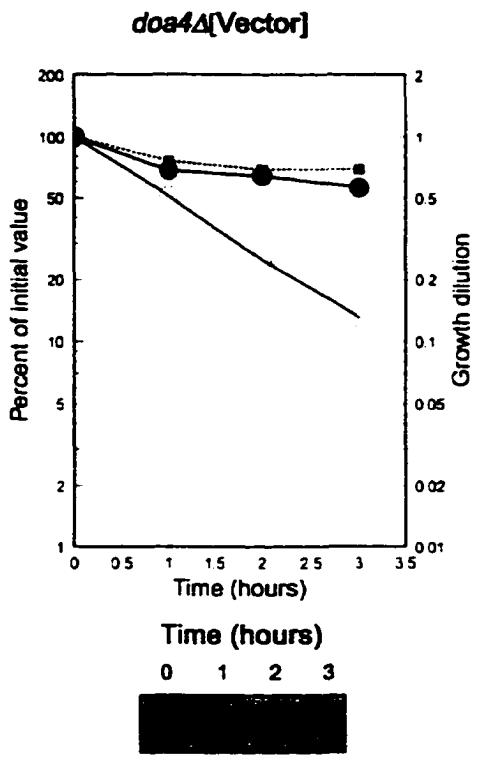
Strain	Genotype	Reference
CMY1001	*MATa MAL61/HA MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 DOA4*	Medintz *et al.*, 1996
CMY1004	*End3-ts* (isogenic to CMY1001)	Medintz *et al.*, 1996
PMY270	*MATα* doa4*Δ1::LEU2 his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 MAL31 MAL32*	P. McGraw
CMY1025	*Doa4Δ1::LEU2 his3-Δ200 ura3-52 lys2-801 trp1 MAL61/HA MAL12 MAL13 MAL31 MAL32*	This study
23346c	*MATa ura3 NPI1*	Hein *et al.*, 1995
27038a	*MATa ura3 npi1*	Hein *et al.*, 1995
Mgg15	*MATa cdc34-2ts ura3-52 his3-Δ200*	Banerjee *et al.*, 1995
MHY501	*MATa his3-Δ200 leu2-3,112 lys2-801 trp1-1*	Chen *et al.*, 1993
MHY498	*ubc4-Δ1::HIS3* (isogenic to MHY 501)	Chen *et al.*, 1993
MHY499	*ubc5-Δ1::LEU2* (isogenic to MHY 501)	Chen *et al.*, 1993
MHY509	*ubc1-Δ1::HIS3*(isogenic to MHY 501)	Chen *et al.*, 1993
MHY508	*ubc4-Δ1::HIS3 ubc5-Δ1::LEU2*(isogenic to MHY 501)	Chen *et al.*, 1993
MHY519	*ubc1-Δ1::URA3 ubc4-Δ1::HIS3*(isogenic to MHY 501)	Chen *et al.*, 1993
FM394	*MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1(am)*	Seufert *et al.*, 1995
FM395	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1(am) ubc9Δ::TRP1 leu2::ubc9Pro-Ser::LEU2*	Seufert *et al.*, 1995

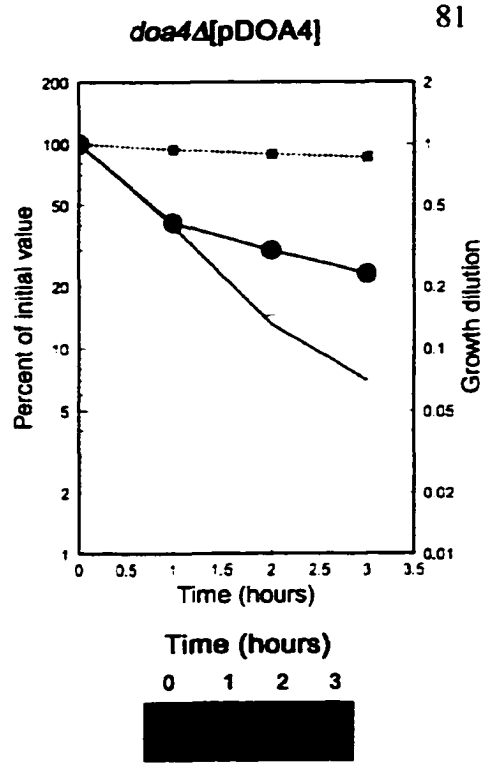
RESULTS

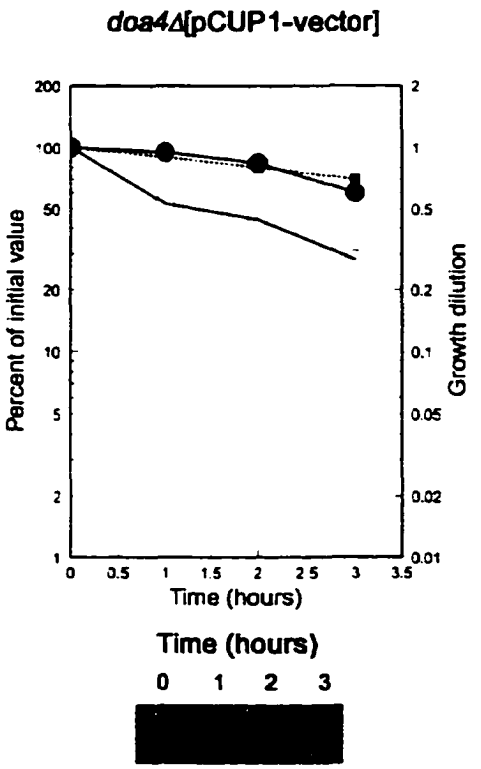
Ubiquitin is required for glucose-induced proteolysis of the maltose permease. The yeast *DOA4* gene encodes a ubiquitin hydrolase enzyme that functions late in the proteasomal degradation pathway by cleaving and recycling ubiquitin from substrate remnants still bound to protease (Papa and Hochstraser, 1993). Although Doa4p is only one of several species of ubiquitin hydrolase enzymes found in *Saccharomyces*, loss of the *DOA4* gene product significantly decreases the rate of ubiquitin recycling and severely decreases levels of available ubiquitin. We used a *doa4Δ* null mutant strain to explore the dependence on ubiquitin of glucose-induced proteolyis of maltose permease.

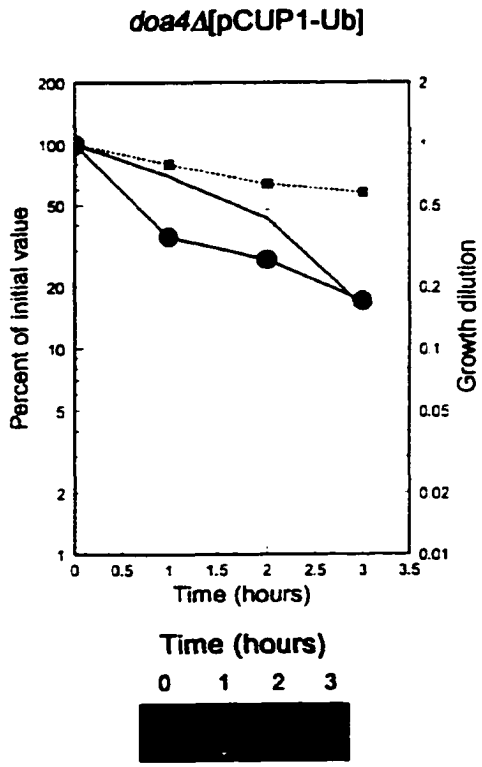
Glucose-induced inactivation of maltose permease was characterized in the *doa4Δ* null strain, CMY1025, and as a control, in strain CMY1025 carrying the wild-type *DOA4* gene on a *CEN* plasmid. As is evident from Figures 1A and 1B, the *doa4* mutant strain exhibits a dramatically decreased rate of glucose-induced 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 2 indicates that the steady state rate of maltose transport in the *doa4Δ* strain is slightly higher, 37%, than that of a strain expressing the wild-type *DOA4* gene which is consistent with the decrease in maltose permease turnover. Interestingly, despite the apparent

Figure 1. Effects of a *doa4Δ* null mutation on glucose-induced inactivation of maltose permease. Strain CMY1025 (*doa4Δ*) was transformed with plasmid pDOA4 carrying the *DOA4* gene or plasmid pUN70 as a vector control. Transformants were grown in selective medium plus 2% maltose, harvested, and the standard inactivation assay performed (as described in Materials and Methods). Plasmid yEP96, carrying a *c-myc* tagged allele of *UBI4* expressed from the copper-inducible *CUP1* promoter (Hochstrasser *et al.*, 1991) or the vector control plasmid yATAG200 lacking the *UBI4* insert were introduced into strain CMY1025. Transformants were grown to early log in selective medium plus 2% maltose, incubated for 4 hours with 0.1mM copper sulfate, harvested, and transferred to nitrogen-starvation medium plus 2% glucose. At the indicated times, the OD_{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 in 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 (O) 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 OD_{600} at time zero divided by the OD_{600} at time x.









lack of glucose-induced proteolysis of maltose permease in the *doa4Δ* strain, glucose stimulates a decrease in maltose transport activity indicating that the inhibition of transport activity occurs by a process that is independent of ubiquitin availability.

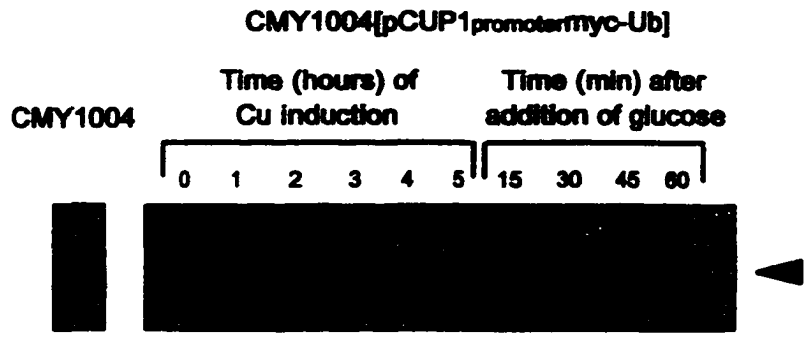
In order to test the possibility that the ubiquitin deficiency in the *doa4Δ* strain is responsible for the decreased rate of glucose-induced proteolysis of maltose permease, we determined whether overexpression of ubiquitin could overcome the loss of active ubiquitin recycling. Plasmid yEP96 (pCUP1-Ub), carrying the ubiquitin gene *UBI4* fused to the copper responsive promoter from *CUP1*, was introduced into the *doa4Δ* mutant strain CMY1025 (Ecker *et al.*, 1987). The standard inactivation assay was carried out except 0.1mM copper sulfate was added to the culture medium 4 hours prior to the transfer to glucose and the initiation of the inactivation assay (Ward *et al.*, 1994). As can be seen in Figure 1, overexpression of ubiquitin in the *doa4Δ* [yEP96] strain suppresses the loss of *DOA4* restoring a more rapid rate of glucose-induced proteolysis of maltose permease than that observed in the *doa4Δ*[yATAG200] control strain. Table 2 shows that the steady state transport rate of maltose in a ubiquitin-overexpressing *doa4Δ* strain, CMY1025[yEP96], is half that seen in the control strain, CMY1025[yATAG200]. 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 (Medintz *et al.*, 1996). *END3* is an early function in the endocytosis process (Raths *et al.*, 1993). 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 (Medintz *et al.*, 1996). Plasmid yEP105, which encodes a *c-myc* tagged allele of ubiquitin, was introduced into strain CMY1004.

Strain CMY1004[yEP105] was grown to very early log phase (OD_{600} 0.2-0.3)in selective media plus 2% maltose at room temperature and 0.1mM $CuSO_4$ was added to induce expression of *c-myc-Ub* (Ward *et al.*, 1994). After a 5 hour ubiquitin induction period, the culture was transferred to 37°C to inhibit endocytosis, and then after an additional 1 hour 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 (Hochstrasser *et al.*, 1991).

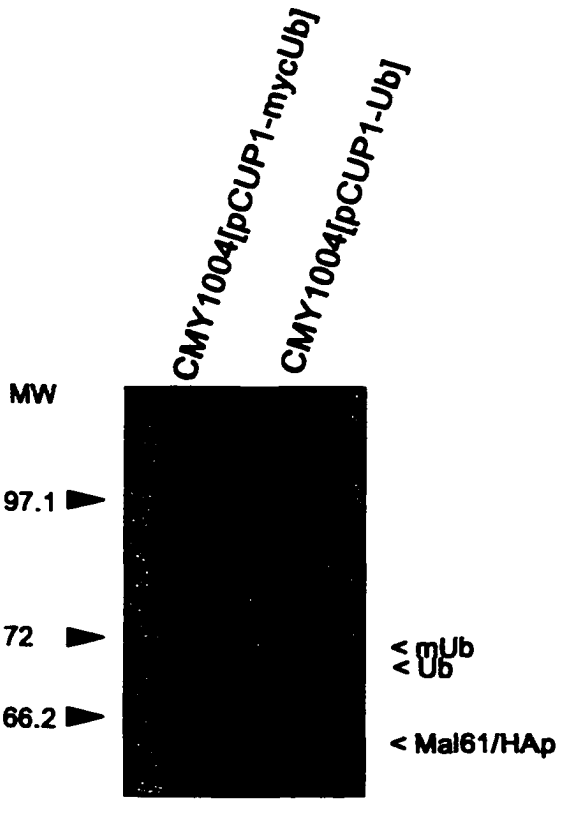
Figure 2. Overexpression of a *c-myc* tagged ubiquitin allele in an *end3-ts* strain. Strain CMY1004, containing an *end3-ts* allele, was transformed with plasmid yEP105 encoding a *c-myc* tagged allele of ubiquitin on a copper inducible promoter, *CUP1*. The resulting strain was grown to early log phase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



As is evident in Figure 2, overexpression of ubiquitin during the Cu++ 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. It should be noted that maltose fermentation generates intracellular glucose. The addition of the 2% glucose to the growth media causes an increase in the abundance of this larger molecular weight band which peaks at about 30 to 45 minutes. 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 (Galan *et al.*,1996; Roth and Davis, 1996; Egner and Kuchler, 1996; Hochstrasser *et al.*, 1991).

Strains CMY1004[yEP105] expressing the Cu++ inducible *c-myc* tagged ubiquitin and CMY1004[yEP96] expressing the Cu++ inducible untagged ubiquitin were both grown at room temperature to early log, and 0.1mM $CuSO_4$ was added to the growth media. After 4 hours the cultures were moved to 37°C for 1 hour prior to the addition of 2% glucose. After the glucose was added cells were allowed to continue growing at 37°C for 1/2 hour and then harvested for Western analysis of Mal61/HAp.

Figure 3. Western blot analysis of an *end3-ts* strain expressing two different molecular weight alleles of ubiquitin. Strain CMY1004 was transformed with plasmid yEP96 carrying *UBI4* expressed from the *CUP1* promoter, or yEP105 encoding a *c-myc* tagged allele of ubiquitin also expressed from the *CUP1* promoter. Both strains were grown to early log at 30°C in selective media plus 2% maltose and then incubated in 0.1mM copper sulfate for 4 hrs, the last hour at 37°C to inhibit endocytosis. Glucose, 2%, was added for 1/2 hr prior to harvesting the cells for Western analysis of Mal61/HA protein.



As is seen in Figure 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 (Hochstrasser *et al.,* 1991). 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 to the major species of Mal61/HAp, and is consistent with a mono-ubiquitinated maltose permease.

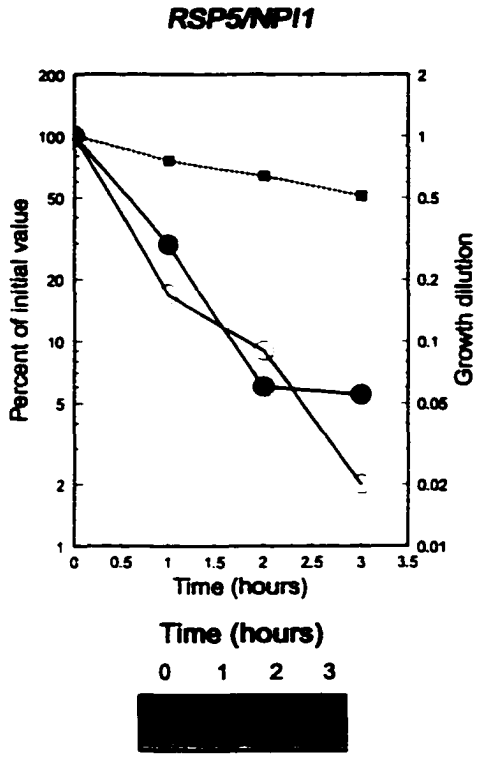
***RSP5/NPI1* plays a role in the glucose-induced proteolysis of maltose permease.** RSP5/*NPI1* encodes a ubiquitin-protein ligase that participates in the induced degradation of at least two permeases, the general amino acid permease, encoded by *GAP1*, and the uracil permease, encoded by *FUR4* (Hein *et al.*, 1995). An *rsp5/npi1* mutant allele was isolated based on its nitrogen repression-resistant phenotype (Grenson, 1983) and has since been shown to be a Ty1 insertion into the *RSP5* promoter (Hein *et al.*, 1995). 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 (Springael and Andre, 1998).

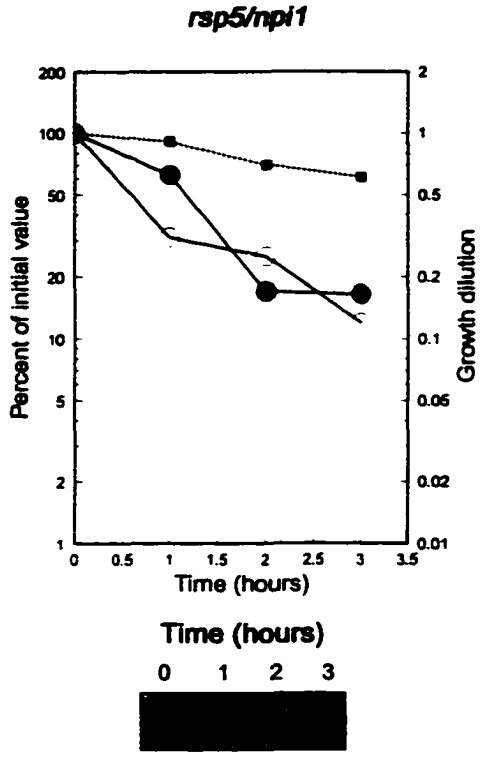
Table 2. Maltose transport rates of *doa4* and *rsp5/npi1* mutant strains.

Strain	Relevant genotype	Transport rate (nmol/mg [dry wt]/min)
CMY1025 [yATAG200]	*doa4Δ::LEU2*	1.57*
CMY1025 [yEP96]	*doa4Δ::LEU2*	0.80*
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

All strains were grown in rich medium with 2% maltose to early log phase at 30°C. Maltose transport rates were determined as described in Materials and Methods.
*Determined following a 4hr incubation in 0.1mM copper sulfate.

Figure 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 (1983) was utilized which expresses significantly reduced levels of Rsp5/Npi1 protein (Springael and Andre, 1998). The standard inactivation assay protocol was carried out. The relative levels of Mal61/HA protein (●) and maltose transport activity (O) compared with the zero time sample are plotted along with the growth dilution (■) as described in Figure 1.





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 Figure 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 to 10-fold) in the *rsp5/npi1* mutant strain. As Table 2 shows the *rsp5/npi1* strain also expresses slightly higher maltose transport activity in maltose-grown cells which is consistent with a decrease in rapid maltose permease turnover. These results indicate that *RSP5/NPI1* plays an important role in the glucose-induced proteolysis of maltose permease.

Role of ubiquitin conjugating enzymes (E2) encoded by *UBC1, UBC4* and *UBC5* in glucose-induced inactivation of maltose permease. The ubiquitin-conjugating enzymes (E2) 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 Hicke, 1997; Hochstrasser, 1996). *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 (Roth and Davis, 1996; Hicke and Riezman, 1996;

Table 3. Effect of *ubc1*, *ubc4*, and *ubc5* mutations c

Strain	Relevant genotype	Fermentation rate in maltose(days)	Maltase activity (nmol PNPG/mg protein/min)	(nm
MHY501	*UBC1 UBC4 UBC5*	1	477	
MHY509	*ubc1Δ::HIS3*	1	559	
MHY498	*ubc4Δ::HIS3*	1	419	
MHY499	*ubc5Δ::LEU2*	1	303	
MHY519	*ubc1Δ::URA3 ubc4Δ::HIS3*	7 - 9	1144	
MHY508	*ubc4Δ::HIS3 ubc5Δ::LEU2*	7 - 9	130	

Strains listed in column 1 were transformed with plasmid pUN30-MAL63 en
MAL61/HA or pADH1-MAL61/HA carrying *MAL61/HA* expressed from the *ADH1*
All strains were grown to early log phase at 30°C in selective medium with 2% m
glycerol/2% lactate (pADH1-MAL61/HA transformants). Maltose transport rates an
Materials and Methods. Relative protein levels are determined by Western blot anal

Table 3. Effect of *ubc1*, *ubc4*, and *ubc5* mutations on *MAL* gene expression

genotype	Fermentation rate in maltose(days)	Maltase activity (nmol PNPG/mg protein/min)	Transport rate (nmol/mg [dry wt]/min)	Relative Mal61/HAp levels	
				MAL61/HA	*ADH1proMAL61/HA*
C4 UBC5	1	477	3.54	100%	100%
::HIS3	1	559	0.68	26	17
::HIS3	1	419	0.84	21	86
::LEU2	1	303	2.22	23	71
ubc4Δ::HIS3	7 - 9	1144	0.34	ND	9
ubc5Δ::LEU2	7 - 9	130	0.18	ND	40

in 1 were transformed with plasmid pUN30-MAL63 encoding the *MAL*-activator and either pRS415-MAL61/HA carrying *MAL61/HA* expressed from the *ADH1* constitutive promoter (results in column 7).
to early log phase at 30°C in selective medium with 2% maltose (pRS415-Mal61/HA transformants) or 3% DH1-MAL61/HA transformants). Maltose transport rates and maltase activity were determined as described in
Relative protein levels are determined by Western blot analysis. ND means not detected.

Hicke, 1997; Hochstrasser, 1996). *UBC6* and *UBC7* also function in the degradation of the Matα2 repressor through a pathway that is distinct from that of *UBC4* and *UBC5* (Chen *et al.*, 1993). 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 Ubc 4/5 ubiquitin conjugating enzymes (E2), has been implicated in the signaling of endocytosis and degradation of many yeast membrane proteins including Ste2p, Gap1p, Fur4p, Ste3p, and Pdr5p (Hein *et al*, 1995; Roth and Davis, 1996, Hicke and Riezman,1996; Kolling and Hollenberg. 1994; Egner and Kuchler, 1996; Hochstrasser, 1996). We explored the role of these E2 enzymes in glucose-induced inactivation of maltose permease.

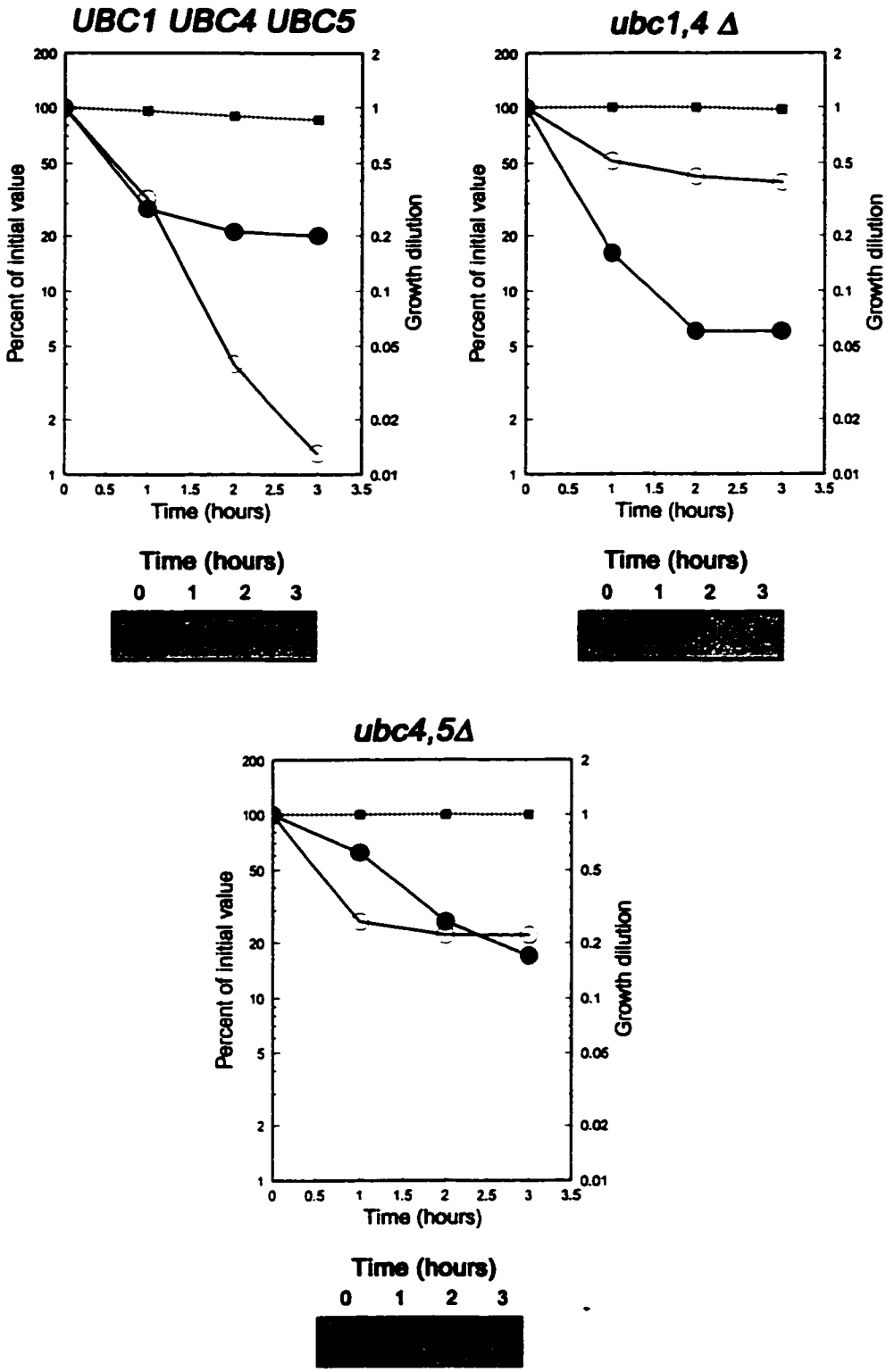
A series of isogenic *ubc* mutant strains, carrying *ubc1Δ*, *ubc4Δ* or *ubc5Δ* alleles were transformed with *CEN* plasmids carrying the *MAL*-activator gene *MAL63* and the HA-tagged maltose permease gene *MAL61/HA*, and the half-life of maltose permease was determined using the standard inactivation assay. The single mutant strains showed no significant change in half-life of the permease compared to the parental strain: *UBC1 UBC4 UBC5* (O.4 hours), *ubc1Δ* (0.5 hours), *ubc4Δ* (0.5 hours), and *ubc5Δ* (0.3 hours). Rather unexpectedly, double mutant strains containing either the *ubc1Δ ubc4Δ* or *ubc4Δ ubc5Δ* double null mutations 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 4. Maltose transport activity of *ubc1*, *ubc4*, and *ubc5* mutant strains expressing constitutive *MAL61/HA*.

Strain	Relevant genotype	Plasmid	Carbon source	Transport velocity (nmol/min/mgdrywt)
MHY501	*UBC1 UBC4 UBC5*	pADH1-MAL61/HA	maltose	0.76
MHY501	*UBC1 UBC4 UBC5*	pADH1-MAL61/HA	glycerol/lactate	5.22
MHY519	*ubc1Δ ubc4Δ*	pADH1-MAL61/HA	maltose	0.06
MHY519	*ubc1Δ ubc4Δ*	pADH1-MAL61/HA	glycerol/lactate	1.21
MHY508	*ubc4Δ ubc5Δ*	pADH1-MAL61/HA	maltose	0.05
MHY508	*ubc4Δ ubc5Δ*	pADH1-MAL61/HA	glycerol/lactate	1.29

Table 3 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, 4, 5, and 6). The single mutant strains exhibit between 20% (*ubc1Δ*) to 35% (*ubc5Δ*) of the level of maltose permease protein expressed by the *UBC1,4,5* parental strain and these levels also are paralleled by a decrease in maltose transport activity. All single mutant strains fermented maltose in one day. The *ubc1Δ ubc4Δ* and *ubc4Δ ubc5Δ* double mutant strains expressed only 5% to 10% of the parental levels of maltose transport activity and took 7 to 9 days to ferment maltose. Interestingly, maltase activity in these strains does not correlate with the levels of maltose permease. The maltase gene is divergently transcribed from a shared promoter with the maltose permease gene and both genes are coordinately regulated. No significant variation from the parental strain is seen in single deletion mutant strains and maltase levels are actually increased in the *ubc1Δ ubc4Δ* double mutant about 2-fold above that of the parental strain. Only in the *ubc4Δ ubc5Δ* strain were the levels of maltase significantly decreased but not to the same extent of maltose transport levels. Expression from *MAL62promoter-LacZ* (*MAL62* encodes maltase) and *MAL61promoter-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

Figure 5. Effects of glucose-induced inactivation on *ubc1,4Δ* and *ubc4,5Δ* 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 12ug/ml of cyclohexamide and the standard inactivation assay was carried out as described in Figure 1 and Materials and Methods. The relative levels of Mal61/HA protein (●) and maltose transport activity (O) compared with the zero time sample are plotted along with the growth dilution (■).



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 3 column 7). Again, reduced levels of Mal61/HA protein are detected in all of the mutant strains but particularly in the *ubc1Δ, ubc1Δubc4Δ,* and *ubc4Δ ubc5Δ* strains. Thus, these results also suggest a role for Ubc 1, 4, and 5 in a post-translational process required for maltose permease expression.

In an effort to determine the rate of glucose-induced proteolysis of maltose permease in the *ubc1Δ ubc4Δ* and *ubc4Δ ubc5Δ* double mutant strains, we used the strains which express *MAL61/HA* from the *ADH1* promoter. These strains, grown on glycerol/lactate as the carbon source, accumulate levels of maltose permease adequate to carry out an inactivation assay (see Table 4). The parental strain and the *ubc1Δ ubc4Δ* and *ubc4Δ ubc5Δ* double mutant strains carrying plasmid pADH1-MAL61/HA were grown to early log in selective media plus 3%glycerol/2% lactate. A standard inactivation assay was carried out with the exception that 12.5 ug/ml cyclohexamide was added to the 2% glucose inactivation media at time zero to stop the continued synthesis of maltose permease. The results shown in Figure 5 do not demonstrate a significant effect on the rate of proteolysis in the mutant strains. The half-life in both the parental strain and the *ubc1Δ ubc4Δ* strain is about 0.5 hours, and in the *ubc4Δ ubc5Δ* double mutant is only increased about 2-fold.

***CDC34/UBC3* and *UBC9* do not function in the glucose-induced proteolysis of maltose permease.** Jiang *et al.* (1997) 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 down-stream component. *Saccharomyces* Grr1p is an F-box protein (Patton *et al.*, 1998). 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 (Patton *et al.*, 1998). 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 (Banerjee *et al.*, 1995; Jentsch, 1992; Goebl *et al.*, 1988; Schneider *et al.*, 1996; Tyers, 1996).

The Ubc enzyme that functions with the Grr1p-containing SCF complex has not been identified (Li and Johnston, 1997). 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 (Banerjee *et al.*, 1995;) was transformed with plasmids carrying the *MAL63 MAL*-activator and *MAL61/HA*. Cells were grown in selective medium plus 2% maltose to early log at the permissive temperature, 23°C, at which time the temperature was raised to 37°C for 2 hours

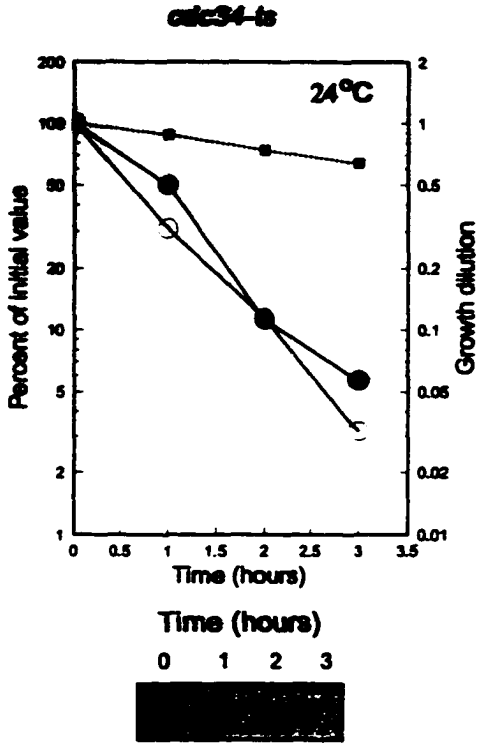
prior to the start of the inactivation assay, which was carried out at 37°C. The control culture was maintained at 23°C throughout the experiment. No significant effect is observed on the kinetics of maltose permease inactivation and proteolysis (Figure 6). Maltose transport activity of this strain grown at the permissive temperature is 4.39 nmol/mg[drywt]/min and is essentially unchanged (4.43 nmol/mg[drywt]/min) after 2 hours at the nonpermissive temperature.

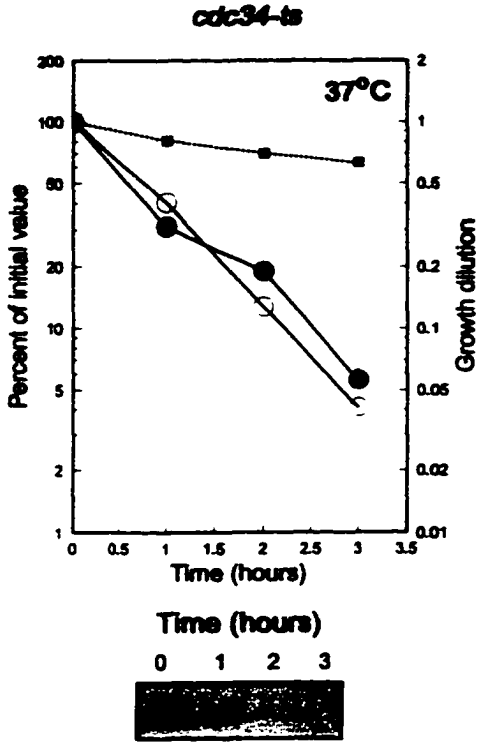
Recent evidence indicates that the ubiquitin-like protein Smt3 of *Saccharomyces* and SUMO-1, its mammalian homolog, are covalently attached to other proteins posttranslationally (Schwarz *et al.*, 1998). *UBC9*, an essential yeast gene, is required for Smt3 conjugation *in vivo* (Schwarz *et al.*, 1998). 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 (Del Pozo *et al.*, 1998). For these reasons, we decided to test the possibility that Ubc9p was involved in glucose-induced proteolysis of maltose permease.

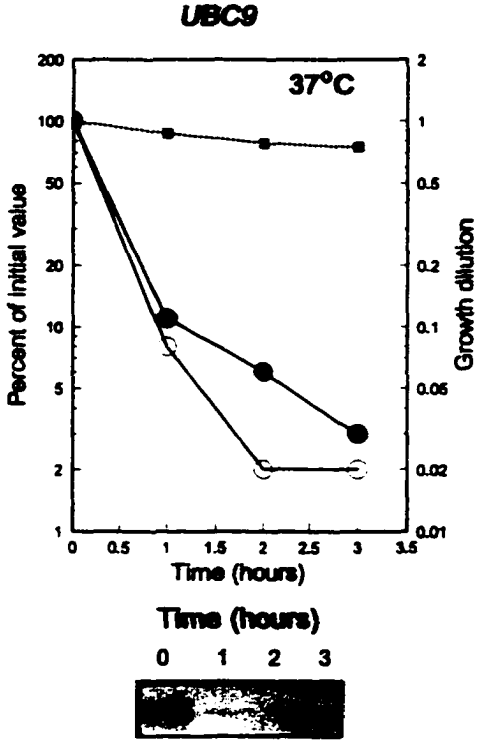
Isogenic *UBC9* and *ubc9-ts* strains were transformed with plasmids containing the *MAL63 MAL*-activator and *MAL61/HA* genes (Seufert *et al.*, 1995). The resulting transformants were grown to early log phase at room temperature in selective media plus 2% maltose and equilibrated at 37°C for 2 hours. Standard inactivation assays were carried out at the nonpermissive temperature of 37°C. The results in Figure 6 demonstrate that loss of Ubc9p function has no

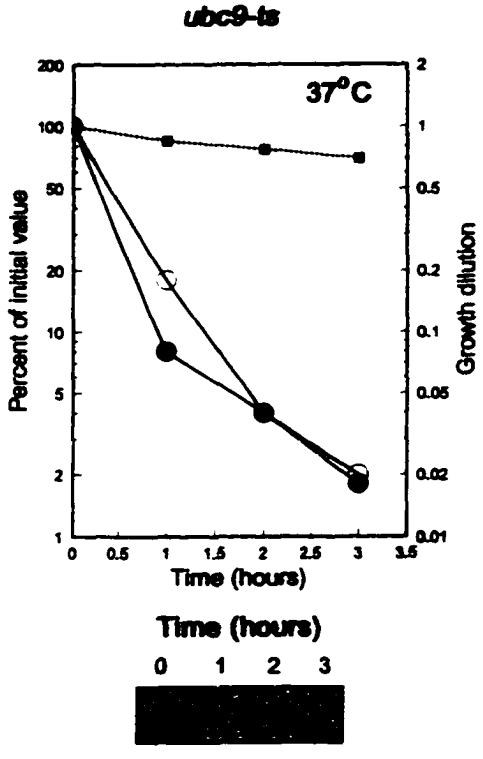
significant effect on glucose-induced inactivation of maltose permease. The rate of loss of maltose transport activity and proteolysis of maltose permease protein are comparable in both strains. Steady state maltose transport activity following 37°C in both strains also was comparable at 2.82 and 2.81 nmol/mg[drywt]/min for the *UBC9* and *ubc9-ts* strains, respectively.

Figure 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 at room temperature, the standard inactivation assay protocol was carried out at the permissive temperature, 23°C, and at the non-permissive 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 hours 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 Figure 1 and Materials and Methods. The relative levels of Mal61/HA protein (●) and maltose transport activity (O) compared with the zero time sample are plotted along with the growth dilution (■).









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 (Hochstrasser *et al.*, 1991), we demonstrated the accumulation of a higher molecular weight species of MAL61/HAp in strains that express this tagged ubiquitin allele (Figure 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 (Figure 2). This technique has been used previously to demonstrate ubiquitin conjugates of the Matα2 transcriptional activator, the ABC transporter Ste6, and the Pdr5 multi-drug transporter (Kolling and Hollenberg, 1994; Egner and Kuchler, 1996; Hochstrasser *et al.*, 1991). We also show that the abundance of this ubiquitinated species of Mal61/HAp dramatically increases upon the addition of glucose to the growth medium (Figure 3).

Second, the rate of glucose induced proteolysis of Mal61p is slowed in a *doa4Δ* strain (Figure 1). *DOA4/UBP4* encodes a ubiquitin-hydrolase that is localized to the 26S proteosome and appears to play an important role in maintenance of free unconjugated ubiquitin pools (Hochstrasser, 1996). Loss of Doa4p affects a variety of physiological functions suggesting decreased levels of available ubiquitin, at least in certain compartments (Papa and Hochstrasser,

1993). The Doa4p deficiency in glucose-stimulated Mal61p turnover can be overcome by overproduction of ubiquitin (Figure 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 (Figure 4). The *RSP5/NPI1* mutation utilized in this study was isolated by Grenson (1983) based on its resistance to ammonium-induced inactivation of the general amino acid permease. This mutant allele contains a *Ty1* insert in the upstream region and is expressed at levels significantly below wild-type (Hein *et al.*, 1995; Springael and Andre, 1998). 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 (Hein *et al.*, 1995; Galan *et al.*, 1996) as well as for the internalization of Ste2p (R. Dunn and L. Hicke, personal communication). *RSP5/NPI1* may be associated with the membrane at certain times (Hein *et al.*, 1995), a fact that is consistent with its role in ubiquitination of maltose permease at the plasma membrane. *RSP5/NPI1* is an essential function and other mutant alleles were isolated as suppressors of *SPT3* mutations, which encodes a TBP-binding factor (Eisenman *et al.*, 1992). Moreover, the large subunit of RNA polymerase II appears to be a substrate of Rsp5p (Huibregste *et al.*, 1997). Thus, Rsp5p plays an important role in regulated gene expression at several levels.

The following findings are also consistent with the conclusion that ubiquitination of maltose permease marks this protein for degradation. The ubiquitin conjugated maltose permease appears to be in relatively low abundance when compared to the level of un-ubiquitinated maltose permease. This is also similar to results demonstrated for the α-factor receptor and the yeast uracil permease (Galan *et al*, 1996; Hicke and Riezman, 1996). 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 non-permissive temperature and this strain even accumulates MAL61/HAp at the membrane at the permissive temperature (Medintz *et al*, 1996).

The ubiquitin-conjugated maltose permease species observed in Figures 2 and 3 has an apparent molecular weight approximately 7-8 kDa higher than the non-ubiquitinated maltose permease when viewed on SDS-PAGE gels. This is likely to correspond to a mono-ubiquitinated form. Studies of a truncated allele of Ste2p demonstrated a ligand-induced mono-ubiquitination sufficient for internalization and vacuolar degradation (Hicke and Riezamn, 1996; Terrell *et al.*, 1998). These authors suggest that a single ubiquitin moiety, as opposed to the polyubiquitin chains preferred by the proteasome, is recognized by the endocytotic machinery. Recent studies (Terrell *et al.*, 1998) suggest that each of the seven lysine residues in the C-terminal cytoplasmic domain of Ste2p is capable of being ubiquitinated and mediating endocytosis, although 2 sites are

preferred. Moreover, studies using the Lys to Arg mutant alleles of ubiquitin unable to form polyubiquitin chains do not indicate a decreased efficiency of degradation (Terrell *et al.*, 1998). This is in contrast with results reported by Galan and Haguenauer-Tsapis (1997) on the uracil permease 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 underway 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 (Hicke and Riezman, 1996; Terrell *et al.*, 1998). Mutation of a "destruction-box" sequence in Fur4p stabilized that permease against stress induced degradation (Galan *et al.*, 1994). Moreover, mutation of a single lysine to alanine (R294A) is resistant to stress-induced degradation (Galan *et al.*, 1996).

Thirteen ubiquitin-conjugating enzymes, UBC (E2), have been identified in *Saccharomyces* with many diverse substrates that control multiple cellular functions such as mitosis and peroxisome biogenesis (Hicke, 1997; Hochstrasser, 1996; Jentsch, 1992). The *UBC1*, *UBC4,* and *UBC5* genes have been implicated as the ubiquitin-conjugation enzymes (E2) required for substrate conjugation of certain yeast plasma membrane proteins including the α-factor receptor, the **a**-factor receptor, and the ABC transporter Ste6p (Roth and Davis,

1996; Hicke and Riezman, 1996; Kolling and Hollenberg, 1994). 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 (Hochstrasser, 1996). 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 (Hicke and Riezman, 1996; Terrell *et al.*, 1998) but similar to findings regarding the inositol permease Itr1p (Robinson *et al.*, 1996) and uracil permease (R. Haugenaur-Tsapis, unpublished results).

Our results add the Mal61/HA maltose permease to the growing list of permeases, receptors, and other plasma membrane proteins which 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 (Hein *et al*, 1995). This makes Npi1p an interesting candidate for a membrane associated ubiquitin-protein ligase. Studies are currently underway 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 result demonstrated that Mal61/HAp is phosphorylated and that the level of phosphorylation is increased by glucose (Medintz *et al.*, 1996). 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.

Acknowledgements

We thank Mark Hochstrasser, Phoebe Johnson for critical discussion and the gift of strains and plasmids. We also thank Pat McGraw and Bruno Andre for the gift of strains and plasmids. We would like to thank Thomas Hradeck, Susan Rotenberg, and Xiao-Gong Sun for technical advice and assistance. This work was supported by a grant to C.A.M. from the National Institute of General Medical Sciences (GM49280). This work was carried out in partial fulfillment of the requirements for the Ph.D. degree from the Graduate School of the City University of New York (I.M. and H.J.).

CHAPTER 3

Mutational analysis of the N-terminal and C-terminal cytoplasmic domains of maltose permease

ABSTRACT

In maltose fermenting *Saccharomyces* cells, maltose permease is required for the transport of maltose into the cell. The addition of glucose to maltose fermenting cells causes the inhibition of maltose transport activity and the degradation of this integral membrane protein by an endocytosis-dependent pathway. We have used mutation analysis to identify target domains of the permease involved in its glucose-induced inhibition and degradation. A series of nonsense mutations were introduced at residues 581, 575, 570, 560, and 553 in the C-terminal cytoplasmic domain of the protein along with several point mutations. Deletions also were constructed in the N-terminal cytoplasmic domain of residues 2-30, 31-60, 61-90, a potential PEST sequence (49-78), as well as point mutations of putative internalization signals. The effects of these alterations on maltose transport activity and glucose-induced inhibition and degradation of the mutant permeases were determined. Results indicate that the PEST sequence is important for the glucose-induced degradation and rapid glucose-induced inhibition of maltose permease transport activity. Glucose-induced inhibition of maltose permease, via the PEST sequence, appears to be mediated by the *RGT2*-dependent glucose-sensing pathway. Mutations that decrease the rate of glucose-induced degradation of maltose permease also decrease the rate of maltose permease ubiquitination in response to glucose.

INTRODUCTION

The addition of glucose to maltose fermenting *Saccharomyces* cells expressing a *MAL61/HA* tagged allele, causes a rapid and irreversible inhibition of maltose permease transport activity followed by a slower degradation of the permease protein. Proteolysis is dependent on endocytosis, vesicular sorting, and vacuolar degradation, and is independent of the proteasome (Medintz *et al.*, 1996). As has been shown for a number of yeast and mammalian plasma membrane proteins, we found that ubiquitin and the ubiquitin conjugating system play an important role in the glucose-induced degradation of maltose permease (Medintz *et al.*, 1998). In this report, we use mutation analysis of the N- and C-terminal cytoplasmic domains of maltose permease to identify sequences required for the glucose-induced proteolysis of this plasma membrane protein.

Mutation analysis has helped to define the endocytosis targeting domains in several yeast membrane proteins. The C-terminal cytoplasmic domain of the Ste2p α-factor receptor contains the sequence SINNDAKSS, which mediates the pheromone-induced ubiquitination of Ste2p in the membrane and leads to receptor endocytosis and vacuolar degradation (Hicke and Riezman, 1996). The SINNDAKSS minimum sequence restores ligand-induced endocytosis to an endocytosis deficient C-terminal truncation Ste2p mutant. Mutation of the lysine of this minimum sequence in truncated Ste2p protein abolishes ubiquitination and endocytosis (Hicke and Riezman, 1996). The ABC-transporter, encoded by *STE6,* can be subdivided into 4 modular units and information regarding final membrane topology is contained within its membrane spanning domain

(Berkower *et al.*, 1996). Deletion analysis demonstrated that the middle-spanning linker region of Ste6p contains a signal that mediates ubiquitination and fast-turnover of this protein (Kolling and Losko, 1997). PEST sequences, regions rich in proline, glutamate, aspartate, serine, and threonine, are associated with rapid protein turnover. A putative PEST-like sequence in the uracil permease is required for phosphorylation and efficient ubiquitination (Marchal *et al.*, 1998). Other internalization signals also have been described in yeast membrane spanning proteins. These include the di-leucine motiff in the Gap1 general amino acid permease and the NPF signal located in the Ste3 a-factor receptor (Springael and Andre, 1998; Tan *et al.*, 1996).

Mal61/HAp contains a PEST region spanning residues 48-79 of the N-terminal cytoplasmic domain. Additionally two other reported endocytosis signals are present, an NPF sequence in the C-terminal cytoplasmic domain and a di-leucine motif found within the N-terminal cytoplasmic PEST domain. We undertook to define regions of Mal61/HAp critical to the glucose-induced inhibition and degradation through mutation analysis of these potential targeting sequences. Our results suggest that the N-terminal PEST region mediates rapid glucose-induced inhibition and degradation of maltose permease while the C-terminal cytoplasmic domain appears to play a role in inducable expression and transport activity. Mutations in maltose permease that affect the rate of glucose-induced degradation also affect the rate of glucose-induced ubiquitination of the permease. Our results suggest that the PEST sequence of maltose permease is the target of the Rgt2p-dependent glucose sensing/signalling pathway.

MATERIALS AND METHODS

Strain construction. A *Mal61/HA* null strain was constructed for this study using the parent strain CMY1001 described in Medintz *et al.* (1996) by a PCR-based gene disruption method (Guldener *et al.*, 1996). The upstream (5') primer consists of 45 bases of sequence complementary to the upstream region of *MAL61/HA* followed by 19 bases of sequence complementary to the *HIS3* gene 5' sequence. Its sequence is 5'GTACTCAGCATATAAAGAG ACACAATATACTCCATACTTGTTGTGAGTGGGCTTGGTGAGCGCTAGGAG3'. The downstream (3') primer contains 45 bases of sequence complementary to sequence 3' of *MAL61/HA* followed by 21 bases 3' to the *HIS3* ORF. The sequence of the downstream primer is 5'CACAACAGATGGGGTG CTTCGCCCTTCATCTACCACAGAAGTTTCCAAATCCACACCGCATAGATCCG TCG3'. These primers were used to amplify the *HIS3* gene from plasmid pRS413 (Sikorsky and Heiter, 1989) for PCR based gene disruption. The amplified product was transformed into strain CMY1001 (Medintz *et al*, 1996), which contains the single *MAL1* locus, for a one-step gene replacement of the *MAL61/HA* sequence present at *MAL1*, creating strain CMY1050. Transformants were selected on minimal media lacking histidine and gene replacement was confirmed by Southern Analysis. Strain CMY1050 has the genotype *MATa mal61Δ::HIS3 MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200.* The same process was used to delete the *MAL61/HA*

Table I. *MAL61/HA in vitro* mutagenesis oligonucl

mal61/HA allele	Oligo sequence (5′-3′)
N-terminal mutations	
mal61/HA-581NS	TGCTGCTTTCTAAGCTGCAAA
mal61/HA-575NS	TGCAAAAGGGTCTCATTTAGTCGCATT
mal61/HA-570NS	TTTAGTCGACTTTCACTTTCTTGCTGG
mal61/HA-560NS	ACCAAGTCTAAACTATTCATTTATCTC
mal61/HA-553NS	CTCAATAAATCACCTGCCAGC
mal61/HA-581NS/P577A	AGCTGCAAAAGCGTCGACTTTAGT
mal61/HA-581NS/P566A	CTTTCTTGCTGCAACACCAAG
mal61/HA-581NS/S572A,T573A	GCAAAAGGGTCGACTTTAGCCGCCTTGAACTTGAACTTTC
mal61/HA-581NS/K569A,K571A, K574A	AGGGTCGACTCTAGTCGATCTGAATCTTCTTGCTGGAACA
mal61/HA-N576A,P577A,F578A	CTGCAGCTGCTGCTTTGGCAGCTGCAGCAGCGGCGACTTT ACTTGAACTTTCTTGCTGG
C-terminal mutations	
mal61/HA-Δ2-30	TTGCTCCTCCATGTCTATCGAGTTCAAAGGACCACCCAAG GTAATCTGG
mal61/HA-Δ31-60	GAGAAGGTCGGGGACTTCTTCATTATTATCTTCGGTAGCG GCCATTCTCGATCTC
mal61/HA-Δ61-90	CCAAGCAGCAGCTTTTGGATATGTCTTCAAAGCTGTGTTT TAGTGAACCTGGACCGTACTC
mal61/HA-Δ49-78	GAGTGGCATTCCCCTCTCACTTTCATCTGCCTCGGAAAGA ATCACTTTTCTTACCTTGCTCCTCC
mal61/HA-Δ49-60	CATCGAGAAGGTCGGGGACTTCTTCATTATTATCGGAAAG AATCACTTTTCTTACCTTGCTCCTCC
mal61/HA-Δ61-78	GAGTGGCATTCCCCTCTCACTTTCATCTGCCTCGTTTGGT TGAACCTGGACCGTACTC
mal61/HA-L69A,L70A	GGCGTCCTGCATAGCTTCATCGGCAGCGTCGGGGACTTCT

*Open reading frame begins with base pair number 1.

.e 1. *MAL61/HA in vitro* mutagenesis oligonucleotide sequences

llele	Oligo sequence (5'-3') .	Annealing site*
utations		
	TGCTGCTTTCTAAGCTGCAAA	1753-1733
	TGCAAAAGGGTCTCATTTAGTCGCATT	1741-1711
	TTTAGTCGACTTTCACTTTCTTGCTGG	1723-1696
	ACCAAGTCTAAACTATTCATTTATCTC	1693-1667
	CTCAATAAATCACCTGCCAGC	1670-1649
7A	AGCTGCAAAAGCGTCGACTTTAGT	1741-1718
6A	CTTTCTTGCTGCAACACCAAG	1707-1687
2A,T573A	GCAAAAGGGTCGACTTTAGCCGCCTTGAACTTGAACTTTCTTGC	1737-1699
9A,K571A,	AGGGTCGACTCTAGTCGATCTGAATCTTCTTGCTGGAACACC	1732-1699
7A,F578A	CTGCAGCTGCTGCTTTGGCAGCTGCAGCAGCGGCGACTTTAGTCG ACTTGAACTTTCTTGCTGG	1761-1696
tations		
	TTGCTCCTCCATGTCTATCGAGTTCAAAGGACCACCCAAGCTAGC GTAATCTGG	118-91,-39 to -12
	GAGAAGGTCGGGGACTTCTTCATTATTATCTTCGGTAGCGTTCAC GCCATTCTCGATCTC	211-181,90-61
	CCAAGCAGCAGCTTTTGGATATGTCTTCAAAGCTGTGTTTGGTAT TAGTGAACCTGGACCGTACTC	307-271,180-152
	GAGTGGCATTCCCCTCTCACTTTCATCTGCCTCGGAAAGATCAAA ATCACTTTTCTTACCTTGCTCCTCC	267-235,145-110
	CATCGAGAAGGTCGGGGACTTCTTCATTATTATCGGAAAGATCAA AATCACTTTTCTTACCTTGCTCCTCC	215-181,145-110
	GAGTGGCATTCCCCTCTCACTTTCATCTGCCTCGTTTGGTATTAG TGAACCTGGACCGTACTC	276-235,180-152
	GGCGTCCTGCATAGCTTCATCGGCAGCGTCGGGGACTTCTTC	232-191

me begins with base pair number 1.

gene in the *end3-ts* strain, CMY1004 (Medintz *et al.*, 1996), and the *rgt2Δ* strain, CMY1009 (Jiang *et al.*, 1997). Strain CMY1051 carries an *end3-ts* allele created by targeted integration (Medintz *et al.*, 1996). The ORF of the *RGT2* gene was replaced by KAN^R using PCR based gene disruption (Jiang *et al.*, 1997) in CMY1052. Strains CMY1051 and CMY1052 are otherwise isogenic to CMY1050

Plasmid construction and mutagenesis. The construction and characterization of the *MAL61/*HA gene has been described previously (Medintz *et* al, 1996). *MAL61/HA* was cloned into the pUN30 vector yielding plasmid pMAL61/HA (Elledge and Davis, 1988). This plasmid was used as the template for *in vitro* mutagenesis using the BIO-RAD Muta-Gene kit [BIO-RAD, CA] (Ausubel *et al.*, 1989). The mutagenic primers are listed in Table 1. Alterations in *MAL61/HA* were confirmed by sequencing. All mutant alleles were constructed with the full length *MAL61/HA* gene as a template except for certain C-terminal cytoplasmic domain mutations which used the 581-stop mutant allele of *MAL61/HA* as a template (see Table 1). Plasmid pRGT2-1 contains a constitutive *RGT2* allele (Ozcan *et al.* 1998; Ozcan *et al.* 1996).

Inactivation assay protocol. The inactivation assay protocol is described in detail in Medintz *et al.* (1996). Briefly, strains are grown at 30°C to very early log phase (OD_{600} of 0.1 to 0.3) in maltose containing selective medium. Cells are harvested, and transferred to nitrogen-starvation medium (yeast nitrogen base without amino acids and ammonium sulfate) plus 2% glucose. Cell samples are collected at the indicated times over a 3 hour period, and for each sample, the maltose transport rate is determined and total cell extracts are prepared for

Western analysis. Growth dilution was calculated as the OD_{600} at time zero divided by OD_{600} at time X. For all assays and determinations performed, standard error was less than 20%.

Western blotting. Western blotting analysis was carried out as described previously (Medintz *et al.*, 1996). The Mal61/HA protein in the extracts is detected using anti-HA specific antibody and the ECL Western blotting kit (Amersham) or the Vistra-ECF kit (Amersham) and the Storm Image analyzer (Molecular Dynamics, CA). The relative amount of each band on the ECL-Hyperfilm or Storm captured image is measured by densitometric comparison to the zero time sample. Western analysis was done in duplicate from duplicate cultures.

Sugar transport assays. Maltose transport was measured as the uptake of 1 mM ^{14}C-maltose as described in Cheng and Michels (1991) and Medintz *et al.* (1996). Assays were done in duplicate on duplicate cultures.

RESULTS

Mutation analysis of the Mal61/HA C-terminal cytoplasmic domain. The cytoplasmic C-terminal regions of several yeast plasma-membrane proteins contain sequences involved in their internalization. These include the Ste2p α-factor receptor, which contains the internalization signal SINNDAKSS. The SINNDAKSS serines are required for receptor phosphorylation, and the lysine is required for ubiquitination and endocytosis (Hicke and Riezman, 1996; Hicke *et al.*, 1998). Gap1p general amino acid permease is rendered resistant to NH_4^+ - induced inactivation by alterations in the C-terminal cytoplasmic domain (Hein and Andre, 1997). Changes that confer resistance include a glutamate to lysine substitution in a sequence similar to the DXKSS sequence of Ste2p and alanine substitutions of a di-leucine motif located in the C-terminal (Hein and Andre, 1997).

In order to determine potential C-terminal target regions in Mal61/HAp involved in glucose-induced inactivation, a series of stop mutations were introduced at codons 581, 575, 570, 560 and 553 in this cytoplasmic domain by site-directed mutagenesis of the plasmid borne *MAL61/HA*. These plasmid encoded mutant *mal61/HA* alleles were then introduced into strain CMY1050 in which the chromosomal copy of the maltose permease gene is replaced with *HIS3* .

The phenotypes of the C-terminal truncation alleles is shown in Table 2 including determination of the rate of glucose-induced proteolysis of the mutant permeases. It should be noted that in the null mutant strain negligible maltose

transport activity is detected, probably representing the reported low-affinity transporter shown to be an artifact of maltose binding (Benito and Lagunas, 1992). Strain CMY1050[pUN30] is unable to ferment maltose or grow on 2% maltose as the sole carbon source, and maltase expression is not induced (X. Wang, I. Medintz, and C.A. Michels, unpublished results). Strains carrying the *mal61/HA-581NS* allele ferment maltose and maltose transport activity is approximately one-third that of strains expressing the wild-type allele. The glucose-induced half-life of mal61/HA-581NS permease is not significantly different from that of the wild-type Mal61/HAp, 2hrs vs. 1.7 hrs. Strains carrying *mal61/HA-575NS* express approximately wild-type levels of permease protein and expression is maltose inducible. However, maltose transport activity of this strain is approximately 5% that of strains carrying the wild-type allele and it is unable to sustain fermentative growth on 2% maltose media. The half-life of mal61/HA-575NS permease under glucose-induced conditions also is not significantly different from that of the wild-type protein, 2.1 hrs. Strains carrying *mal61/HA-570NS*, *mal61/HA-560NS*, and *mal61/HA-553NS* are unable to induce the expression of either the truncated permease or maltase. Studies of the truncated mal61/HA-570NS and mal61/HA-560NS permeases expressed from the constitutive *ADH1* promoter are in progress

Additional mutations were constructed using the *mal61/HA-581NS* allele. These included *mal61/HA-581NS(P577A)*, *mal61/HA-581NS(P566A)*, *mal61/HA-581NS(S572A,T573A)* and *mal61/HA-581NS(K569A,K571A,K574A)*. All are expressed at approximately normal levels and capable of supporting

fermentative growth on maltose media. Transport activity of strains expressing these mutant permeases ranged from 12% of wild-type levels for *mal61/HA-581NS(S572A,T573A)* to 30% for *mal61/HA-581NS(P577A)* suggesting that specific activity is partially affected. Glucose-induced half-life of these permease mutants also is not significantly altered from that of the wild-type protein, and in some cases the rate of glucose-induced degradation appears to be increased. Particularly, *mal61/HA-581NS(P577A)* mutant permease exhibits a glucose-induced half-life of 0.5hrs, approximately 3-fold faster than that of the wild-type protein.

A novel plasma-membrane protein endocytosis signal recently has been identified in *Saccharomyces*. A chimeric protein consisting of the trans-golgi network protein Kex2p cytoplasmic domain fused to an endocytosis defective α-factor receptor Ste2p was constructed and shown to undergo rapid endocytosis (Tan *et al.*, 1996). The sequence NPFSD, located 37 residues from the C-terminus, was shown to be essential for endocytosis of this fusion protein. This sequence is similar to the NPFSTD sequence located in the cytoplasmic C-terminal domain of the Ste3 a-factor receptor. Mutation of the NPFSTD sequence of Ste3p prevents pheromone-stimulated endocytosis of a truncated Ste3p (Tan *et al.*, 1996). Mal61/HAp contains a similar sequence, NPFAA, located at residues 576-80 of the C-terminal cytoplasmic domain, 36 residues from the C-terminus (Cheng and Michels, 1989).

In order to test whether the NPF sequence of Mal61/HAp contributes to the glucose-induced inactivation, endocytosis and degradation of maltose

permease, the NPF residues were simultaneously changed to alanine. CMY1050 transformants carrying *mal61/HA-(N576A,P577A,F578A)* ferment maltose and express maltose transport activity to levels approximately 10% that of strains carrying the wild-type allele (Table 2). The glucose-induced half-life of this mutant permease is slowed slightly to 2.8 hours.

Table 2. Phenotypes of maltose permease C-terminal cytoplasmic domai

MAL61 Allele	Maltose fermentation	Maltose –induced permease expression	Maltose transport activity (nM/min/mg)
MAL61/HA	+	+	5.96
mal61/HA-581NS	+	+	1.71
mal61/HA-575NS	-	+	0.40
mal61/HA-581NS(P577A)	+	+	1.82
mal61/HA-581NS(P566A)	+	+	1.30
mal61/HA-581NS(S572A,T573A)	+	+	0.71
mal61/HA-581NS(K569A,K571A, K574A)	+	+	0.95
mal61/HA(N576A,P577A,F578A)	+	+	0.51
Vector control	-	-	0.06

ND - not determined
MAL61/HA mutant alleles carried on pUN30 vector (Elledge and Davis, 1988) were introduced into CMY1050(*mal61Δ::HIS3*).

pes of maltose permease C-terminal cytoplasmic domain mutants.

Maltose fermentation	Maltose –induced permease expression	Maltose transport activity (nM/min/mg)	Half-life of maltose permease protein (hrs)
+	+	5.96	1.7
+	+	1.71	2
-	+	0.40	2.1
+	+	1.82	0.5
+	+	1.30	2
+	+	0.71	2.4
+	+	0.95	1.2
+	+	0.51	2.8
-	-	0.06	ND

on pUN30 vector (Elledge and Davis, 1988) were introduced into strain

Mutation analysis of the Mal61/HA permease N-terminal cytoplasmic domain. The N-terminal cytoplasmic domain of the yeast uracil permease, Fur4p, contains a PEST-like sequence shown to be essential for turnover (Marchal *et al.*, 1998). Internalization of Fur4p was reduced when some of the serine residues in the PEST-like sequence were converted to alanines and severely impaired when all serines in this region were mutated or when this region was deleted (Marchal *et al.*, 1998). A di-leucine motif found to be required for internalization of *GLUT4* also has been described to play a similar role in the yeast Gap1 general amino acid permease and the eukaryotic β_2-adrenergic receptor (Springael and Andre, 1998; Gabilondo *et al.*, 1997). Mal61/HAp contains a N-terminal cytoplasmic PEST sequence which spans residues 49-78 of this domain (Cheng and Michels, 1989). Located within this PEST sequence is a di-leucine motif at residues 69 and 70.

To investigate whether the N-terminal cytoplasmic domain of Mal61/HAp contains residues critical for its glucose-induced inactivation, a series of deletion mutations of this domain along with specific point mutations were constructed. These include deletions of residues 2-30, 31-60, 61-90, 49-78, 49-60, 61-78, and point mutations of di-leucine 69 and 70 to alanine (see Table 1). Plasmid-borne mutant alleles were transformed into strain CMY1050 and the phenotypes determined (see Table 3). Only strains carrying *mal61/HA(Δ61-90)* were unable to sustain growth on maltose and exhibited maltose transport activity less than 2% of wild-type strains. Strains carrying the other mutant alleles all exhibited reduced levels of maltose transport activity, ranging from 7% to 35% of wild-type

levels. The relative amounts of Mal61/HAp, as determined by densitometer, also are presented in Table 3. These do not vary more than 25-30% from wild-type expression levels indicating that the N-terminal alterations reduce the specific activity of the encoded permease.

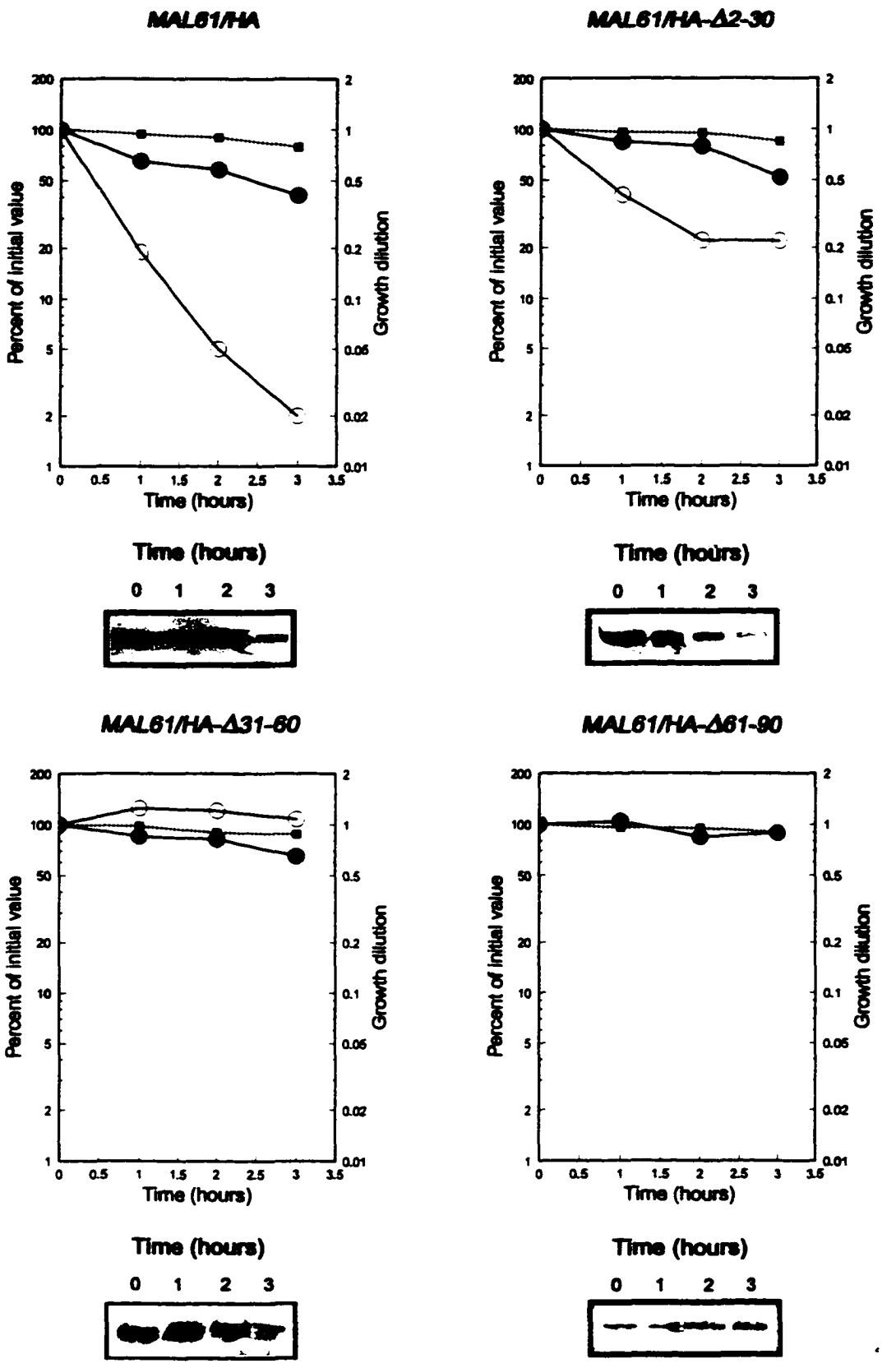
Figures 1 and 2 present the results of glucose-induced inactivation assays carried out on these N-terminal mutants. It should be noted that the kinetics of glucose-induced proteolysis of maltose permease in strain CMY1050 carrying plasmid-borne *MAL61/HA* alleles is slightly slower than rates reported previously for strains expressing solely a chromosomal copy of the gene (Medintz *et al.*, 1996). We feel that this is a result of increased expression of maltose permease perhaps to levels that tax the endocytic machinery. As can be seen in Figure 1 the kinetics of inactivation for the Mal61/HA(Δ2-30) permease are not significantly different from those of the wild-type Mal61/HA permease. In contrast, the Mal61/HA(Δ31-60) permease which lacks a portion of the PEST sequence, is relatively insensitive to glucose-induced inhibition of maltose permease and exhibits a much reduced rate of glucose-induced degradation. Strains expressing Mal61/HA(Δ61-90) permease are unable to ferment maltose despite the presence of approximately 35% of normal protein levels. Maltose transport activity is very low and at nearly background levels (see Table 3). Glucose-induced degradation of the Mal61/HA(Δ61-90) permease is much

TABLE 3. Phenotypes of *MAL61/HA* maltose permease N-terminal cytoplasmic mutants

MAL61 allele	Maltose transport activity (nM/min/mg)	Maltose fermentation	Relative level of Mal61/HAp
mal61/HA	5.96	+	100
mal61/HA(Δ2-30)	0.71	+	85
Mal61/HA(Δ31-60)	2.07	+	76
Mal61/HA(Δ61-90)	0.07	-	34
Mal61/HA(Δ49-78)	0.43	+	71
Mal61/HA(Δ49-60)	0.83	+	126
Mal61/HA(Δ61-78)	0.53	+	81
Mal61/HA(L69A,L70A)	0.53	+	111
Vector control	0.06	-	-

MAL61/HA mutant alleles carried on pUN30 vector (Elledge and Davis, 1988) were introduced into the maltose permease null strain CMY1050

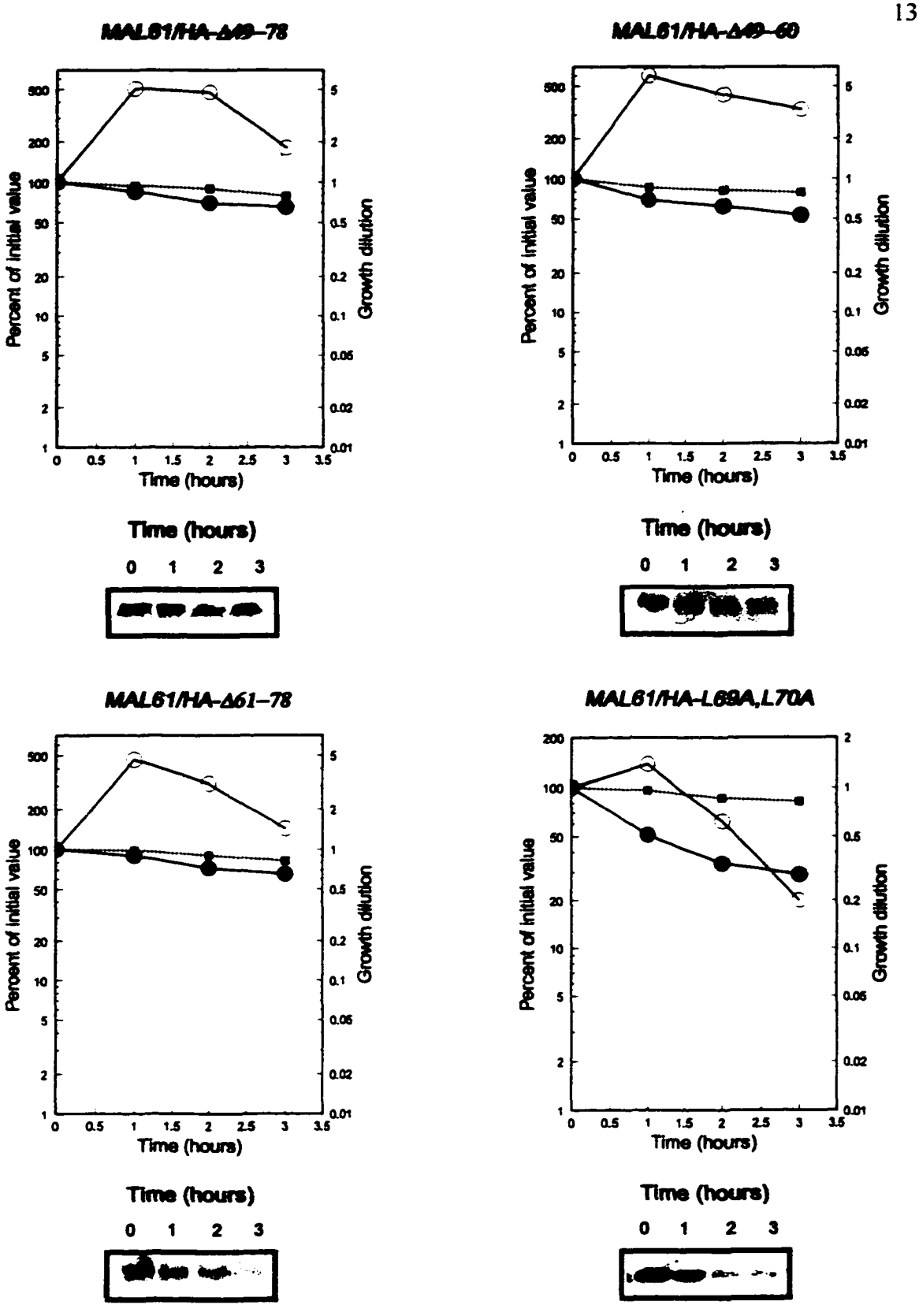
Figure 1. Glucose-induced inactivation of maltose permease mutant alleles with alterations in the N-terminal cytoplasmic domain. Strain CMY1050 carrying either plasmid pMAL61/HA, pMAL61/H(Δ2-30), pMAL61/HA(Δ31-60), or pMAL61/HA-(Δ60-90) was grown on selective medium plus 2% maltose, harvested and transferred to nitrogen-starvation medium plus 2% glucose. At the indicated times the OD_{600} was determined and aliquots of the culture were removed for maltose transport assays and the preparation of total protein extracts for Western analysis of Mal61/HA protein levels, as described in Materials and Methods. The Vistra-ECF Western Blotting Visualization System (Amersham) and the Molecular Dynamics Storm Image Analyzer were used to capture the Western image and quantitate the relative density of each time point. Representative Western blots are shown, but the quantitation data used in the graph was obtained from the average of at least two experiments, each run on duplicate gels, and scanned twice. The relative levels of Mal61/HA protein (●) and maltose transport activity (○) compared to the zero time sample are plotted along with the growth dilution (■, dotted line). Growth dilution represents the growth of the culture during the course of the experiment and is calculated as the OD_{600} at time zero divided by the OD_{600} time X.



reduced, but the significance of this result is uncertain given the possibility that the protein may not assume the normal topology. It is interesting to note that expression of this nonfunctional deleted permease is maltose inducible. Figure 2 presents the results of inactivation assays carried out on strains expressing the *mal61/HA(Δ49-78)*, *mal61/HA(Δ49-60)*, and *mal61/HA(Δ61-78)* permease mutant alleles lacking all or part of the putative PEST sequence. As can be seen in Figure 2, these deletion mutations all result in permeases that are insensitive to glucose-induced inhibition and exhibit much reduced rates of glucose-induced proteolysis. Rather surprisingly, in contrast to the rapid inhibition of transport activity observed in the other maltose permease alleles even in the absence of endocytosis and degradation, addition of glucose to the media stimulates an immediate increase in maltose transport activity, up to 5-fold, that is only slowly reduced over the course of the experiment.

The strain expressing the mal61/HA(L69A,L70A) permease demonstrates a modest increase in maltose transport activity upon glucose addition that drops to 20% of initial levels by the third hour (see Figure 2). Glucose-induced degradation of this di-leucine motif mutant permease appears to be normal.

Figure 2. Glucose-induced inactivation of additional maltose permease mutant alleles with alterations in the N-terminal cytoplasmic domain. Strain CMY1050 carrying either plasmid pMAL61/HA(Δ49-78), pMAL61/HA(Δ49-60), pMAL61/HA(Δ61-78), or pMAL61/HA(L69A,L70A) were grown as described in Figure 1, harvested, and transferred to nitrogen-starvation medium containing 2% glucose. Samples were taken at the indicated times, and the growth dilution (■), maltose transport activity (○), and relative Mal61/HA protein levels (●) were determined as described in Materials and Methods and Figure 1. Note the difference in scales on some of the axes.



Glucose-induced ubiquitination of mutant maltose permease alleles.

Two mutant permease alleles, *Mal61/HA-581NS(S572A,T573A)* and *Mal61/HA(Δ49-78)*, were selected as examples of C-terminal and N-terminal mutant alleles exhibiting reduced rates of glucose-induced proteolysis. Our previous studies demonstrated that glucose stimulates ubiquitination of maltose permease and that this ubiquitination is involved in the glucose-induced degradation of maltose permease (Medintz *et al.*, 1996). If either of the altered sequences affect a target site required for ubiquitin conjugation we should expect significantly reduced rates or no ubiquitination of these mutant proteins. We tested this prediction.

Ubiquitinated maltose permease is most effectively demonstrated in an endocytosis deficient strain expressing an overabundance of a *c-myc* tagged ubiquitin allele (Medintz *et al.*, 1998). *C-myc* tagged ubiquitin-conjugated species have a greater abundance because they are more resistant to ubiquitin hydrolases than proteins conjugated to wild-type ubiquitin (Medintz *et al.*, 1998; Hochstrasser *et al.*, 1991). Strain CMY1052 contains an *end3-ts* allele which results in deficient endocytosis at the non-permissive temperature of 37°C (Medintz *et al.*, 1996; Raths *et al.*, 1993). Ubiquitin-conjugated maltose permease accumulates in this strain at the non-permissive temperature and is thus more abundant and easily detected (Medintz *et al.*, 1998).

Strain CMY1052 was transformed with a plasmid carrying the *c-myc* tagged ubiquitin gene expressed from the *CUP1* copper responsive promoter (Ward *et al.*, 1994) and either *MAL61/HA*, *mal61/HA/581NS(S572A,T573A)*, or

mal61/HA(Δ49-78). Strains were grown overnight at 30°C in maltose selective media to early log phase (OD $_{600}$ 0.1 to 0.3). The cultures were then supplemented with copper sulfate and grown a further 3 hours at 30°C. The temperature was then raised to 37°C for another 1 hour at which time 2% glucose was added to stimulate ubiquitination. After an additional 0.5 hrs the cells were harvested and total protein extracts prepared. These conditions produce the highest abundance of ubiquitinated maltose permease in this strain under these conditions (Medintz *et al.*, 1998). Samples containing approximately equal amounts of maltose permease protein were analyzed by Western blot. As can be seen in Figure 3, the ubiquitinated maltose permease species is clearly visible in the control lane containing Mal61/HAp. Significantly reduced levels of the ubiquitinated species of Mal61/HA-581NS(S572A,T573A)p and Mal61/HA(Δ49-78) permease are seen relative to the amounts for the wild-type. This correlates with the reduced rates of glucose-induced turnover that these alterations cause (see Table 2 and Figure 3).

Figure 3. Relative ubiquitination of wild-type and mutant alleles of Mal61/HAp. All strains were transformed with the pC-myc-Ub plasmid (Medintz *et al.*, 1998) that encodes a *c-myc* tagged ubiquitin allele on a copper inducible promoter. Strains were also transformed with the maltose permease allele indicated. Strains were grown overnight in maltose selective media and then incubated in 0.1mM $CuSO_4$ for 3 hours at 30°C. Cultures were then transferred to 37°C for 1 hour, 2% glucose was added to the growth media for an additional 30 minutes prior to harvesting. Samples were prepared and analyzed as described in Materials and Methods and Figure 1.

Inactivation of Mal61/HA maltose permease mutants in Pathway 1 and *Pathway* 2 strains. Jiang *et al* (1997) identified two glucose sensing/signaling pathways that are used to monitor glucose levels and to stimulate glucose-induced degradation of maltose permease. Pathway 1, which is independent of glucose transport, appears to be modulated by Rgt2p, a glucose sensor present in the plasma membrane. Rgt2p and Snf3p are homologous membrane localized proteins, which have been identified as extracellular glucose sensors (Ozcan *et al.*, 1996). Rgt2p senses the presence of high extra-cellular glucose concentrations, while Snf3p is a low glucose sensor (Ozcan *et al.*, 1996). The *RGT2-1* allele, which was identified as a dominant mutation causing constitutive expression of *HXT1* encoding a low-affinity glucose transporter, also causes the constitutive proteolysis of maltose permease in the absence of glucose (Jiang *et al.*, 1997). The model of glucose-induced-inactivation of maltose permease proposes that a high concentration of extracellular glucose is sensed by Rgt2p and transduction is mediated by Grr1p and Glc7p/Reg1p protein phosphatase type-1 (Jiang *et al.*, 1997; Jiang *et al.*, submitted). Pathway 2 uses high rates of glucose transport and phosphorylation (or other fermentable sugars) to generate a signal (Jiang *et al.*, 1997; Jiang *et al.*, submitted [b]).

In order to distinguish effects of these two glucose sensing/signaling pathways on specific mutant Mal/61/HA maltose permeases, strains were constructed with alterations in Pathway 1 or Pathway 2 signaling and either plasmid-borne *MAL61/HA*, mal61/HA-581(S572A,T573A), or mal61/HA(Δ49-78) was introduced into these strains.

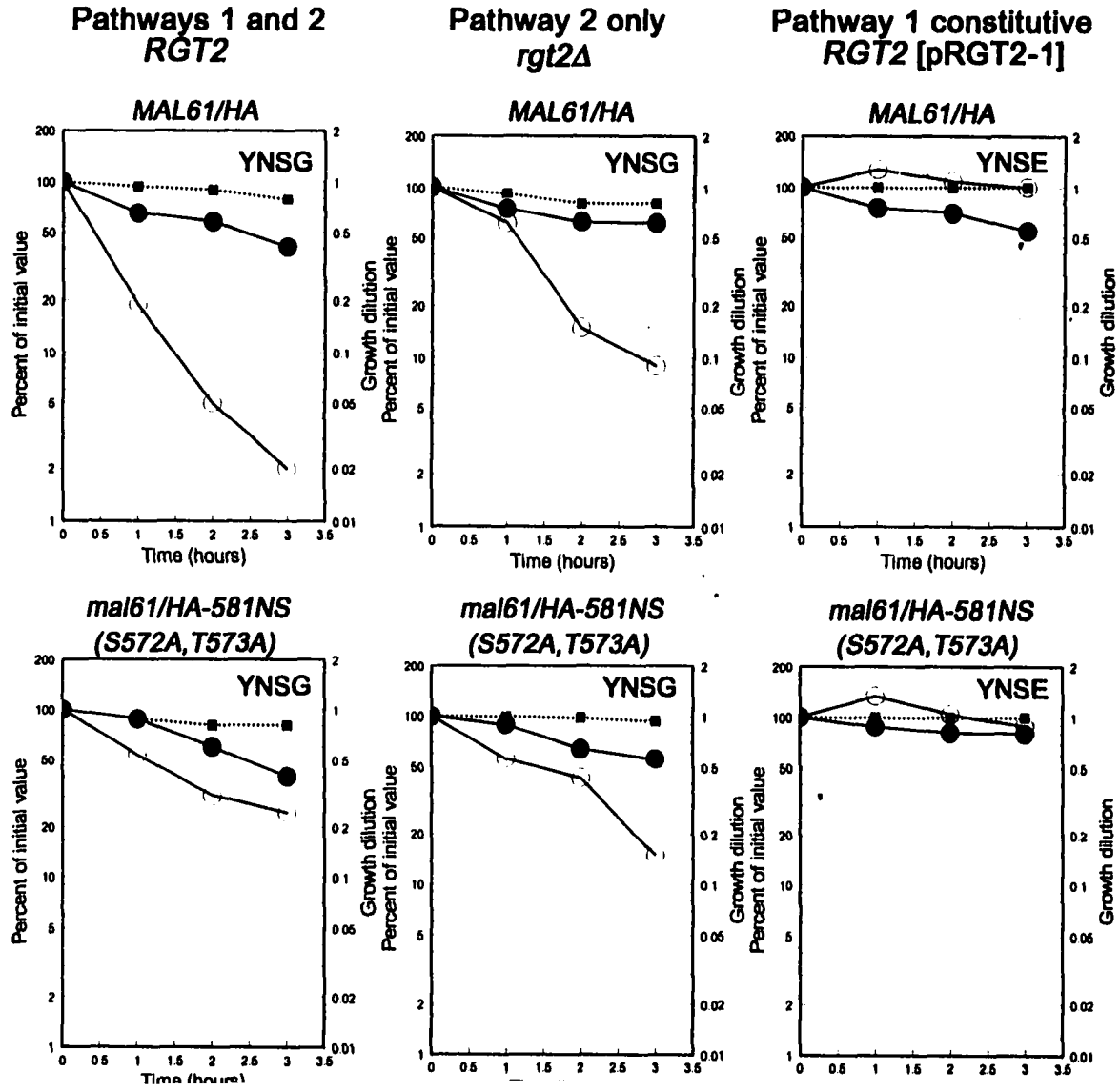
Both pathways are intact in strain CMY1050, which carries a null mutation in the chromosomal maltose permease gene. Results from this strain are shown in the left-hand column of panels in Figure 4. Strain CMY1051 is isogenic to CMY1050 except that the *RGT2* ORF was replaced with a KAN^R gene using a PCR based one step gene replacement (Jiang *et al.*, 1997; Guldener *et al.*, 1996). Since the glucose sensor of Pathway 1, *RGT2*, has been removed strain CMY1051 is deficient in Pathway 1 signaling and only has a functional Pathway 2. Results for this strain are presented in the middle column of panels in Figure 4. By introducing the constitutive *RGT2-1* allele carried by plasmid pRGT2-1 into strain CMY1050 we are able to activate Pathway 1 in the absence of glucose and without any contribution from Pathway 2 since glucose is not present to be transported or phosphorylated (Jiang *et al.*, 1997). Results for this strain are shown in the right hand column of panels in Figure 4. Inactivation of maltose permease was characterized and the results are shown in figure 4. The top row of graphs presents the results for strains expressing wild-type Mal61/HA permease, the middle row for strains expressing Mal61/HA-581NS(S572A,T573A) permease, and the bottom row for strains expressing Mal61/HA(Δ49-78)p.

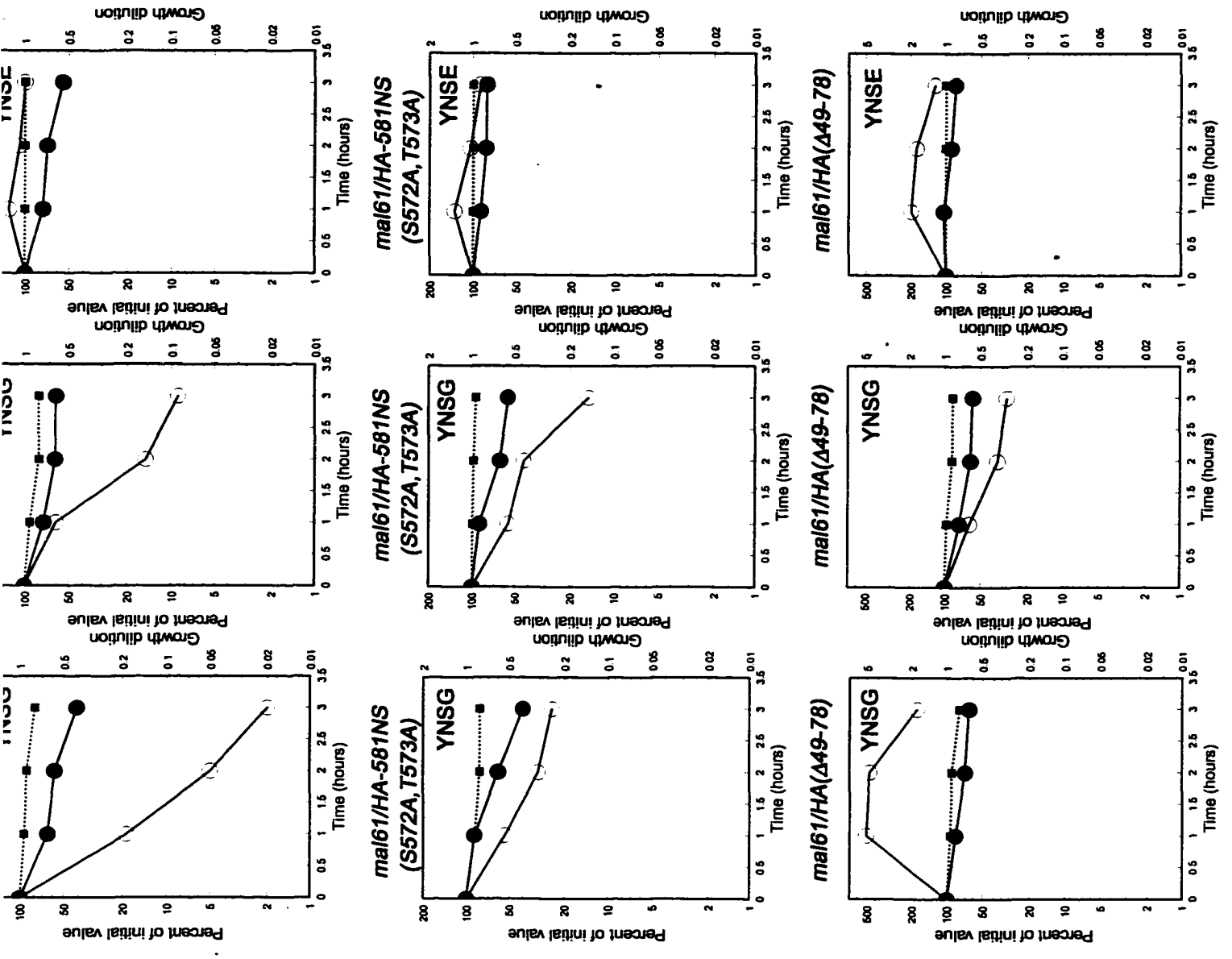
As shown by Jiang *et al* (1997), the results in the top row of panels confirm that two glucose sensing/signaling pathways stimulate proteolysis of maltose permease, but Pathway 2 appears to be predominantly responsible for glucose-induced inhibition of maltose transport activity. Truncation and mutations of the C-terminal cytoplasmic domain, as in Mal61/HA-

581NS(S572A,T573A) permease, modestly reduces the rate of glucose-induced proteolysis. Results with both the *RGT2* and *rgt2Δ* strains give similar inactivation profiles, while Rgt2p-stimulated degradation of this mutant protein is significantly compromised. This suggests that Pathway 1 acts on C-terminal sequences with some effects mediated by N-terminal sequences while Pathway 2 largely targets the N-terminal domain.

Loss of residues 49-78 has a major impact on maltose permease proteolysis and this is most clearly evident in the constitutive Pathway 1 strain where little or no turnover of Mal61/HA(Δ49-78) permease is seen. As mentioned previously, a glucose-stimulated activation of maltose transport activity is seen in the *RGT2* strain expressing the PESTΔ mal61/HA(Δ49-78) permease allele. This transport activation appears to be Pathway 1 mediated since it is constitutively exhibited in the strain carrying *RGT2-1* but absent in the glucose-stimulated *rgt2Δ* strain.

Figure 4. Glucose-induced inactivation of Pathway 1 and Pathway 2 deficient strains expressing mutant Mal61/HA maltose permease. Results of inactivation assays carried out in the parental CMY1050 (*RGT2*) or the CMY1051(*rgt2Δ)* strain, and CMY1050[pRGT2-1] expressing *MAL61/HA*, *mal61/HA/581NS(S572A,T573A)* or *mal61/HA(Δ49-78)* alleles of maltose permease are presented. Left-hand and middle column of panels are glucose-induced inactivation assays. The right-hand column of panels presents inactivation assays carried out in 2% ethanol as the carbon source and represents constitutive effects of *RGT2*. YNSG indicates inactivation media containing 2% glucose while YNSE indicates inactivation media containing 2% ethanol. Samples were taken at the indicated times, and the growth dilution (■), maltose transport activity (○), and relative Mal61/HA protein levels (●) were determined as described in Materials and Methods and Figure 1.





DISCUSSION

Several recent studies have revealed target sequences of yeast membrane proteins that are required for efficient ubiquitination. The SINNDAKSS sequence in the C-terminal tail of the Ste2p α-factor receptor is necessary and sufficient for ubiquitination and internalization of a truncated α-factor receptor (Hicke and Riezman, 1996; Hicke, 1997). The full length α-factor receptor tail carries 7 redundant lysine residues each of which can serve as potential ubiquitin-conjugation sites (Hicke and Riezman, 1996). Upon binding of α-factor, Ste2p is hyperphosphorylated which in turn promotes downregulation by positively controlling ubiquitination and internalization of this G-protein coupled receptor (Hicke *et al.,* 1998). Recently, it has been demonstrated that monoubiquitination of α-factor receptor itself serves as the internalization signal (Terrell *et al.*, 1998). The ABC peptide transporter Ste6p contains a 100 residue "linker region" in an intracellular loop, which is required for ubiquitination and rapid turnover of the protein. The acidic stretch DAKTI, which is similar to SINNDAKSS, is required for ubiquitination and turnover of Ste6p (Kolling and Losko, 1997).

The C-terminal tail of the Gap1 general amino acid permease contains sequences, including a di-leucine motif, which are required for NH_4^+ -induced internalization and degradation of this permease. Although mutant Gap1 permeases altered in these sequences still form ubiquitin-conjugates, it is at a

significantly reduced rate compared to the full-length protein (Springael and Andre, 1998). When located at the cell surface, the Fur4p uracil permease is subject to phosphorylation and ubiquitination. Ubiquitination is necessary prior to permease endocytosis and degradation. A PEST-like sequence located within the cytoplasmic N-terminus of this protein is essential for uracil permease turnover. The serine residues within the PEST-like sequence are required for phosphorylation and ubiquitination of Fur4p (Marchal *et al.*, 1998). Mal61/HAp contains numerous residues with homology to some of these ubiquitination target domains. These include a N-terminal PEST sequence, numerous lysine residues and a potential protein kinase C serine/threonine phosphorylation site in the C-terminus cytoplasmic domain of the protein (Cheng and Michels, 1989; Harma *et al.*, 1998).

The results reported here indicate that the putative PEST sequence located in residues 49-78 of Mal61 maltose permease acts as an important targeting sequence for the degradation of this protein. Mutant permease proteins lacking this sequence, or portions of this sequence, exhibit significantly reduced rates of glucose-induced proteolysis. While we have not demonstrated the site(s) of ubiquitin conjugation, we do show a dramatic reduction in the relative amount of the ubiquitin-conjugated form of the Mal61(Δ49-78) protein relative to the wild-type protein. Thus, these results suggest that residues 49-78 are required for efficient glucose-induced ubiquitination of maltose permease. There are several lysine residues in and around the PEST sequence which could serve as ubiquitin conjugation sites.

Alterations in other sequences throughout Mal61 permease have variable minor effects on the rate of glucose-induced proteolysis. Mutations in the C-terminal domain of Mal61/HA permease result in loss of specific activity of maltose transport but only modest alterations in the half-life of glucose-induced degradation (see Table 2). These changes include mutation of proline residues at 566 and 577 to alanine, *mal61/HA-581NS(P566A)* and *mal61/HA-581NS(P577A)*, mutating a potential protein kinase C phosphorylation site at S572, T573 to alanine, *mal61/HA-581NS(S572A,T573A*), and mutating lysine residues at 569, 571, and 574 to alanine, *mal61/HA-581NS(K569A,K571A, K574A*). Interestingly, two of these mutants alleles, *mal61/HA-581NS(P577A)* and *mal61/HA-581NS(K569A,K571A,K574A)*, actually result in proteins with a more rapid glucose–induced turnover rate than the wild-type Mal61/HAp. Mutation of a potential endocytosis signal, NPF 576-578 to alanine, in the full-length MAL61/HAp C-terminal, resulted in a mutant permease, mal61/HA-581NS(N576A,P577A,F578A)p, with a longer half life, 2.8 hrs. These results suggest that while the NPF endocytosis signal and other sequences in the C-terminal cytoplasmic domain may contribute to the rate of glucose-induced degradation of Mal61/HAp, this region is not the sole or most important determinant regulating this process.

Medintz *et al* (1996) showed that glucose induces two processes that both lead to the loss of maltose permease activity. The initial event is a rapid decrease in maltose transport activity, which is proposed to result from a modification event (such as phosphorylation) because it occurs more rapidly than

can be explained by protein loss. This rapid inhibition of maltose transport activity is distinct from endocytosis and ubiquitination since it occurs in strains deficient for ubiquitin conjugation, such as a *doa4Δ* strain, and in endocytosis defective strains, such as *end3-ts* strains (Medintz *et al.*, 1998; Medintz *et al.*, 1996). We feel that rapid phosphorylation is a possible explanation for the rapid glucose-induced loss of maltose transport activity. Mal61/HAp is phosphorylated and becomes hyper-phosphorylated in glucose-grown conditions (Medintz, *et al.*, 1996). Moreover, Harma *et al* (1998) demonstrated that mutating potential phosphorylation sites in Mal61p resulted in significantly reduced rates of inactivation in the presence of glucose. Two Mal61p mutants, S295A and S487A, also resulted in slower rates of degradation as well.

Mutation of all the serine residues to alanine within the PEST-like sequence of Fur4p resulted in the very poor phosphorylation of the protein and elimination of ubiquitination (Marchal *et al.*, 1998). The phosphorylation and degree of ubiquitination of variant Fur4 permeases was inversely correlated with the number of serines replaced by alanines. Mutating these same serine residues to glutamate residues restored efficienct turnover of Fur4p suggesting that these particular residues act as phosphoacceptors (Marchal *et al.*, 1998). Given our demonstration of the importance of the PEST sequence in Mal61HAp, we suggest that phosphorylation of residues in this region also may initiate the events to the glucose-induced inactivation of this protein. However, it appears that there may be other phosphorylation sites throughout the protein that regulate transport activity and may contribute to turnover rates.

It is interesting to note that in the absence of residues 49-78, Pathway 1 stimulation, but not Pathway 2 stimulation, activates transport activity. This suggests that both pathways target multiple sites throughout the permease. The effects on transport activity are variable. A major target of Pathway 1 appears to be the PEST sequence and the initiation of the inactivation process. In the absence of the PEST sequence, other direct or indirect effects of Pathway 1 increase the specific activity of the permease in response to glucose, probably by phosphorylation. The mechanism of this process is now being studied.

ACKNOWLEDGEMENTS

We wish to thank Sara Danzi for technical assistance with *in-vitro* mutagenesis and Jeannette Schaefer for assistance with figure preparations. This work was supported by a grant to C.A.M. from the National Institute of General Medical Sciences (GM49280). This work was carried out in partial fulfillment of the requirements for the Ph.D. degree form the Graduate School of the City University of New York (I.M.).

CONCLUSION

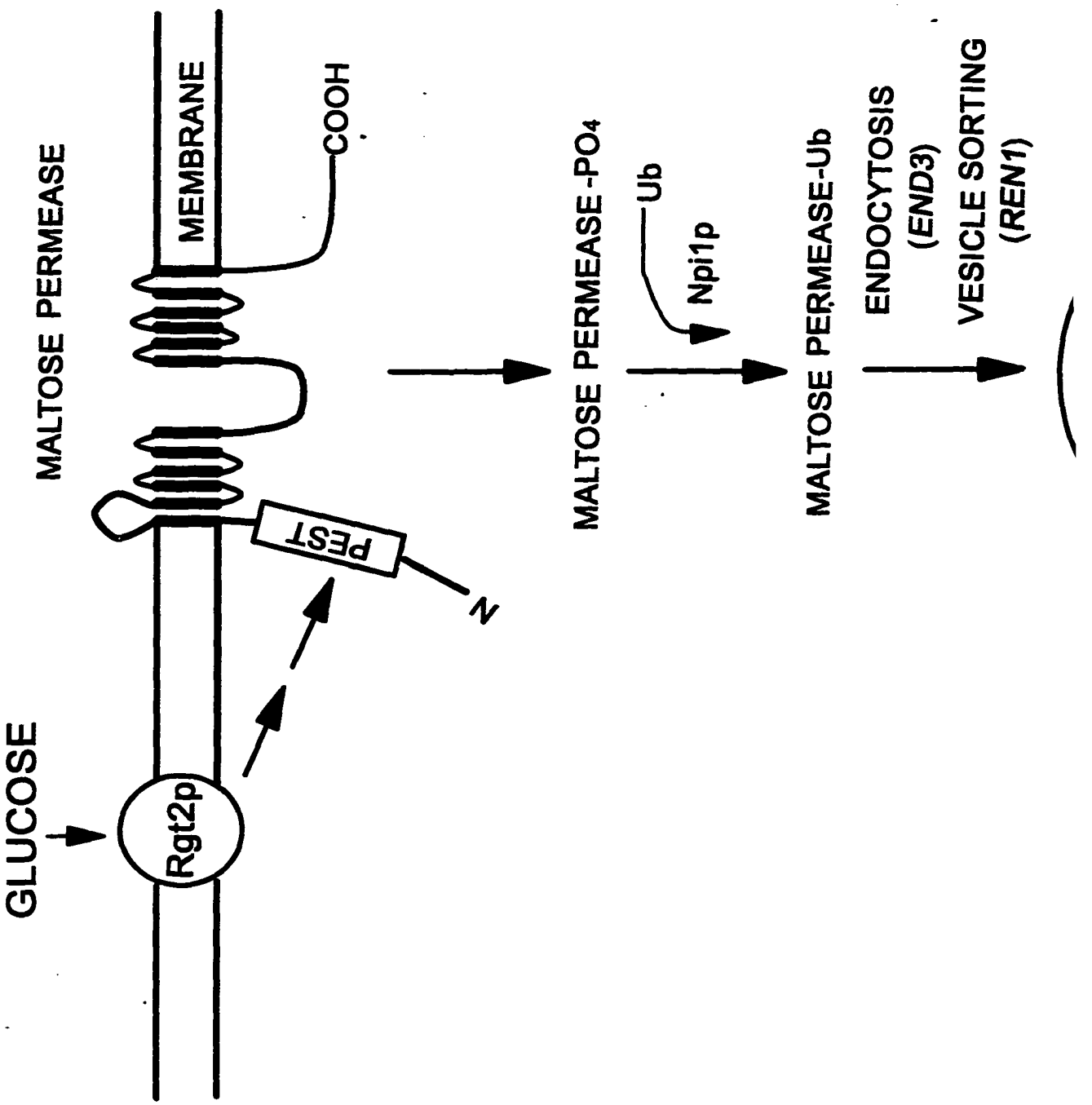
A model is presented here to describe the mechanism whereby the addition of glucose to the growth media results in an increased rate of proteolytic degradation of maltose permease (see Figure 1 following). Chapter 1 demonstrates that this is dependent on endocytosis, vesicle sorting and vacuolar proteolysis. Rgt2p has been implicated as the most upstream component of a signal transduction pathway that is primarily responsible for targeting maltose permease for degradation (Jiang *et al.*, 1997). Rgt2p is a sensor of high concentrations of extracellular glucose (Ozcan *et al.*, 1996a, b). The thesis project of Jiang suggests that the net downstream effect of this Rgt2p-dependent signal is the phosphorylation of the maltose permease. We have shown that the maltose permease exists in differentially phosphorylated forms depending upon the carbon source present, see Chapter 1 (Medintz *et al.*, 1996).

This model further proposes that phosphorylation of maltose permease is the signal that targets maltose permease for ubiquitin conjugation by a complex that includes the ubiquitin protein-ligase (E3) encoded by *RSP5/NPI1*. Ubiquitin conjugation allows the endocytosis machinery to target maltose permease for removal from the membrane. Similar results have been demonstrated and a similar conclusion has been proposed for many other yeast membrane proteins (Hicke, 1997). After undergoing rapid inhibition and ubiquitination, maltose permease undergoes endocytosis that is dependent on the *END3* gene product. The permease is then transported to the vacuole, via endocytotic vesicles (which include the *REN1* gene product), where it undergoes proteolytic degradation that

is dependant upon the *PEP4* gene product. This occurs independent of the proteasome. This is consistent with other findings indicating that almost all yeast membrane proteins studied to date undergo vacuolar degradation (Hicke, 1997). A similar model has also been proposed for the yeast uracil permease encoded by *FUR4*. A PEST-like sequence located within the cytoplasmic N-terminus of Fur4p is essential for efficient phosphorylation, subsequent ubiquitination (via Rsp5p) and endocytotic downregulation of this protein (Marchal *et al.*, 1998; Galan *et al.*, 1996).

It is proposed that the phosphorylation of maltose permease results in its irreversible rapid inhibition. In response to glucose, maltose permease undergoes a loss of transport activity more rapidly than can be explained by the slower degradation process and this is true even when endocytosis is disrupted and the protein remains at the membrane as in an *ends-ts* strain (Medintz *et al.*, 1996). Similar glucose-induced inhibition studies of maltose permease also have been reported and the same modification, phosphorylation, has been suggested as the cause of rapid inhibition in response to glucose by Harma *et al.* (1998). The model of glucose-induced inactivation of maltose permease established here will allow further elucidation of this process.

Figure 1. Proposed model describing the mechanism of glucose-induced inactivation and degradation of maltose permease. A diagram describing the model whereby the presence of high glucose is sensed and results in the inactivation of maltose permease. A high glucose concentration is sensed by the membrane located Rgt2p and a signal is transduced to the maltose permease which targets and phosphorylates the N-terminal cytoplasmic PEST region. This results in the inhibition of maltose transport activity of the permease and leads to the subsequent ubiquitination of the permease. Ubiquitin-conjugation signals the endocytosis of the permease, which is transported via vesicles to the vacuole where it is degraded. Where appropriate genes that were mutated in order to determine their contribution are indicated.



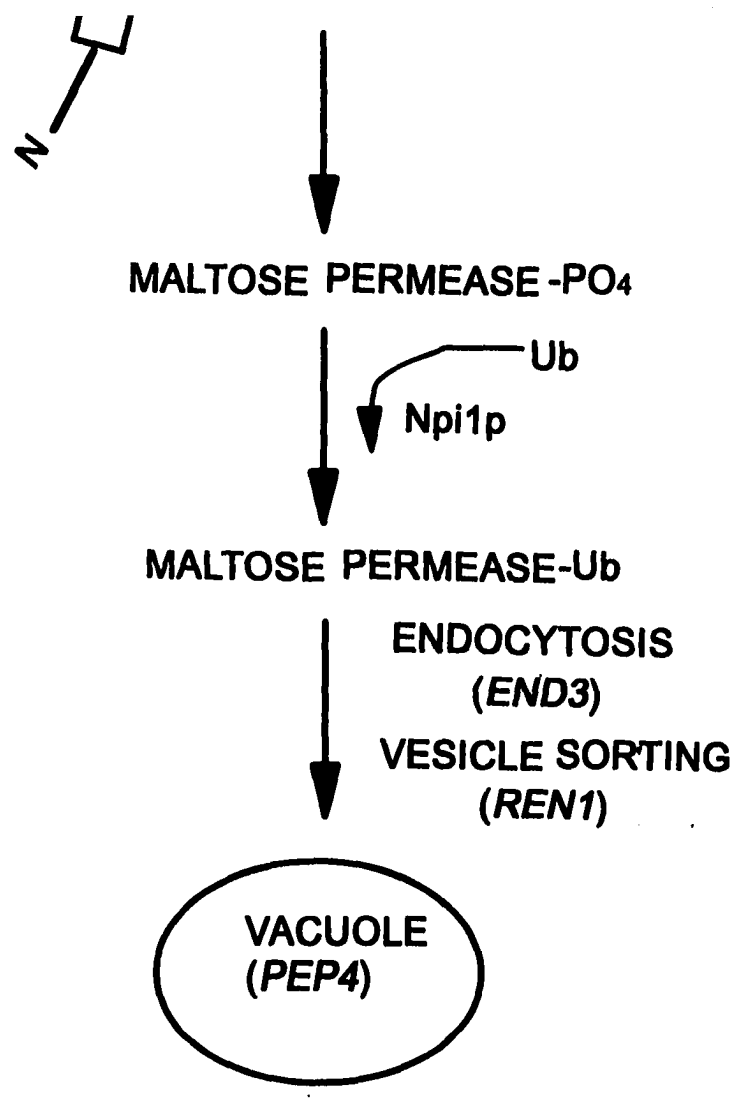


Figure 1. Proposed model describing the mechanism of glucose-induced inactivation and degradation of maltose permease.

BIBLIOGRAPHY

Ammerer. G., C.P. Hunter, J.H. Rothman, G.C. Saari, L.A. Valls, and T.H. Stevens. 1986. *PEP4* gene of *Saccharomyces cerevisiae* encodes proteinase A, a vacuolar enzyme required for processing of vacuolar precursors. *Mol. Cell. Biol.* 6:2490-2499.

Ausubel, F.M., R. Brent, R.E. Kingston, D.E. Moore, J.G. Seidman, J.A.Smith, and K. Struhl. 1995. *Current protocols in molecular biology* p8.1.1-6 & .p13.13.7. Greene Publishing Associates, Inc. and John Wiley and Sons, Inc., New York.

Banerjee, A., Deshaies, R.J., and Chau, V. 1995. Characterization of a dominant negative mutant of the cell cycle ubiquitin-conjugating enzyme Cdc34. *J. Biol. Chem.* 44, 26209-26215.

Bansal, A. and L.M. Gierasch, 1991. The NPXY internalization signal of the LDL receptor adopts a tight-turn conformation. *Cell* 67: 1195-1201.

Benito, B., and R. Lagunas, 1992. The low-affinity component of *Saccharomyces cerevisiae* maltose transport is an artifact. *J. Bact.* 174:3065-3069.

Benito, B., E. Moreno, and R. Lagunas. 1991. Half-life of the plasma membrane ATPase and its activating system in resting cells. *Biochem. Biophy. Acta* 1063:265-268.

Berkower, C., Taglicht, D., and S. Michaelis. 1996. Functional and physical interactions between partial molecules of STE6, a yeast ATP-Binding cassette protein. *J. Bio. Chem.* 271:22983-22989.

Berkower, C., and S. Michaelis. 1991. Mutational analysis of the yeast a-factor transporter STE6, a member of the ATP binding cassette (ABC) protein superfamily. *EMBO J.* 10:3777-3785.

Boeke, J., F. Lacroute, and G. Fink. 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet.* 197:345-346.

Bustaria, A., and R. Lagunas. 1985. Identification of two forms of the maltose transport system in *Saccharomyces cerevisiae* and their regulation by catabolite inactivation. *Biochim. Biophys. Acta.* 820:324-236.

Bustaria, A., and R. Lagunas. 1986. Catabolite inactivation of the glucose transport system in *Saccharomyces cerevisiae*. J. *Gen. Microbio.* 132:379-385.

Canfield, W.M., Johnson, K.F., Ye, R.D., Gregory, W. and S. Kornfeld, 1991. Localization of the signal for rapid internalization of the bovine cation-dependent mannose 6-phosphate/insulin-like growth factor-II receptor to amino acids 24-29 of the cytoplasmic tail. *J. Bio. Chem.* 266: 5682-5688.

Charron, M.J., Read, E., Haut, S.R., and C.A. Michels, 1989. Molecular evolution of the telomere-associated *MAL* loci of *Saccharomyces*. *Genetics* 122: 307-316.

Charron, M.J., R.A. Dubin, and C.A. Michels. 1986. Structural and functional analysis of the *MAL1* locus of *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 6:3891-3899.

Chen, P., Johnson, P., Sommer, T., Jentsch, S., and Hochstrasser, M. 1993. Multiple ubiquitin-comjugating enzymes perticipate in the in vivo degradation of the yeast MATα2 repressor, *Cell* 74, 357-369.

Chen, W.J., Goldstein, J.L. and M.S. Brown, 1990. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J. Bio. Chem.* 265: 3116-3123.

Cheng, Q., and C.A. Michels. 1989. The maltose permease encoded by the *MAL61* gene of *Saccharomyces cerevisiae* exhibits both sequence and structural homology to other sugar transporters. *Genetics* 123:477-484.

Cheng, Q., and C.A. Michels. 1991. *MAL11* and *MAL61* encode the high-affinity maltose transporter of *Saccharomyces cerevisiae*. *J. Bacteriol.* 171:1817-1820.

Chiang, H., and R. Schekman. 1991. Regulated import and degradation of a cytosolic protein in the yeast vacuole. *Nature* 350:313-318.

Chiang, H., and R. Schekman. 1994. Letter. *Nature* 369:284.

Ciechanover, A., 1994. The ubiquitin-proteasome proteolytic pathway. *Cell* 79: 13-21.

Collawn, J.F., Lai, A., Domingo, D., Fitch, M., Hatton, S. and I.S. Trowbridge, 1993. YTRF is the conserved internalization signal of the transferrin receptor, and a second YTRF signal at position 31-34 enhances endocytosis. *J. Biol. Chem.* 268: 21686-21692.

Corvera, S., Chawla, A., Chakrabarti, R., Joly, M., Buxton, J. and M.P. Czech, 1994. A double leucine within the GLUT4 glucose transporter COOH-terminal domain functions as an endocytosis signal. *J. Cell. Bio.*, 126: 979-989.

Dargemount, C., Le-Bivic, A., Rothenberger, S., Iacopetta, B. and L.C. Kuhn, 1993. The internalization signal and the phosphorylation site of transferrin receptor are distinct from the main basolateral sorting information. *EMBO J.* 12: 1713-1721.

Davis, C.G., van Driel, I.R., Russel, D.W., Brown, M.S. and J.L. Goldstein, 1987. The low density lipoprotein receptor. *J. Bio. Chem.* 262: 4075-4082.

Davis, N.G., J.L. Horecka, and G.F. Sprague, Jr. 1993. *Cis* and *trans*-acting functions required for endocytosis of the yeast pheromone receptors. *J. Cell Bio.* 122:53-65.

DeJuan, C., and R. Lagunas. 1986. Inactivation of the galactose transport system in *Saccharomyces cerevisiae*. *FEBS Letters*207:258-261.

Del Pozo, J.C., Timpte, C., Tan, S., Callis, J., and Estelle, M. 1998. The ubiquitin-related protein RUB1 and auxin response in *Arabidopsis. Science* 280, 1760-1763.

Dittrich, E., C.R. Haft, L. Muys, P.C. Heinrich, and L. Graeve. 1996. A di-leucine motif and an upstrean serine in the interleukin -6 (IL-6) signal transducer gp130 mediate ligand-induced endocytosis and down-regulation of the IL-6 receptor. *J. Bio. Chem.* 271:5487-5494.

Dubin, R.A., M.J. Charron, S.R. Haut, R.B. Needleman, and C.A. Michels. 1988. Constitutive expression of the maltose fermentation enzymes in *Saccharomyces carlsbergensis* is dependent upon the mutational activation of a nonessential homolog of *MAL63. Mol. Cel. Biol.* 8:1027-1035.

Dubin, R.A., Needleman, R.B., Gossett, D., and Michels, C.A. 1985. Identification of the structural gene encoding maltase within the *MAL6* locus of *Saccharomyces carlsbergensis. J. Bact.* 164: 605-610.

Ecker, D.J., Khan, M.I., Marsh, J., Butt, T.R., and Crooke, S.T. 1987. Chemical synthesis and expression of a cassette adapted ubiquitin gene. *J. Biol. Chem.* 262, 3524-3527.

Egner, R. and K. Kuchler, 1996. The yeast multidrug transporter Pdr5 of the plasma membrane is ubiquitinated prior to endocytosis and degradation in the vacuole. *FEBS Letters.* 378:177-181.

Egner, R., Thumm, M., Straub, M., Simeon, A., Schuller, H.J. and D.H. Wolf, 1993. Tracing intracellular proteolytic pathways. *J. Bio. Chem.* 268: 27269-27276.

Eisenman, D.M., Arndt, K.M., Ricupero, S.L., Tooney, J.W., and Winston, F. (1992). SPT3 interacts with TFIID to allow normal transcription in *Saccharomyces cerevisiae. Genes Dev.* 6:1319-1331.

Elledge, S.J. and R.W. Davis, 1988. A family of versatile centromeric vectors designed for use in the sectoring-shuffle mutagenesis assay in *Saccharomyces cerevisiae. Gene* 70: 303-312.

Fallon, R.J., Danaher, M., Saylors, R.L. and A. Saxena, 1994. Defective asialoglycoprotein receptor endocytosis mediated by tyrosine kinase inhibitors. *J. Bio. Chem.*269: 11011-11017.

Fallon, R.J., M. Danaher, R.L. Saylors, R.L., and A. Saxena 1992. Defective asialoglycoprotein receptor endocytosis mediated by tyrosine kinase inhibitors. *Gene* 70:303-312.

Gabilondo, A.M., J. Hegler, C. Krasel, V. Boivin-Jahns, L. Hein, and M.J. Lohse. 1997. A dileucine motif in the C terminus of the β_2 –adrenergic receptor is involved in receptor internalizaiton. *Proc. Nat. Acad. Sci.* 94:12285-12290.

Galan, J.M., and Haguenauer-Tsapis, R. 1997. Ubiquitin Lys63 is involved in ubiquitination of a yeast plasma membrane protein. *EMBO J.* 16, 5847-5854.

Galan, J.M., V. Moreau, B. Andre, C. Volland, and R. Haguenauer-Tsapis. 1996. Ubiquitination mediated by the Npi1/Rsp5 ubiquitin-protein ligase is required for endocytosis of the yeast uracil permease. *J. Bio. Chem.* 271:10946-10952.

Galan, J.M., Volland, C., Urban-Grimal, D., and Haguenauer-Tsapis, R. 1994. The yeast plasma membrane uracil permease is stabilized against stress induced degradation by a point mutation in a cyclin-like "destruction box. *Biochem. Biophys. Res. Commun.* 201, 769-775.

Garippa, R.J., Judge, T.W., James, D.E. and T.E. McGraw, 1994. The amino terminus of GLUT4 functions as an internalization motif but not an intracellular retention signal when substituted for the transferrin receptor cytoplasmic domain. *J. Cell Bio.* 124: 705-715.

Girones, N., Alvarez, E., Seth, A., Lin, I.M., Latour, A. and R.J. Davis, 1991. Mutational analysis of the cytoplasmic tail of the human transferrin receptor. *J. Bio.Chem.* 266: 19006-19012.

Goebl, M., Yochem, J., Jentsch, S., McGrath, J.P., Varxhavsky, S., and Byers, B. 1988. The yeast cell cycle gene *CDC34* encodes a ubiquitin-conjugating enzyme. *Science* 241, 1331-1335.

Gorts, C.P.M. 1989. Effect of glucose on the activity and the kinetics of the maltose-uptake system and of -glucosidase in *Saccharomyces cerevisiae. Biochim. Biophys. Acta*. 184:299-305.

Grenson, M. 1983. Study of the positive control of the general amino-acid permease and other ammonia-sensitive uptake systems by the product of the NPR1 gene in the yeast *Saccharomyces cerevisiae. Eur. J. Biochem* 133, 135-139.

Guldener, U., S. Heck, T.Fiedler, J. Beinhauer and J.H. Hegemann. 1996. A new efficient disruption cassette for repeated use in gene deletion in budding yeast. *Nucl. Acid Res.* 24:2519-2534.

Haft, C.R., R.D. Klausner, and S.I. Taylor. 1994. Involvement of dileucine motifs in the internalization and degradation of the insulin receptor. *J. Biol.Chem.* 269:26286-26294.

Harma, T., C. Brondijk, M.E. van der Rest. D. Plum Y. de Vries, D. Stingl, B. Poolman, and W.N. Konings. Catabolite inactivation of wild-type and mutant maltose transport proteins in *Saccharomyces cerevisiae. J. Biol. Chem.* 273:15352-15357.

Han, E.K., F. Cotty, C. Sottas, H. Jiang, and C.A. Michels. 1995. Characterization of *AGT1* encoding a general -glucoside transporter from *Saccharomyces. Mol. Microbio.* 17:1093-1107.

Hare, J.H., 1990. Mechanisms of membrane protein turnover. *Biochim. Biophys. Acta* 1031: 71-90.

Hein, C. and B. Andre. 1997. A C-terminal di-leucine motiff and nearby sequences are required for NH_4+ - induced inactivation and degradation of the general amino acid permease, Gap1p, of *Saccharomyces cerevisiae. Mol. Micrbio.* 24:607-616.

Hein, C., J.-Y. Springael, C. Volland, R. Haguenauer-Tsapis, and B. Andre. 1995. *NPI*, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. *Mol. Micrbio.* 18:77-87.

Heinemeyer, W., Gruhler, A., Mohrle, V., Mahe, Y. and D.H. Wolf, 1993. *PRE2*, highly homologous to the human major histocompatibility complex-linked *RING10* gene, codes for a yeast proteasome subunit necessary for chymotryptic activity and degredation of ubiquitinated proteins. *J. Bio. Chem.* 268: 5115-5120.

Hemmings, B.A., G.S. Zubenko, A. Hasilik, and E.W. Jones. 1981. Mutant defective in processing of an enzyme located in the lysosome-like vacuole of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* USA 78:435-439.

Hicke, L., B. Zanolari, and H. Riezman. 1998. Cytoplasmic tail phosphorylation of the α-factor receptor is required for its ubiquitination and internalization. *J Cell Bio.* 141:349-358.

Hicke, L.1997. Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins. *FASEB J.* 11:1215-1226.

Hicke, L. and H. Riezman. 1996. Ubiquitination of a yeast plasma membrane receptor signals its ligand stimulated endocytosis. *Cell.* 84:277-287.

Hochstrasser, M. 1996. Protein degradation of regulation: Ub the judge. *Cell.* 84:813-815.

Hochstrasser, M. 1996. Ubiquitin-dependent protein degradation. *Ann. Rev. Genet.* 30:405-439.

Hochstrasser, M., Ellison, M.J., Chau, V. and A. Varshavsky, 1991. The short-lived MATα2 transcriptional regulator is ubiquitinated *in vivo*. *Proc. Natl. Acad. Sci. USA* 88: 4606-4610.

Holzer, H. 1976. Catabolite inactivation in yeast. *Trends in Bio. Sci.* 8:178-181.

Horak, J. and D.H. Wolf, 1997. Catabolite inactivation of the galactose transporter in the yeast *Saccharomyces cerevisiae*: ubiquitination, endocytosis, and degradation in the vacuole. *J. Bact.* 179:1541-1549.

Hu, Z., J.O. Nehlin, H. Ronne, and C.A. Michels. 1995. *MIG1*-dependent and *MIG1*-independent glucose regulation of *MAL* gene expression in *Saccharomyces cerevisiae*. Curr. Genet. 28:258-266.

Hubbard, A.L., 1989. Endocytosis. *Current Opinion in Cell Biology* 1: 675-683.

Huibregtse, J.M., Yang, J.C., and Beaudenon, S.L. 1997. The large subunit of RNA polymerase II is a substrate of the Rsp5 ubiquitin-protein ligase. *Proc. Natl. Acad. Sci. U.S.A.* 94: 3656-3661.

Jadot, M., Canfield, W.M., Gregory, W. and S. Kornfeld, 1992. Characterization of the signal for rapid internalization of the bovine mannose 6-phosphate/insulin-like growth factor-II receptor. *J. Bio. Chem.* 267: 11069-11077.

James, D.E., J. Hiken, and J. C. Lawrence, Jr. 1989. Isoproterenol stimulates phosphorylation of the insulin-regulatable glucose transporter in rat adipocytes. *Proc. Natl. Acad. Sci.* 86:6368-8372.

Jentsch, S., 1992. The ubiquitin-conjugation system. *Annu. Rev. Genet.* 26: 179-207.

Jiang, H., I. Medintz, Zhang, B., and C.A. Michels. 1998. Metabolic signals trigger glucose-induced inactivation of maltose permease in *Saccharomyces*. *Mol. Cell. Bio.* Submitted.

Jiang, H., K. Tatchell, and C.A. Michels. 1998. Protein phosphatase type-1 regulatory subunits Reg1p and Reg2p are signal transducers in the glucose-induced proteolysis of maltose permease in *Saccharomyces*. *Mol. Cell. Bio.* Submitted.

Jiang, H., I. Medintz, and C.A. Michels. 1997. Two glucose sensing/signalling pathway for glucose-induced inactivation of maltose permease in *Saccharomyces*. *Mol. Biol. Cell.* 8:1293-1304.

Jing, S., Spencer, T., Miller, K., Hopkins, C., and I.S. Trowbridge, 1990. Role of the human transferrin receptor cytoplasmic domain in endocytosis: localization of a specific signal sequence for internalization. *J. Cell. Bio.* 110: 283-294.

Johnston, M. and M. Carlson. 1992. Regulation of carbon and phosphate utilization. *The molecular and cellular biology of the yeast Saccharomyces:gene expression.* p193-281. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Jones, E.W., 1991. Three proteolytic systems in the yeast *Saccharomyces cerevisiae*. *J. Bio. Chem.* 266: 7963-7966.

Kolling, R., and S. Losko. 1997. The linker region of the ABC-transporter Ste6 mediates ubiquitination and fast turnover of the protein. *EMBO J.* 16:2251-2261.

Ktistakis, N.T., Thomas, D. and M.G. Roth, 1990. Characteristics of the tyrosine recognition signal for internalization of transmembrane surface glycoproteins. *J. Cell Bio.* 111: 1393-1407.

Knop, M., Schiffer, H.H., Rupp, S. and D.H. Wolf, 1993. Vacuolar/lysosomal proteolysis: proteases, substrates, mechanisms. *Current Opinion in Cell Bio.* 5: 990-996.

Kolodziej, P.A. and R.A. Young. 1991. Epitope tagging and protein surveillance. *Methods Enzymol.* 194:508-519.

Kublaoui, B., J. Lee, and P.F. Pilch. 1995. Dynamics of signalling during insulin-stimulated endocytosis of its receptor in adipocytes. *J. Bio. Chem.* 170:59-65.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assemble of the head of bacteriophage T4. *Nature* 227:680-685.

Lagunas, R.,1993. Sugar Transport in *Saccharomyces cerevisiae*. *FEMS Microbiology Rev.* 104: 229-242.

Lai, K., P. Bolognese, S. Swift, and P. McGraw. 1995. Regulation of inositol transport in *Saccharomyces cerevisiae* involves inositol-induced changes in permease stability and endocytic degradation in the vacuole. *J. Bio. Chem.* 270:2525-2534.

Lehmann, L.E., Eberle, W., krull, S., Prill, V., Schmidt, B., Sander, C., von Figura, K. and C. Peters, 1992. The internalization signal in the cytoplasmic tail of lysosomal acid phosphatase consists of the hexapeptide PGYRHV. *EMBO J.* 11: 4391-4399.

Lehrman, M.A., Goldstein, J.L., Brown, M.S., Russell, D.W. and W.J. Schneider, 1985. Internalization-defective LDL receptors produced by genes with nonsense and frameshift mutations that truncate the cytoplasmic tail. *Cell* 41: 735-743.

Letourneur, F. and R.D. Klausner. 1992. A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. *Cell.* 69:1143-1157.

Li, F.N., and Johnston, M. 1997. Grr1 of *Saccharomyces cerevisiae* is connected to the ubiquitin proteolysis machinery through Skpi: coupling glucose sensing to gene expression and the cell cycle. *EMBO J.* 16: 5629-5638.

Loayza, D., and S. Michaelis, 1998. Role of the ubiquitin-proteasome system in the vacuolar degradation of Ste6p, the a-factor transporter in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 18:779-789.

Lucero, P., and Lagunas, L. 1997. Catabolite inactivation of the yeast maltose transporter requires ubuiquitin-ligase npi/rsp5 and ubiquitin-hydrolase npi2/doa4. *FEMS Microbio. Lett.* 147, 273-277.

Lucero, P., M. Herweijer, and R. Lagunas. 1993. Catabolite inactivation of the yeast maltose transporter is due to proteolysis. *FEBS* 333:165-168.

Marchal, C., R. Haguenauer-Tsapis, and D. Urban-Grimal, 1998. A PEST-like sequence mediates phosphorylation of yeast uracil permease. *Mol. Cell Bio.* 18:314-321.

Marcus, F., J. Rittenhouse, L. Moberly, I. Edelstein, E. Hiller, and D.T. Rogers. 1988. Yeast (*Saccharomyces cerevisiae*) fructose-1,6-bisphosphatase. *J. Biol. Chem.* 263:6058-6062.

Martinez, P., Zvyagilakaya, R., Allard, P., and Persson, B.L. 1998. Physiological regulation of the derepressible phosphate transporter in *Saccharomyces cerevisiae*. *J. Bact.* 180:2253-2256.

Matern, H., and H. Holzer. 1977. Catabolite inactivation of the galactose uptake system in yeast. *J. Biol. Chem.* 252:6399-6402.

Mazon, M.J., J.M. Gancedo, and C. Gancedo. 1982. Inactivation of yeast Fructose-1,6-bisphosphatase. *J. Bio. Chem.* 257:1128-1130.

Medintz, I., H. Jiang, and C.A. Michels, 1998. The role of ubiquitin conjugation in glucose-induced proteolysis of *Saccharomyces* maltose permease. *J. Biol. Chem.* In press.

Medintz, I., H. Jiang, E.-K. Han, W. Cui, and C.A. Michels, 1996. Characterization of the glucose-induced inactivation of maltose permease in *Saccharomyces cerevisiae*. *J. Bact.* 178:2245-2254.

Mellman, I. 1996. Endocytosis and molecular sorting. *Ann Rev Cell. Dev. Biol.* 12:575-625.

Miyazawa, K., Toyama, K., Gotoh, A., Hendrie, P.C., Mantel, C., and Broxmeyer, H.E. 1994. Ligand-dependent polyubiquitination of c-kit gene product: a possible mechanism of receptor down modulation in M07E cells. *Blood* 83:137-145.

Mori, S., Heldin, C.H., Claesson-Welsh, L. 1992. Ligand-induced polyubiquitination of the platelet-derived growth factor beta-receptor. *J. Biol. Chem.* 127:6429-6434.

Muller, D., and H. Holzer. 1981. Regulation of fructose-1,6-bisphosphatase in yeast by phosphorylation / dephosphorylation. *Biochem. Biophys. Res. Commun.* 103:926-933.

Naim, H.Y. and M.G. Roth, 1994. Characteristics of the internalization signal in the Y543 Influenza virus hemagglutinin suggests a model for recognition of internalization signals containing tyrosine. *J. Bio. Chem.* 269: 3928-3933.

Needleman, R. 1991. Control of maltase synthesis in yeast. *Mol. Microbiol.* 5:2079-2084.

Needleman, R.B., Kaback, D.B., Dubin, R.A., Perkins, E.L., Rosenberg, N.G., Sutherland, K.A., Forrest, D.B. and C.A. Michels, 1984. *MAL6* of *Saccharomyces*. A complex genetic locus containing three genes required for maltose fermentation. *Proc. Natl. Acad. Sci.* 81: 2811-2815.

Needleman, R.B. and C.A. Michels, 1983. Repeated families of genes controlling maltose fermentation in *Saccharomyces carlsbergensis*. *Mol. Cell. Biol.* 3: 796-802.

Neeff, J., E. Hagele, J. Nauhaus, U. Heer, and D. Mecke. 1978. Evidence for catabolite degradation in the glucose-dependent inactivation of yeast cytoplasmic malate dehydrogenase. *Eur. J. Biochem.* 87:489-495.

Ozcan, S., J. Dover, A.G., and M. Johnston. 1998. Glucose sensing and signaling by two glucose receptors in the yeast *Saccharomyces cerevisiae*. *EMBO J.* 17:2566-2573.

Ozcan, S., J. Dover, A.G. Rosenwald, S. Wolfl, and M. Johnston, 1996a. Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. *Proc. Natl. Acad. Sci.* : 12428-12432.

Ozcan, S., T. Leong, and M. Johnston, 1996b. Rgt1p of *Saccharomyces cerevisiae*, a key regulator of glucose-induced genes, is both an activator and a repressor of transcription. *Mol. Cell. Biol.* 16:6419-6426.

Paolini, R., and Kinet, J.P. 1993. Cell surface control of the multiubiquitination and deubiquitination of high-affinity immunoglobulin E receptors. *EMBO J.* 12:779-786.

Papa, F.R. and M. Hochstrasser, 1993. The yeast *DOA4* gene encodes a deubiquitinating enzyme related to a product of the human *tre-2* oncogene. *Nature* 366: 313-319.

Patton, E.E., Willems, A.R., and Tyers, M. 1998. Combinatorial control in ubiquitin-dependent proteolysis: don't skip the F-box hypothesis. *Trends in Genet.*14:236-243.

Peinado, J.M. and M.C. Loureiro-Dias. 1986. Reversible loss of affinity induced by glucose in the maltose-H^+ symport of *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* 856:198-192.

Peters, C., Braun, M., Weber, B., Wendland, M., Schmidt, B., Pohlmann, R., Waheed, A. and K. von Figura, 1990. Targeting of a lysosomal membrane protein: a tyrosine-containing endocytosis signal in the cytoplasmic tail of

lysosomal acid phosphatase is necessary and sufficient for targeting to lysosomes. *EMBO J.* 9: 3497-3506.

Quelle, D.E., Quelle, F.W. and D.M. Wojchowski, 1992. Mutations in the WSAWSE and cytosolic domains of the erythropoietin receptor affect signal transduction and ligand binding and internalization. *Mol. Cell. Bio.* 12: 4553-4561.

Ramos, J., K. Szkutnicka, and V.P. Cirillo. 1988. Relationship between low- and high-affinity glucose transport systems of *Saccharomyces cerevisiae. J. Bacteriol.* 170:5375-5377.

Ramos, J., and V.P. Cirillo. 1989. Role of cyclic-AMP-dependent protein kinase in catabolite inactivation of the glucose and galactose transporters in *Saccharomyces cerevisiae. J. Bacteriol.* 171:3545-3548.

Raths, S., J. Rohrer, F. Crausaz, and H. Riezman. 1993. *end3* and *end4*: two mutants defective in receptor-mediated and fluid-phase endocytosis in *Saccharomyces cerevisiae. J. Cell Bio.* 120:55-65.

Rechsteiner, M., and S.W. Rogers. 1996. PEST sequences and regulation by proteolysis. *TIBS.* 21:267-271.

Reneke, J.E., Blumer, K.J., Courchesne, W.E. and J. Thorner, 1988. The carboxy-terminal segment of the yeast α-factor receptor is a regulatory domain. *Cell* 55: 221-234.

Riballo, E., M. Herwijer, D.H. Wolf, and R. Lagunas. 1995. Catabolite inactivation of the yeast maltose transporter occurs in the vacuole after internalization by endocytosis. *J. Bacteriol.* 177:5622-5627.

Rittenhouse, J., P.B. Harrsch, J.N. Kim, and F. Marcus. 1986. Amino acid sequence of the phosphorylation site of yeast (*Saccharomyces cerevisiae*) fructose-1,6-bisphosphatase. *J. Biol. Chem.* 261:3939-3943.

Robinson, K.S., Lai, K., Cannon, T.A., and McGraw, P. 1996. Inositol transport in *Saccharomyces cerevisiae* is regulated by transcriptional and degradative endocytotic mechanisms during the growth cycle that are distinct from inositol-induced regulation. *Mol.Bio.Cell* 7, 81- 89.

Robinson, M.S., 1992. Adaptins. *Trends Cell Bio.* 3: 272-277.

Rohrer, J., Benedetti, H., Zanolari, B. and Riezman, H., 1993. Identification of a novel sequence mediating regulated endocytosis of the G protein coupled α-pheromone receptor in yeast. *Mol. Bio. of the Cell* 4: 511-521.

Roth, A.F. and N.G. Davis, 1996. Ubiquitination of the yeast a-factor receptor. *J. Cell Bio.* 134:661-674.

Sandoval, I.V., and O. Bakke. 1994. Targeting of membrane proteins to endosomes and lysosomes. *Trends Cell Biol.* 4:292-297.

Schmid.S.L. 1997. Calthrin – coated vesicle formation and protein sorting: an integrated process. *Ann. Rev. Biochem.* 66:511-548.

Schneider, B.L., Yang, Q-H., and Futcher, A.B. 1996. Linkage of replication to start by the Cdk inhibitor Sic1. *Science* 272:560-562.

Schork, S.M., G. Bee, M. Thumm, and D.H. Wolf. 1994. Letter. *Nature* 369:283-284.

Schwarz, S.E., Matuschewski, K., Liakopoulos,, D., Scheffner, M., and Jentsch, S. 1998. The ubiquitin-like proteins SMT3 and SUMO-1 are conjugated by the UBC9 E2 enzyme. *Proc. Natl. Acad. Sci. U.S.A.* 95, 560-564.

Serrano, R., 1977. Energy requirements for maltose transport in yeast. *Eur. J. Biochem.* 80: 97-102. Seufert, W. and S. Jentsch, 1990. Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins. *EMBO J.* 9: 543-550.

Seufert, W., Futcher,B., and Jentsch, S. 1995. Role of a ubiquitin-conjugating enzyme in degradation of S- and M-phase cyclins. *Nature* 373, 78-81.

Seufert, W. and S. Jentsch. 1990. Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins. *EMBO J.* 9:543-550.

Shin, J., R.L. Dunbrack, Jr., S. Lee, and J.L. Strominger. 1991. Phosphorylation-dependent down-modulation of CD4 requires a specific structure within the cytoplasmic domain of CD4. *J. Biol. Chem.* 266:10658-10665.

Siegelman, M., Bond, M.W., Gallatin, W.M., St.John, T., Smith, H.T., Fried, V.A., and Weissman, I.L. 1986. Cell surface molecule associated with lymphocyte homing is ubiquitinated branched-chain glycoprotein. *Science* 23: 823-829.

Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122:19-27.

Simeon, A., van der Klei, I.J., Veenhuis, M. and D.H. Wolf, 1992. Ubiquitin, a central component of selective cytoplasmic proteolyis, is linked to proteins residing at the locus of non-selective proteolysis, the vacuole. *FEBS letters* 301: 231-235.

Singer, B. and H. Riezman, 1990. Detection of an intermediate compartment involved in transport of α-factor from the plasma membrane to the vacuole in yeast. 110: 1911-1922.

Springael, J-I., and B. Andre. 1998. Nitrogen-regulated ubiquitination of the Gap1 permease of *Saccharomyces cerevisiae*. *Mol. Biol. Cell.* 9:1253-1263.

Stanbrough, M., and B. Magasanik. 1995. Transcriptional and posttranslational regulation of the general amino acid permease of *Saccharomyces cerevisiae*. *J. Bacteriol.* 177:94-102.

Strous, G.J., P. van Kerkhof, R. Govers, A. Ciechanover, and A.L. Schwartz, 1996. The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor. *EMBO J.* 15:3806-3812.

Tan, P.K., J.P. Howard, and G.S. Payne. 1996. The sequence NPFXD defines a new class of endocytosis signal in *Saccharomyces cerivisiae*. *J. Cell. Bio.* 135:1789-1800.

Teichert, U., Mechler, B., Muller, H. and D.H. Wolf, 1989. Lysosomal (vacuolar) proteinases of yeast are essential catalysts for protein degredation, differentiation, and cell survival. *J. Bio. Chem.* 264: 16037-16045.

Terrell, J., Shih, S., Dunn, R., and Hicke, L. 1998. A function for monoubiquitination in the internalization of a G protein-coupled receptor. *Mol. Cell* 1, 193-202.

Tortora, P., M. Birtel, A-G. Lenz, and H. Holzer. 1981. Glucose-dependent metabolic interconversion of fructose-1,6-bisphosphatase in yeast. *Biochem. and Biophys. Res. Commun.* 100: 688-695.

Trowbridge, I.S., J.F. Collawn, and C.R. Hopkins. 1993. Signal-dependent membrane protein trafficking in the endocytic pathway. *Ann. Rev. Cell Biol.* 9:129-161.

Trowbridge, I.S., 1991. Endocytosis and signals for internalization. *Current Opinion in Cell Biology* 3: 634-641.

Tyers, M. 1996. The cyclin-dependent kinase inhibitor p40SIC1 imposes the requirement for Cln G1 cyclin function at Start. *Proc. Natl. Acad. Sci. U.S.A.* 93:7772-7776.

Van den Broek, P.J.A., C.C.M. Van Leeuwen, R.A. Weusthuis, E. Postma, J.P. Van Dijken, R.H. Karssies, and R. Amons. 1994. Identification of the maltose transport protein of *Saccharomyces cerevisiae. Biochem. Biophys. Res. Comm.* 200:45-51.

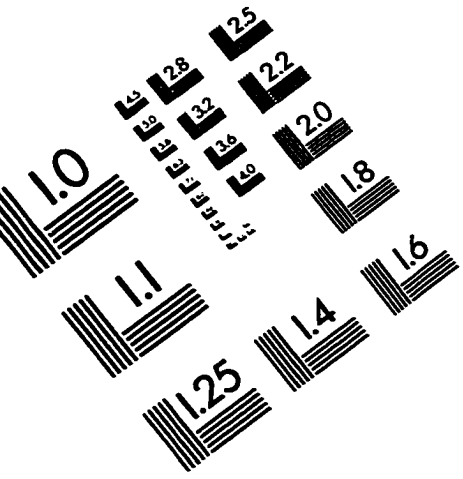
Volland, C., D. Urban-Grimal, G. Geraud, and R. Haguenauer-Tsapis. 1994. Endocytosis and degradation of the yeast uracil permease under adverse conditions. *J. Bio. Chem.* 269:9833-9841.

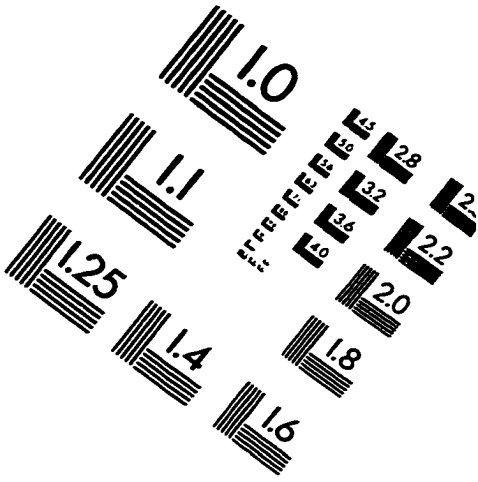
Ward, A.C., Castelli, L.A., MaCreadie, I.G., and Azad, A. 1994. Vectors for Cu(2+)-inducible production of glutathione S-transferase-fusion proteins for single-step purification from yeast. *Yeast* 10:441-449.

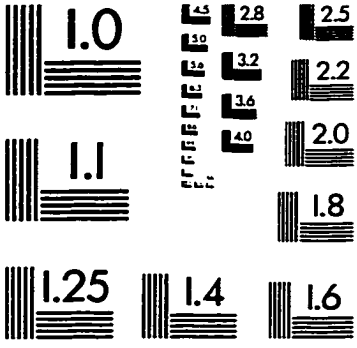
Wolf, D.H., and G.R. Fink. 1975. Proteinase C (carboxypeptidase Y) mutants of yeast. *J. Bacteriol.* 123:1150-1156.

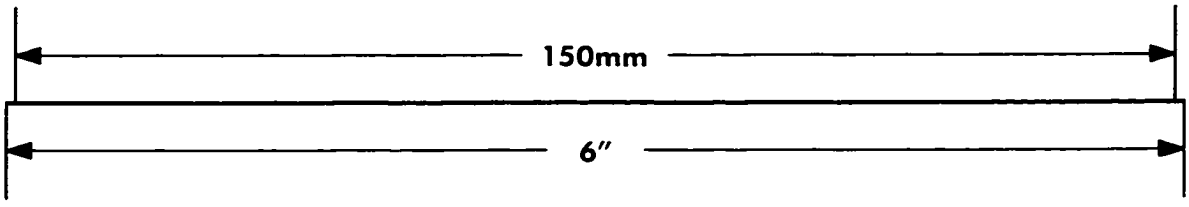
Zanolari, B., S. Raths, B. Singer-Kruger, and H. Riezman. 1992. Yeast pheromone receptor endocytosis and hyperphosphorylation are independent of G protein-mediated signal transduction. *Cell* 71:755-763.

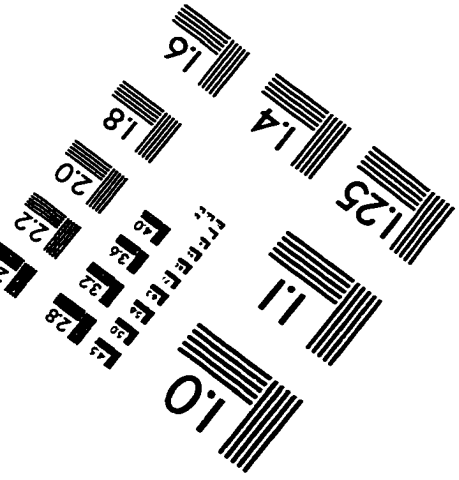
IMAGE EVALUATION TEST TARGET (QA-3)

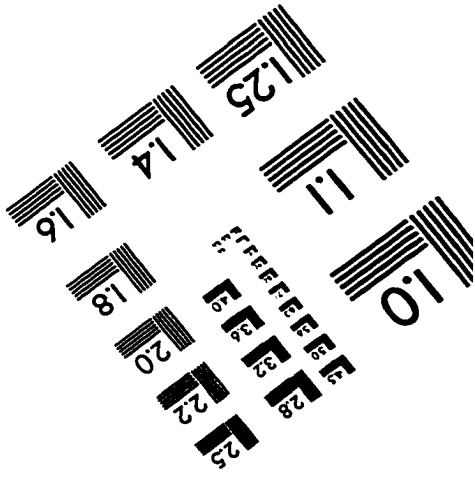












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989