Alg13p, the Catalytic Subunit of the Endoplasmic Reticulum UDP-GlcNAc Glycosyltransferase, Is a Target for Proteasomal Degradation

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Submitted October 25, 2007; Revised February 20, 2008; Accepted February 28, 2008

Monitoring Editor: Reid Gilmore

The second step of dolichol-linked oligosaccharide synthesis in the N-linked glycosylation pathway at the endoplasmic reticulum (ER) membrane is catalyzed by an unusual hetero-oligomeric UDP-N-acetylglucosamine transferase that in most eukaryotes is comprised of at least two subunits, Alg13p and Alg14p. Alg13p is the cytosolic and catalytic subunit that is recruited to the ER by the membrane protein Alg14p. We show that in *Saccharomyces cerevisiae*, cytosolic Alg13p is very short-lived, whereas membrane-associated Alg13 is relatively stable. Cytosolic Alg13p is a target for proteasomal degradation, and the failure to degrade excess Alg13p leads to glycosylation defects. Alg13p degradation does not require ubiquitin but instead, requires a C-terminal domain whose deletion results in Alg13p stability. Conversely, appending this sequence onto normally long-lived β -galactosidase causes it to undergo rapid degradation, demonstrating that this C-terminal domain represents a novel and autonomous degradation motif. These data lead to the model that proteasomal degradation of excess unassembled Alg13p is an important quality control mechanism that ensures proper protein complex assembly and correct N-linked glycosylation.

INTRODUCTION

N-linked glycosylation is an essential protein modification required for the structure and function of glycoproteins. The attachment of N-linked glycans to proteins in eukaryotes involves the assembly of an oligosaccharide on the lipid anchor dolichol at the endoplasmic reticulum (ER) membrane. During lipid-linked oligosaccharide (LLO) synthesis, two N-acetylglucosamines (GlcNAc) and five mannoses (Man) are added sequentially to dolichol (dol) on the cytosolic face of the ER from nucleotide sugar donors that are synthesized in the cytosol (Snider and Rogers, 1984; Perez and Hirschberg, 1986; Abeijon and Hirschberg, 1992). This Man₅GlcNAc₂-PP-dol intermediate flips across the ER membrane and is the acceptor for the addition of the next four Man and three glucoses (Glc), which are added in the lumen of the ER from dol-linked sugar substrates to form Glc₃Man₉GlcNAc₂-PP-dol (reviewed in Weerapana and Imperiali, 2006). After assembly, the oligosaccharide is transferred to protein and immediately modified by the removal of some sugars. Failure to assemble, transfer, or modify this oligosaccharide results in glycoproteins that often misfold and are targeted for degradation by ER quality control systems (Frickel *et al.*, 2004).

The second step of LLO synthesis, in which GlcNAc from UDP-GlcNAc is added to GlcNAc-PP-dol to form GlcNAc₂-PP-dol, is catalyzed by an unusual hetero-oligomeric glycosyltransferase (GTase), recently identified in *Saccharomyces cerevisiae*. Unlike other GTases that are membrane proteins comprised of single polypeptides, in the vast majority of eukaryotes this enzyme consists of at least two polypeptides, Alg13p and Alg14p. In yeast, both subunits are essential and required for UDP-GlcNAc transferase activity (Bickel *et al.*, 2005; Chantret *et al.*, 2005; Gao *et al.*, 2005; Averbeck *et al.*, 2007). Alg13p lacks any membrane-spanning domains but contains the predicted catalytic domain. Alg13p's predicted tertiary structure is highly similar to the *Escherichia coli* MurG UDP-GlcNAc transferase involved in peptidoglycan synthesis, as well as other UDP-GlcNAc transferases, but just within the catalytic domain (Bickel *et al.*, 2005; Chantret *et al.*, 2005; Gao *et al.*, 2005). The ER membrane protein Alg14p does not contain any obvious similarity to other GTases, but its predicted structure is related to the putative lipid-acceptor domain at the N-terminal domain of MurG (Bickel *et al.*, 2005; Chantret *et al.*, 2005). Alg14p physically interacts with Alg13p and is required to recruit Alg13p to the cytosolic face of the ER where GlcNAc₂-PP-dol synthesis occurs (Bickel *et al.*, 2005; Gao *et al.*, 2005; Averbeck *et al.*, 2007). Overproduction of Alg13p leads to its cytosolic partitioning, as does reduction of Alg14p levels (Gao *et al.*, 2005). In the absence of Alg14p, Alg13p is inactive, suggesting that the Alg13p/Alg14p interaction at the ER membrane is required for normal enzyme activity or stability (Bickel *et al.*, 2005; Averbeck *et al.*, 2007). Together, these data have led to the model that Alg13p and Alg14p together form the ER GTase complex, in which Alg13p catalyzes the

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07-10-1077>) on March 12, 2008.

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Abbreviations used: ER, endoplasmic reticulum; GlcNAc, N-acetylglucosamine; LLO, lipid-linked oligosaccharide; v-ATPase, vacuolar ATPase; GTase, glycosyltransferase.

addition of GlcNAc from UDP-GlcNAc to GlcNAc-PP-dol to form GlcNAc₂-PP-dol.

The unique hetero-oligomeric nature of the Alg13p/Alg14p GTase complex has raised the question of why this enzyme has evolved such an unusual configuration compared with other eukaryotic GTases. This Alg13p/14 UDP-GlcNAc GTase complex is a well-suited target for regulation of LLO assembly because it catalyzes one of the earliest steps in this pathway. One possible explanation is that this “split” subunit arrangement provides a mechanism to regulate its activity. There are data that suggest subunits of multiprotein complexes are more susceptible to degradation when unassembled, but the molecular mechanisms for this phenomenon are largely unexplored. Here we present evidence that the cytosolic component Alg13p is a target for proteasomal degradation. This degradation does not appear to depend on ubiquitin. Degradation is mediated by the C-terminus of Alg13p, which acts as an autonomous motif that can confer instability to a normally stable reporter. Failure to degrade excess Alg13p causes a glycosylation defect, supporting the model that the proteolytic degradation of unassembled Alg13p contributes to the regulation of glycosylation.

MATERIALS AND METHODS

Yeast Strains, Growth Conditions, and Plasmids

S. cerevisiae strains used in this study are listed in Table 1. Standard yeast media, growth conditions, and genetic techniques were used (Guthrie and Fink, 1991). Yeast strains were maintained in YEA (1% yeast extract, 2% peptone, 50 mg/l adenine sulfate) or in selective minus histidine (–His) medium, supplemented with glucose (2%) or galactose (2%). Strains in which chromosomal *ALG13* or *ALG14* expression is controlled by the *GAL1* promoter (XGY160, NAY13, NAY19, NAY20, XGY151, NAY105-1) were constructed by PCR-mediated recombination using the pFA6a-plasmid series as templates (Longtine *et al.*, 1998). In XGY160, this recombination leads to a replacement of 202 base pairs upstream of *ALG13* (position –1 to –202 relative to the start codon of *ALG13*) with the *Schizosaccharomyces pombe* *his5⁺* selectable marker and *GAL1* promoter. In NAY13, NAY19, and NAY20, the *GAL1* promoter and *his5⁺* were inserted at –1 (relative to the start codon of *ALG13*), leaving the *ALG13/RPT6* intergenic region intact. NAY26 is an offspring of a cross between the *uba1-2 gal2* mutant, MHY1409, and the wild-type WCG4a *GAL2*, which was generated to cross out the *gal2* mutant allele, whose presence prevents uptake of galactose and induction of the *GAL1* promoter. The NAY110 series of strains contain the *uba1-2* allele and in addition express the *P_{TP11}-lacZ* gene. These haploid strains were isolated by

dissection of a diploid produced by crossing the *uba1-2 GAL2* mutant NAY26 with W303-1A transformed with the integrative pTi-lacZ plasmid. pTi-lacZ was linearized with XhoI to target recombination at the *ura3-52* locus. The NAY111 series of strains are similar and also contain the *uba1-2* allele, but in addition, express the *P_{TP11}-lacZ-alg13Δ1-101* gene. These strains were isolated by dissection of a diploid produced by crossing NAY26 and W303-1A transformed with pTi-lacZ-alg13Δ1-101.

Plasmids used in this study and their relevant features are listed in Table 2. Fragments obtained by PCR and cloned into vectors were verified by DNA sequence analysis. Sequences of any plasmids or primers used in this study are available upon request. To construct pALG13, the C-terminal FLAG tagged *ALG13* allele was amplified by PCR of genomic DNA from XGY155 (Gao *et al.*, 2005) and cloned into Sall/SmaI of the integrative *LEU2* vector, pRS305. To construct pCEN-ALG13 the *GAL1* promoter and HA-*ALG13* were amplified by colony PCR of XGY160; the amplified fragment included the *GAL1* promoter (position –1 to –541 upstream of *GAL1*) and the HA-*ALG13* open reading frame (ORF). This fragment was cloned into the SmaI site of the *CEN/HIS3* vector, pRS313. pCEN-ALG13Δ101aa, pCEN-ALG13Δ60aa, and pCEN-ALG13Δ20aa were obtained by “deletion” PCR from pCEN-ALG13. The primers for these PCR amplifications were chosen so that the base pairs 304–606, 427–606, and 547–606 of the *ALG13* ORF in pCEN-ALG13, respectively, were not amplified within the PCR product and were therefore deleted. The amplified products, which include all of the vector sequences, were ligated to produce each of the plasmids that carry truncated HA-*ALG13* alleles. pTi-lacZ contains *lacZ*, driven by the constitutive *TP11* promoter in an integrative *URA3* plasmid. To construct it, the *lacZ* ORF was obtained by PCR from pSJ101 (Liu *et al.*, 1995) as template. The PCR product was cloned into the 4.5-kb SmaI fragment of pTαO (Dean and Pelham, 1990). To construct pTi-lacZ-alg13Δ1-101, the *lacZ* ORF and the *ALG13* ORF were first “fused” in frame by fusion PCR, using nested primers. In a next step base pairs 1–303 of the *ALG13* ORF were deleted by PCR, in which the primers were chosen so that the area to be deleted was not amplified. The ligated PCR product represents pTi-lacZ-alg13Δ1-101. To integrate pTi-lacZ or pTi-lacZ-alg13Δ1-101 into the *URA3* locus the plasmids were linearized with XhoI. To construct pUb-R-lacZ the 8.3-kb EcoRI fragment of pUB23-Arg (Bachmair *et al.*, 1986) was ligated resulting in pUb-R-lacZ that lacks a 2μ fragment.

Protein Isolation and Immunoanalyses

To extract whole cell protein, 1–5 OD₆₀₀ units of logarithmic phase cells (harvested at an OD₆₀₀ of 0.2–1) were lysed by bead bashing in 100 μl ice-cold JR buffer (0.1 M sorbitol, 50 mM potassium acetate, 2 mM EDTA, 20 mM HEPES-KOH, pH 7.4, 1 mM PMSF; Gao and Dean, 2000) by five repeated cycles of vortexing (30 s) and incubating on ice (30 s). Cell debris was removed by centrifugation (2 min, 3300 × g, 4°C), and the supernatant was transferred to a new tube. The beads were rinsed with 100 μl JR buffer and centrifuged, and the combined supernatant was clarified by centrifugation (2 min, 3300 × g, 4°C). To separate soluble, cytosolic Alg13p from ER-associated Alg13p, this whole cell lysate was subjected to differential centrifugation. Membranes were sedimented by centrifugation at 100,000 × g for 30 min at 4°C. The resulting supernatant (S100) contained soluble proteins, including Alg13p. Protein concentration was determined by the Bradford method (Bradford, 1976).

Table 1. Yeast strains used in this study

Strain	Genotype	Source
W303-1A	<i>MATa his3-11 leu2-3,112 ura3-1 trp1-1 ade2-1 can1-100 ssd1-d</i>	Thomas and Rothstein (1989)
W303-1B	<i>MATα his3-11 leu2-3,112 ura3-1 trp1-1 ade2-1 can1-100 ssd1-d</i>	Thomas and Rothstein (1989)
MHY1409	<i>MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2 uba1-2::mTnURA3</i>	Swanson and Hochstrasser (2000)
XGY151	As in W303-1A but <i>his5⁺-P_{GAL1}-HA₃-ALG14</i>	Gao <i>et al.</i> (2005)
XGY160	As in W303-1A but <i>his5⁺-P_{GAL1}-HA₃-ALG13 rpt6</i>	This study
NAY13	As in W303-1B but <i>his5⁺-P_{GAL1}-HA₃-ALG13</i>	Aeverbeck <i>et al.</i> (2007)
WCG4a	<i>MATa his3-11,15 leu2-3,112 ura3</i>	Heinemeyer <i>et al.</i> (1993)
WCG4-11/22a	As in WCG4a but <i>pre1-1 pre2-2</i>	Heinemeyer <i>et al.</i> (1993)
NAY19	As in WCG4a but <i>his5⁺-P_{GAL1}-HA₃-ALG13</i>	This study
NAY20	As in WCG4a but <i>pre1-1 pre2-2 his5⁺-P_{GAL1}-HA₃-ALG13</i>	This study
NAY26	<i>MATa his3 leu2-3,112 ura3 uba1-2::mTnURA3</i>	This study
NAY105-1	As in W303-1A but <i>his5⁺-P_{GAL1}-ALG14</i>	This study
NAY110-10.1b	<i>MATa his3 leu2-3,112 trp1-1 ura3::pTi-lacZ uba1-2::mTnURA3</i>	This study
NAY110-10.5d	<i>MATα his3 leu2-3,112 ade2-1 trp1-1 ura3::pTi-lacZ uba1-2::mTnURA3</i>	This study
NAY110-12.5b	<i>MATa his3 leu2-3,112 ade2-1 trp1-1 ura3::pTi-lacZ uba1-2::mTnURA3</i>	This study
NAY111-11.9a	<i>MATα his3 leu2-3,112 ura3::pTi-lacZ-alg13Δ1-101 uba1-2mTnURA3</i>	This study
NAY111-11.9d	<i>MATα his3 leu2-3,112 ade2-1 trp1-1 ura3::pTi-lacZ-alg13Δ1-101 uba1-2::mTnURA3</i>	This study
NAY111-13.7c	<i>MATa his3 leu2-3,112 ura3::pTi-lacZ-alg13Δ1-101 uba1-2::mTnURA3</i>	This study

Table 2. Plasmids used in this study

Name	Relevant features	Reference
pRS304	<i>TRP1</i> integration plasmid	Sikorski and Hieter (1989)
pRS305	<i>LEU2</i> integration plasmid	Sikorski and Hieter (1989)
pRS313	<i>HIS3</i> centromeric plasmid	Sikorski and Hieter (1989)
pRS304RPT6	<i>RPT6</i> expressed from its own promoter in pRS304	Gao <i>et al.</i> (2005)
pALG13	<i>ALG13-FLAG</i> expressed from its own promoter in pRS305	This study
pCEN-ALG13	<i>HA-ALG13</i> expressed from the <i>GAL1</i> promoter in pRS313	This study
pCEN-ALG13Δ101aa	<i>HA-alg13Δ304-606</i> expressed from the <i>GAL1</i> promoter in pRS313	This study
pCEN-ALG13Δ60aa	<i>HA-alg13Δ427-606</i> expressed from the <i>GAL1</i> promoter in pRS313	This study
pCEN-ALG13Δ20aa	<i>HA-alg13Δ547-606</i> expressed from the <i>GAL1</i> promoter in pRS313	This study
pUb-R-lacZ	<i>Ulb-Arg-lacZ</i> expressed from <i>GAL1</i> promoter; <i>URA3</i> integration vector	This study
P415-GPD-UB-L-lacZ	<i>Ulb-Leu-lacZ</i> expressed from the <i>TDH3</i> promoter; <i>LEU2</i> CEN plasmid	Swanson and Hochstrasser (2000)
pTpi-lacZ	<i>lacZ</i> expressed from <i>TPI1</i> promoter; <i>URA3</i> integration vector	This study
pTpi-lacZ-alg13Δ1-101	<i>lacZ-alg13Δ1-303</i> expressed from <i>TPI1</i> promoter; <i>URA3</i> integration vector	This study

For immunoblot analyses, equal amounts of protein (from 2.5 to 10 μ g) were separated on 12 or 13.5% SDS-PAGE gels, blotted on Immobilon-P PVDF membrane (Millipore, Bedford, MA) and processed as described (Gao and Dean, 2000). Proteins were detected using these antibodies: 12CA5 to detect HA-Alg13p (culture supernatants, 1:10); mouse anti-Vma2 to detect the 60-kDa fragment of v-ATPase (Molecular Probes, Eugene, OR; 1:2000). Primary antibodies were visualized with secondary anti-mouse antibody conjugated to horseradish peroxidase (Amersham, Piscataway, NJ) followed by chemiluminescence detection (ECL, GE Healthcare, Waukesha, WI).

Invertase and β -Galactosidase Assays

The analysis of invertase mobility by gel electrophoresis was performed by an *in situ* activity assay as described using 6% stackless SDS-polyacrylamide gels (Alvarado *et al.*, 1990; Odani *et al.*, 1997).

Liquid β -galactosidase (β -gal) activity assays of yeast lysed by the addition of chloroform and SDS (Keleher *et al.*, 1988) were performed as described (Miller, 1972), where the hydrolysis of ortho-nitrophenol β -D-galactopyranoside to O-nitrophenol and galactose by β -gal is measured spectroscopically. One Miller unit of β -gal produces 1 nmol of O-nitrophenol per minute at pH 7.5 at 37°C.

RESULTS

Excess Alg13p Causes an N-linked Glycosylation Defect

ALG13 is an essential gene, so its mutant phenotype was studied by placing hemagglutinin (HA)-tagged *ALG13* under the control of the glucose-repressible *GAL1* promoter. This HA-tagged *ALG13* allele is fully functional; it complements both the inviability and glycosylation defect of an *alg13* mutant (Gao *et al.*, 2005b). As expected, this strain failed to grow in media containing glucose. However, it also displayed a severe N-linked glycosylation defect in media containing galactose, as seen by an increased electrophoretic mobility of invertase (Figure 1A, lane 2). Invertase is a glycoprotein exclusively modified by N-linked glycans whose truncations decrease the molecular weight. The increased gel mobility of invertase from the strain that expressed *HA-ALG13* from the *GAL1* promoter was due to a defect in glycosylation and not proteolysis as shown by endo H digestion, which removes N-linked glycans; invertase from wild-type and the *GAL1* promoter-driven *HA-ALG13* strain migrated with the same electrophoretic mobility after endo H digestion (data not shown).

To our surprise, overexpression of *ALG13* from another promoter in an otherwise wild-type strain did not cause this phenotype (data not shown). This showed that the glycosylation defect was not simply due to the *GAL1* promoter-induced overexpression of *ALG13*. Instead, as shown below, this phenotype was due to the replacement of *ALG13* upstream sequences with the *GAL1* promoter, which in turn reduced expression of the adjacent *RPT6* gene. *ALG13* and

RPT6, which lie on opposite strands, are unusual because they are separated by only 266 base pairs (Figure 1B, top panel). Most of this intergenic region (202 base pairs) was replaced with the *GAL1* promoter during the strain construction (XGY160, Figure 1B, middle panel). The invertase glycosylation defect was rescued when *RPT6* expression was restored, either by complementation with a plasmid-borne copy of *RPT6* (Figure 1A, lane 3) or in a strain containing the *GAL1* promoter integrated at position -1 relative to the *ALG13* start codon (NAY13; Figure 1, B, bottom panel, and A, lane 4). These results suggested that impaired expression of the adjacent *RPT6* gene is responsible for the invertase glycosylation defect in the strain where the *GAL1* promoter replaces 202 base pairs upstream of *ALG13*.

This was surprising since a role in glycosylation for *RPT6*, which encodes an essential ATPase of the regulatory particle of the 26S proteasome (Rubin *et al.*, 1998), has not been reported. To examine if the overproduction of Alg13p in this *rpt6* strain contributes to the glycosylation defect, another copy of *ALG13* driven by its own promoter was introduced into the *P_{GAL1}-HA-ALG13 rpt6* strain. This additional copy of *ALG13*, whose expression is independent of a carbon source, allowed us to study glycosylation in this *rpt6* strain as a function of *ALG13* expression. In contrast to *rpt6* cells that overexpress *ALG13* (growth on galactose; Figure 1A, lane 5), *rpt6* cells that express *ALG13* at a wild-type level (growth on glucose to repress the *GAL1* promoter-driven *ALG13* allele) did not have an N-linked glycosylation defect because they produced invertase whose glycosylation appeared no different from wild-type cells (Figure 1A, lane 6). Taken together, these results demonstrate that the observed glycosylation defect depends on two concomitant factors: the impairment of *RPT6* expression and overexpression of *ALG13*.

Because *RPT6* is required for proteasome function (Rubin *et al.*, 1998), we hypothesized that proteasome impairment allows an accumulation of excess Alg13p, which causes the glycosylation defect. If this idea is correct, then Alg13p should be more stable in the *P_{GAL1}-HA-ALG13 rpt6* strain (XGY160). To test this, Alg13p stability in this strain was compared with the control strain that we constructed that overexpresses *ALG13* but is not defective in *RPT6* expression, because the insertion of the *GAL1* promoter does not affect the *RPT6* promoter (NAY13; Figure 1A, lane 4). To compare the relative half-life of Alg13p in these strains, translation was arrested by treatment with 100 μ g/ml cycloheximide (CHX). At various times after CHX addition, aliquots of cells were removed, protein was extracted, and

Figure 1. ALG13 overexpression causes an *RPT6*-dependent N-linked glycosylation defect. (A) The mobility of invertase from different yeast strains (described below) was analyzed by 6% PAGE using an in situ activity assay. A strain in which *ALG13* is under the control of the *GAL1* promoter that replaces 202 base pairs upstream of *ALG13* (XGY160, lane 2) was transformed with a plasmid-borne *RPT6* allele controlled by its own promoter (XGY160/pR5304RPT6, lane 3) or with a plasmid-borne *ALG13* allele controlled by its own promoter (XGY160/pALG13); the latter transformant was grown on medium supplemented with galactose (lane 5) or glucose (lane 6). All other strains and transformants used in this experiment were grown on medium containing galactose. Lane 4 shows invertase from a strain in which *ALG13* is driven by the *GAL1* promoter inserted immediately upstream of *ALG13* (NAY13). Wild-type (W303-1A) served as a control (lane 1). (B) Schematic map of the chromosomal loci of *ALG13* and *RPT6* and their intergenic region before (top) and after integration of the *GAL1* promoter-containing fragment used to construct P_{GAL1} -HA-ALG13 *rpt6*, in which the *RPT6* promoter is replaced (XGY160, middle), and P_{GAL1} -HA-ALG13, in which the *RPT6* promoter region is intact (NAY13, bottom).

Alg13p in these extracts was quantified by Western analysis. It should be noted that HA-Alg13p occasionally runs as a doublet in certain strain backgrounds, including *rpt6*, but we experimentally confirmed that this altered mobility was not due to proteolysis, ubiquitination, or phosphorylation (data not shown). The 60-kDa vacuolar ATPase subunit (v-ATPase) is not degraded by the proteasome and served as a loading control. As seen in Figure 2A, most of Alg13p was degraded after 20 min ($t_{1/2} \sim 10$ min) in an *RPT6* wild-type strain (P_{GAL1} -HA-ALG13, NAY13, bottom panel). In contrast to its instability in the *RPT6* wild-type strain background, a significant amount of Alg13p was still detectable even after 5 h ($t_{1/2} \sim 300$ min) in the *rpt6* mutant strain (P_{GAL1} -HA-ALG13 *rpt6*, XGY160; Figure 2A, top panel). Thus, the Alg13p half-life is increased significantly if *RPT6* expression is impaired. In agreement with these CHX results, we observed a strong correlation between Alg13p stability and growth rate; cells whose P_{GAL1} -driven *ALG13* expression was repressed by growth on glucose but whose Alg13p stability was increased by impairment of *RPT6* grew better than those in which Alg13p was labile (Figure 2B). Taken together, we conclude that Alg13p accumulates in the P_{GAL1} -HA-ALG13 *rpt6* strain, and it is this accumulation that causes the glycosylation defect.

Alg13p Degradation Depends on the Proteasome But Not on Ubiquitin

Rpt6p also plays nonproteolytic roles in transcriptional elongation (Ferdous *et al.*, 2001; Gonzalez *et al.*, 2002). To confirm that the accumulation of Alg13p in the *rpt6* strain is due to an impaired proteasome, we examined if this strain is defective for proteasomal degradation. This was assayed by monitoring the stability of the model proteasome substrate, Ub-Arginine- β -galactosidase (Ub-R- β -gal). This reporter

protein has a destabilizing Arg residue at its N-terminus and according to the N-end rule is very short-lived because it is rapidly degraded by the proteasome ($t_{1/2} \sim 2$ min; Bachmair *et al.*, 1986). An integrative plasmid expressing *Ub-R-lacZ* under the control of the *GAL1* promoter (pUb-R-lacZ) was introduced into wild-type (W303-1A), the P_{GAL1} -HA-ALG13 *rpt6* mutant (XGY160) with or without plasmid-borne *RPT6*

Figure 2. Alg13p degradation is impaired in an *rpt6* strain. (A) Alg13p stability was measured after arresting translation with cycloheximide (CHX). Strains were grown in YPA + 2% galactose and harvested for protein isolation before (0 min) and at variable times after addition of 100 μ g/ml CHX: P_{GAL1} -HA-ALG13 *rpt6*, XGY160 (top) and P_{GAL1} -HA-ALG13 *RPT6*, NAY13 (bottom). Western analysis was performed with 12CA5 antibody to detect HA-Alg13p and with anti-Vma2 antibody to detect v-ATPase that serves as a loading control because it is not degraded by the proteasome. (B) The stability of HA-Alg13p correlates with the growth rate on glucose medium, which represses HA-ALG13 expression from the *GAL1* promoter. Tenfold serial dilutions of wild type (W303-1A) and the strains described in A were spotted on YPA plates with glucose or galactose and incubated at 30°C for 2 d.

Figure 3. Alg13p degradation depends on the proteasome. (A) Replacement of the *RPT6/ALG13* intergenic DNA with the *GAL1* promoter results in a defective proteasome. Wild type (W303-1A), a P_{GAL1} -HA-ALG13 *rpt6* strain (XGY160), the latter strain expressing plasmid-borne *RPT6* (XGY160/pRS304RPT6), and the P_{GAL1} -HA-ALG13 strain (NAY13) were transformed with a Ub-R-lacZ plasmid, pUb-R-lacZ. These transformants were cultivated in YPA + 2% galactose. The stability of Ub-R-β-gal was measured using a liquid β-gal assay. The average of measurements of three independent transformants is shown. (B) Alg13p accumulates in a *pre1-1 pre2-2* mutant. The stability of HA-Alg13p was assayed in the proteasome double mutant *pre1-1 pre2-2* P_{GAL1} -HA-ALG13 (NAY20) and the isogenic wild-type strain P_{GAL1} -HA-ALG13 (NAY19) in a Western analysis as performed in Figure 2A.

(pRS304RPT6), and into the *RPT6* wild-type strain (NAY13). The steady-state level of Ub-R-β-gal was determined by β-gal assays. Although β-gal levels were similar to the wild-type control in strains expressing *RPT6* normally, the P_{GAL1} -HA-ALG13 *rpt6* strain (XGY160) showed at least 10 times higher values (Figure 3A). Hence, this strain is impaired in proteasomal degradation. Furthermore, this impairment depends on *RPT6* because the isogenic strain, in which *RPT6* was expressed from a plasmid (XGY160/pRS304RPT6), and the P_{GAL1} -HA-ALG13 strain, in which the *RPT6* promoter was intact (NAY13), both behaved like the wild-type control in this assay.

Because the P_{GAL1} -HA-ALG13 *rpt6* strain (XGY160) is impaired in proteasomal degradation and accumulates Alg13p, we assumed that Alg13p is degraded by the proteasome. To verify this, the stability of Alg13p was examined in another proteasome mutant, *pre1-1 pre2-2*, whose degradation defect is *RPT6*-independent. Pre1 and Pre2 are essential endopeptidase subunits of the 20S proteasome; the *pre1-1 pre2-2* double mutant is partially defective for proteasomal degradation (Heinemeyer *et al.*, 1993). Alg13p stability in a *pre1-1 pre2-2* mutant strain (NAY20) and an isogenic wild-type strain (NAY19) was measured by monitoring Alg13p levels after a CHX translational arrest as described above. Similar to the *rpt6* strain, Alg13p was about three times more stable in the *pre1-1 pre2-2* strain ($t_{1/2} \sim 30$ min) than in the control strain (NAY19; Figure 3B). Thus, we conclude that Alg13p degradation depends on the proteasome.

Although our data established that Alg13p degradation is dependent on the proteasome, we did not observe any higher molecular weight poly-ubiquitinated Alg13p intermediates that were expected to accumulate in these proteasome mutants (data not shown). If Alg13p is degraded by the ubiquitin pathway, then it should be stabilized if ubiquitination is blocked. To test this idea, we studied Alg13p stability in a strain that is impaired for ubiquitination because it encodes a defective E1 ubiquitin activating enzyme Uba1p. In the *uba1-2* mutant, ubiquitination is defective and thus, proteins whose proteasomal degradation depends on

ubiquitin accumulate (Swanson and Hochstrasser, 2000). Alg13p stability was examined in the *uba1-2* mutant strain (NAY26) and the isogenic wild-type strain (WCG4a) harboring a centromeric plasmid in which HA-ALG13 is expressed from the *GAL1* promoter (pCEN-ALG13). The amount of Alg13p that accumulated was determined in *uba1-2* and wild-type cells harvested at various times after a CHX translational arrest (Figure 4A). As a control, we also measured the stability of a reporter protein, Ub-L-β-gal, whose rapid degradation strictly depends on ubiquitination (Bachmair *et al.*, 1986; Swanson and Hochstrasser, 2000) and should therefore display increased stability in the *uba1-2* strain. To study the stability of Ub-L-β-gal, we determined its steady-state levels in β-gal assays. The results of these experiments demonstrated that the relative $t_{1/2}$ of Alg13p was unaffected by reduced ubiquitination as Alg13p did not accumulate in an *uba1-2* mutant (Figure 4A). In contrast, Ub-L-β-gal activity was four- to fivefold higher in the *uba1-2* mutant than in wild-type (Figure 4B), confirming that this *uba1-2* strain is indeed phenotypically defective in ubiquitin-dependent proteasomal degradation. Taken together, these results support the model that Alg13p does not require ubiquitination for its proteasomal degradation. Consistent with these results, Alg13p isolated from proteasome mutants did not cross-react with anti-ubiquitin antibodies nor could it be detected in an enriched pool of accumulated ubiquitinated proteins isolated from proteasome mutants. Further more, the decay rate of Alg13p was unaffected by chemicals or mutations that inhibit deubiquitination, or ubiquitin ligases (data not shown). Although these data do not rule out the possibility that Alg13p can be modified by ubiquitin, they demonstrate that proteasomal degradation of Alg13p does not require ubiquitin.

Degradation of Alg13p Requires its C-Terminus, Which Can Serve as an Autonomous Degradation Signal

Degradation of Alg13p does not depend on ubiquitin. This raises the question of how Alg13p is targeted to the protea-

accordingly. SDs were calculated based on normalized triple data sets. (B) The *uba1-2* mutant used in this study accumulates ubiquitinated substrates. Wild-type (WCG4a) and the *uba1-2* strain (NAY26) were transformed with a Ub-L-lacZ plasmid, p415-GDP-Ub-L-lacZ. The stability of Ub-L-β-gal was measured using a liquid β-gal assay. The average of the measurements of three independent transformants is shown.

some. An earlier result showed that Alg13p that is FLAG-tagged at its C-terminus instead of HA-tagged at its N-terminus is not degraded, even after 5 h of CHX treatment (data not shown). This suggested that the C-terminus of Alg13p might play a role in targeting this protein to the proteasome, an idea for which there is a precedent. Only a few proteins that do not rely on ubiquitin modification for proteasome targeting have been identified. The best-characterized example of such a protein is ornithine decarboxylase (ODC), which contains a C-terminal proteasome targeting motif (Li and Coffino, 1993). To determine if the C-terminus of Alg13p is required for its degradation, yeast strains were constructed that encode HA-tagged Alg13p proteins with

successively longer deletions of the C-terminus (Figure 5A). The relative amount of each of these truncated HA-Alg13p proteins in wild-type cells was measured at various times after a CHX translational arrest, by Western blotting with anti-HA antibody. The 60-kDa v-ATPase subunit served as a loading control and was detected with anti-Vma2 antibody. The results of this deletion analysis are shown in Figure 5B. Although Alg13p with a C-terminal truncation of 20 amino acids was degraded at the same rate as the wild-type Alg13p ($t_{1/2}$ between 10 and 20 min), a truncation of 60 amino acids increased Alg13p's half-life by at least ninefold ($t_{1/2}$ between 90 and 300 min), and a truncation of 101 amino acids increased the half-life even further ($t_{1/2} > 300$ min). We noted

Figure 5. The C-terminal region of Alg13p is necessary for its degradation. (A) A schematic diagram of the truncated Alg13p proteins lacking the C-terminal 20, 60, or 101 amino acids. The region within Alg13p that contains the predicted UDP-GlcNAc binding and catalytic domain is indicated in gray. (B) Analysis of the stability of truncated Alg13p. Wild-type yeast (W303-1A) expressing plasmid-borne wild-type HA-ALG13 (pCEN-Alg13p) or HA-*alg13Δ* C-terminal deletion alleles (20 amino acids deleted: pCEN-Alg13pΔ20aa; 60 amino acids deleted: pCEN-Alg13pΔ60aa; 101 amino acids deleted: pCEN-Alg13pΔ101aa) were grown in selective minimal (–His) medium supplemented with 2% galactose. Cells were harvested before (0 min) and at variable times after translational arrest by the addition of CHX (100 μg/ml). Equal amounts of protein were examined by Western analysis with anti-HA antibody and anti-Vma2 to detect v-ATPase, which serves as a loading control.

that the long-lived Alg13p truncated proteins all lacked the predicted UDP-GlcNAc transferase catalytic domain (Figure 6A), suggesting there may be a correlation between sugar catalysis and Alg13p protein half-life. However, this is unlikely because a catalytically inactive Alg13p protein, which contains a single amino acid change in the predicted UDP-GlcNAc binding domain, is as short-lived as the wild-type Alg13p (data not shown). These results demonstrated that

Figure 6. Appending the C-terminal region of Alg13p to β -galactosidase causes it to be short-lived in a proteasome-dependent but ubiquitin-independent manner. (A) A schematic diagram of the β -gal/Alg13p chimera containing β -gal fused at its C-terminus to the C-terminal 101 amino acids of Alg13p. (B) Liquid β -gal assay to compare the activity of β -gal-Alg13p Δ 1-101 and β -gal. Wild-type (WCG4a) yeast were transformed with an integrative plasmid bearing a *TP11* promoter-driven *lacZ* (pTi-LacZ) or *lacZ*-alg13 Δ 1-101 (pTi-LacZ-Alg13p Δ 1-101). Cells were grown in YPA + 2% glucose, and β -gal activity was measured using a liquid, colorimetric assay. The average of measurements from three independent transformants is shown. (C) Relative activity of β -gal-Alg13p Δ 1-101 in wild type, a ubiquitination mutant (*uba1-2*), and a proteasome mutant (*pre1-1 pre2-2*). Wild type (WCG4a), *uba1-2* mutants (NAY110, NAY111), and a *pre1-1 pre2-2* mutant (WCG4-11/22a) expressing the *TP11*-driven *lacZ* or *lacZ*-alg13 Δ 1-101 genes were obtained by targeted integration of plasmids containing *lacZ* (pTpi-lacZ) or *lacZ*-alg13 Δ 1-101 (pTpi-lacZ-alg13 Δ 1-101). Cells were grown in YPA + 2% glucose and harvested, and liquid β -gal assays were performed (see Materials and Methods). To obtain the relative β -gal-Alg13p Δ 1-101 activity in each strain background, the resulting β -gal-Alg13p Δ 1-101 activity was divided by the activity of β -gal. For each strain background the β -gal and β -gal-Alg13p Δ 1-101 activities of three to five independent transformants were measured and averaged.

amino acids in the C-terminus of Alg13p, between amino acid 101 and 183, are necessary for its degradation.

To determine if this C-terminal domain can act as an autonomous degradation signal, we asked if attaching this domain to a normally long-lived heterologous protein would cause it to be short-lived. As a reporter, we chose to analyze β -gal because it is a very stable protein; β -gal is not targeted to the proteasome unless it is engineered to contain proteasome-targeting sequences, for instance ubiquitin or the ODC-targeting domain (Bachmair *et al.*, 1986; Chau *et al.*, 1989; Hoyt *et al.*, 2005; Matsuzawa *et al.*, 2005). A gene fusion was constructed that encodes a β -gal/Alg13p chimera, in which the C-terminal 101 amino acids of Alg13p were fused to the C-terminus of β -gal (Figure 6A). An integrative plasmid (pTi-lacZ-alg13 Δ 1-101) containing this *TP11* promoter-driven *lacZ*-alg13 Δ 1-303 gene was introduced into yeast and confirmed to be single copy in the chromosome. The steady-state level of β -gal was measured by an activity assay. The results of this experiment demonstrated that the presence of the Alg13p C-terminal domain on β -gal (β -gal-Alg13p Δ 1-101) decreased β -gal's activity by almost 10-fold compared with the unmodified β -gal (Figure 6B). We confirmed that this decrease in β -gal activity was a reflection of a decrease in β -gal concentration by Western blot analysis (data not shown). This ~10-fold difference in protein stability was similar in its extent to that seen when comparing Alg13p to Alg13p Δ 101 that lacks this C-terminal motif (Figure 5). Importantly, the instability of this β -gal-Alg13p Δ 1-101 protein is proteasome- and ubiquitin-independent, because it is significantly more stable in a *pre1-1 pre2-2* mutant but is unaffected in a *uba1-2* mutant (Figure 6C). These results demonstrate that the C-terminal motif of Alg13p can autonomously confer proteasome-mediated degradation to an otherwise stable protein.

Cytosolic Alg13p Is Degraded Rapidly, Whereas Membrane-bound Alg13p Is Stable

How might the C-terminal Alg13p domain mediate proteolysis? One possibility is that this domain acts as proteasome targeting signal that is exposed in the Alg13p monomer but that is masked in the Alg13p/Alg14p hetero-oligomer. Such a model has been hypothesized to explain the selective degradation of free subunits of multimeric enzymes (Buchler *et al.*, 2005; Asher *et al.*, 2006). Alg13p exists in two pools: one bound to the ER membrane through its association with Alg14p and another pool that is soluble in the cytosol (Gao *et al.*, 2005). If the association of Alg13p with Alg14p at the ER membrane protects or delays Alg13p proteolysis, then the cytosolic pool of Alg13p should turn over more rapidly than membrane bound Alg13p. To explore this idea, we compared the stability of cytosolic Alg13p versus membrane-associated Alg13p. Cells expressing HA-ALG13 were harvested at various time after the addition of CHX to arrest translation and lysed in the absence of detergent. These lysates were subjected to differential centrifugation to separate ER membranes from soluble, cytosolic proteins (see Materials and Methods). Proportionally equal amounts of protein in each of these fractions were separated by SDS-PAGE and immunoblotted with anti-HA antibody to detect Alg13p and anti-Vma2 to detect the v-ATPase loading control. From this analysis, we found that membrane-associated Alg13p displayed a markedly increased half-life (more than 300 min) compared with the cytosolic pool (<10 min; Figure 7). These results demonstrated that cytosolic Alg13p is a target for rapid degradation while membrane associated Alg13p is relatively stable. These data are consistent with the model that the hetero-oligomeric association of Alg13p with Alg14p at the ER membrane protects Alg13p from degradation.

Figure 7. Membrane-association stabilizes Alg13p. Yeast cells expressing *HA-ALG13* (NAY13) were grown in YPA + galactose and harvested before (0 min) and at variable times after addition of CHX (100 μ g/ml) to arrest translation. At each indicated time, cells were harvested, lysates were prepared and subjected to differential centrifugation to enrich for ER membranes and soluble proteins (S100) as described in *Materials and Methods*. Proportionally equal amounts of protein from each fraction were separated by SDS-PAGE and analyzed by Western blotting, with anti-HA antibody to detect HA-Alg13p and anti-Vma2 to detect the v-ATPase loading control.

Inviability Due to Loss of Protein Function Occurs More Rapidly by Depleting Alg13p than Alg14p

Alg13p and Alg14p interact with one another at the ER membrane, and this interaction is required for UDP-GlcNAc transferase activity (Bickel *et al.*, 2005). We considered the possibility that the degradation of Alg13p represents a potentially relevant mechanism for regulating this enzyme. Several results were obtained that are consistent with this idea.

First, a “shut-off” experiment, in which the expression of *GAL1*-promoter-driven *ALG13* or *ALG14* was turned off by growth in glucose, suggested that the amount of cellular Alg13p, but not Alg14p, is rate limiting for enzyme function. Both Alg13p and Alg14p are essential genes, so the rate at

which cells stop growing after a shift into glucose-containing medium is a reflection of protein and/or RNA stability. In this experiment, we compared the growth of *P_{GAL1}-HA-ALG13* and *P_{GAL1}-HA-ALG14* isogenic yeast strains (NAY13 and XGY151) after a shift into glucose-containing medium. In contrast to the *P_{GAL1}-HA-ALG13* strain, whose growth in glucose ceased within 12 h of log-phase growth, the *P_{GAL1}-HA-ALG14* survived much longer; kept under the same conditions, it did not stop growing until 45.5 h. This marked contrast in growth rate was also seen when these strains were grown on solid glucose-containing medium. Here, tagged and untagged Alg14p were studied to make certain that the terminal tag does not influence Alg14p stability, as is the case with Alg13p. The *P_{GAL1}-HA-ALG13* strain was inviable after a single streaking on glucose-containing medium, whereas the strains expressing *ALG14* (NAY105-1) or *HA-ALG14* (XGY151) from the *GAL1* promoter only ceased to grow after at least three consecutive restreakings on glucose (Figure 8A). No effect on growth rate was observed whether or not Alg14p was epitope tagged (Figure 8A and data not shown). These results demonstrated that in contrast to Alg13p, whose loss-of-function phenotype is rapidly observed, there is a marked phenotypic lag associated with loss of Alg14p function.

Both *ALG13* and *ALG14* are required for viability because both subunits are required for GTase activity (Bickel *et al.*, 2005; Chantret *et al.*, 2005; Gao *et al.*, 2005b). One interpretation of the relatively long phenotypic lag associated with Alg14p depletion compared with that of Alg13p is that Alg14p is a relatively stable protein. This idea was confirmed by a direct comparison of the relative half-life of Alg13p and Alg14p. Isogenic *P_{GAL1}-HA-ALG14* and *P_{GAL1}-HA-ALG13* yeast strains were grown in galactose and har-

Figure 8. Death occurs more rapidly by depletion of Alg13p than Alg14p. (A) Survival of cells depleted for Alg13p or Alg14p. Strains expressing *GAL1* promoter-driven *HA-ALG13*, *HA-ALG14*, or *ALG14* (untagged; NAY13, XGY151, and NAY105-1) or isogenic wild-type yeast (W303-1A) were grown on YPA plates supplemented with galactose or glucose. Fresh colonies that were grown on galactose-containing plates were streaked onto glucose-containing plates and incubated overnight at 30°C (first streaking). Resulting colonies were repeatedly streaked onto glucose-containing plates and incubated overnight at 30°C until only wild type remained growing. This occurred after three consecutive streakings (third streaking). (B) Alg14p is much more stable than Alg13p. The relative stability of Alg13p and Alg14p was compared in isogenic strains expressing *HA-ALG13* or *HA-ALG14* (NAY13, XGY151). Cells were harvested before (0 min) and at variable times after CHX addition (100 μ g/ml). Equal amounts of protein from each time point were analyzed by Western analysis with anti-HA to detect HA-Alg13p or HA-Alg14p, and anti-Vma2 to detect the v-ATPase protein loading control.

vested at various times after addition of CHX, and the amount of Alg13p or Alg14p remaining after a CHX translational arrest was assayed by Western blot analysis (Figure 8B). This analysis revealed that Alg13p is more labile than Alg14p. The $t_{1/2}$ of Alg13p is <10 min, whereas that of Alg14p is at least three to six times longer. It should be noted that these data on the relative stability of Alg13p and Alg14p are not in accord with those generated by yeast proteomic analyses in the database, which report far fewer molecules of Alg14p per cell than Alg13p (339 vs. 1950 molecules per cell, respectively; Ghaemmaghami *et al.*, 2003). In those proteomic studies, protein half-lives were measured by analyzing C-terminally epitope-tagged proteins. Thus, this discrepancy can be explained by our observation that addition of sequences at the C-terminus of Alg13p dramatically increases its half-life and serves as a cautionary note when using proteomic data without experimental confirmation. Together with our finding that there is a correlation between growth rate and Alg13p stability (Figure 2B), these data on the relative half-life of Alg13p and Alg14p support the model that the modulation of Alg13p subunit levels restricts Alg13p/Alg14p GTase *in vivo* activity.

DISCUSSION

The Alg13p/Alg14p UDP-GlcNAc transferase is a key enzyme that is required for one of the earliest steps in the N-linked protein glycosylation pathway. This UDP-GlcNAc transferase is the only eukaryotic GTase thus identified that is a hetero-oligomer, in which the acceptor binding site and the catalytic domain reside on separate polypeptides. With the recent identification of the Alg13p and Alg14p subunits, an important goal is to better define how the interaction between these two proteins at the ER membrane is regulated. In this study, we report that, unlike Alg14p, which is quite stable, the Alg13p catalytic subunit is a very short-lived protein when not assembled with its partner, and its cellular levels are restricted by proteasomal degradation.

Alg13p Is Degraded by the Proteasome But Does Not Require Ubiquitin

We showed that Alg13p is degraded rapidly by a proteasome-dependent mechanism (Figures 2 and 3). Strikingly, this degradation does not require ubiquitin (Figure 4). Although a number of proteins have been identified that do not require ubiquitin for proteasomal degradation, most of them use the ubiquitin-dependent pathway as the primary route for their removal (reviewed in Orłowski and Wilk, 2003; Hoyt and Coffino, 2004). Despite an extensive search, we found no evidence for any ubiquitinated Alg13p, even in proteasome mutants. Moreover, the *UBA1*-independent degradation of Alg13p (Figure 4A) provides direct evidence that ubiquitin is not required for targeting to the proteasome. Although we cannot completely rule out the possibility that Alg13p is ubiquitinated for proteasomal degradation, our data in Figure 4 show that ubiquitination does not enhance Alg13p degradation (Figure 4). Thus, like ODC and human thymidylate synthase, which are exclusively degraded independently of ubiquitin (Bercovich *et al.*, 1989; Gandre *et al.*, 2003; Forsthoefel *et al.*, 2004), Alg13p may represent another rare example of a protein whose proteasomal degradation does not involve ubiquitin at all.

An interesting question raised by these studies is how Alg13p is targeted to the proteasome in an ubiquitin-independent manner. The apparent ubiquitin-independent degradation of Alg13p implied that an alternative signal is used for proteasome targeting, as is the case with ODC and hu-

man thymidylate synthase (Li and Coffino, 1993; Peña *et al.*, 2006). We found that a domain consisting of at least 40 amino acids in the C-terminal 101 amino acids of Alg13p is required for its degradation (Figure 5). Moreover, these C-terminal 101 amino acids of Alg13p can serve as an autonomous degradation signal. Attaching them to β -gal caused this stable reporter to be rapidly degraded in a proteasome-dependent but ubiquitin-independent manner (Figure 6). Whether or not this C-terminal domain is directly recognized by the cell's degradative machinery is unclear. There is increasing evidence that many proteins that normally function as part of a multiprotein complex contain natively "disordered" domains that only become folded upon binding to their target (Dyson and Wright, 2005). It has been proposed that these unstructured domains are recognition signals for ubiquitin-independent proteasome targeting of the free monomer (Asher *et al.*, 2006). According to this "default" degradation pathway, these disordered domains enable unpaired monomers to gain direct access into to the 20S catalytic core, allowing them to bypass the 19S regulatory particle that is involved in ubiquitin recognition. Although we do indeed find that cytosolic, unbound Alg13p is selectively degraded compared with the ER-bound form (Figure 7), Alg13p degradation is entirely dependent on Rpt6p, a component of the 19S regulatory particle. Therefore, this "default" degradation model mentioned above cannot explain how the C-terminal domain of Alg13p is recognized and mediates degradation. Further experiments are required to determine if the Alg13p C-terminal domain is directly recognized by the proteasome or if Alg13p degradation requires its association with an accessory factor, as is the case with ODC.

Alg13p Is a Target for the Regulation of N-Glycosylation

An important result of this study is that either too little or too much Alg13p has a deleterious effect on glycosylation. This finding underscores the notion that the cell must tightly regulate Alg13p levels to maintain optimal levels of protein glycosylation. Controlling Alg13p levels through proteolysis could provide the cell with a means of modulating Alg13p/Alg14p GTase activity, but such a proposed regulatory mechanism posits that Alg13p is the limiting subunit of the Alg13p/Alg14p GTase, and that Alg14p is in excess. Several pieces of data support the idea that Alg13p is indeed the limiting subunit for the Alg13p/Alg14p GTase. Alg13p, when unbound, is exceedingly labile, whereas its partner, Alg14p is quite stable (Figure 8B). In addition, there is a rapid onset of lethality when Alg13p is depleted compared with Alg14p (Figure 8A), and the onset of lethality that is associated with Alg13p depletion is slowed down in a proteasome mutant (Figure 2B). Alg13p is the catalytic portion of the Alg13p/Alg14p UDP-GlcNAc transferase, so the conditional degradation of Alg13p represents an economical way to control flux through the LLO pathway, dependent on the functional requirements of the cell. Although these data are consistent with the model that Alg13p is the target for regulation, an alternative model that we have not ruled out is that Alg14p is actually limiting and thus plays the major regulatory role. If Alg13p was produced constitutively in excess but unassembled Alg13p molecules were degraded, regulating the levels of Alg14p levels could be sufficient to fine tune UDP-GlcNAc transferase activity. Further experiments that investigate the steady-state relationship between Alg13p and Alg14p will be required to test these ideas.

Although our data support the idea that the cell can modulate Alg13p/Alg14p GTase activity by restricting Alg13p levels, they do not explain why or how an excess of

Alg13p leads to glycosylation defects. This phenotype is unlikely to result from a direct affect of Alg13p, for instance by perturbation of the stoichiometry of GTase subunits; indeed we find no evidence that Alg13p/Alg14p UDP-GlcNAc transferase itself is inhibited by too much Alg13p (N. Aeverbeck, unpublished observation). Instead, a more likely explanation is that excess cytosolic Alg13p, normally kept in check by the proteasome, interferes with N-glycosylation through another mechanism, possibly by titrating a molecule that is required for this process. An important future goal will be to understand how an excess of Alg13p can inhibit glycosylation.

ACKNOWLEDGMENTS

We thank M. Hochstrasser, A. Varshavsky, and D. Wolf for yeast strains and plasmids and Sabine Keppler-Ross for technical support. This work was supported by National Institutes of Health Grant RO1-GM048467 to N.D.

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