Glucosidase II is essential for sequential removal of two glucose residues from N-linked glycans during glycoprotein biogenesis in the endoplasmic reticulum. The enzyme is a heterodimer whose α-subunit contains the glycosyl hydrodase active site. The function of the β-subunit has yet to be defined, but mutations in the human gene have been linked to an autosomal dominant form of polycystic liver disease. Here we report the identification and characterization of a Saccharomyces cerevisiae gene, GTB1, encoding a polypeptide with 21% sequence similarity to the β-subunit of human glucosidase II. The Gtb1p protein was shown to be a soluble glycoprotein (96–102 kDa) localized to the endoplasmic reticulum lumen where it was present in a complex together with the yeast α-subunit homologue Gls2p. Surprisingly, we found that Δgtb1 mutant cells were specifically defective in the processing of monoglucosylated glycans. Thus, although Gls2p is sufficient for cleavage of the penultimate glucose residue, Gtb1p is essential for cleavage of the final glucose. Our data demonstrate that Gtb1p is required for normal glycoprotein biogenesis and reveal that the final two glucose-trimming steps in N-glycan processing are mechanistically distinct.

In higher eukaryotic cells, there is a close link between protein processing and ER quality control (for reviews, see Refs. 1 and 2). Cleavage of the middle glucose by glucosidase II generates monoglucosylated glycans that are required for productive interactions with the ER-resident chaperones calnexin and calreticulin (4–6). Cleavage of the final glucose residue then prevents further association with calnexin/calreticulin, allowing correctly folded proteins to proceed through the secretory pathway. In contrast, incorrectly folded glycoproteins can be reglucosylated by UDP-glucose:glycoprotein glucosyltransferase (Fig. 1, G7), which acts as a folding sensor (7), permitting another cycle of calnexin/calreticulin interaction. This enables unfolded substrates to go through multiple rounds of interaction with the chaperones of the cycle until the native conformation is reached, when recognition by glucosidase II (but no longer by glucosyltransferase) allows exit from the cycle and the ER. If the native conformation is not achieved, the slower acting ER mannosidase I cleaves a specific terminal mannose, resulting in the targeting of the glycoprotein for degradation (1).

Glucosidase II has been extensively characterized from mammalian tissues as a soluble luminal enzyme composed of two tightly associated α and β glycoprotein chains (8, 9). The α-subunit (GIIα) is a 107-kDa protein sharing sequence identity with other glucosidase enzymes and has a glycosyl hydrolase activity in vitro that is independent of the β-subunit (9). The function of the β-subunit (GIIβ) is less well defined, but it has been suggested that it may be required to retain the heterodimer in the ER via its C-terminal HDEL motif (10–12). More recently, it has been proposed that GIIβ interacts with N-glycans via a carbohydrate recognition domain to stimulate the GIIα trimming of both the middle and innermost glucose residues (13). The importance of a physiological role for GIIβ is underlined by the finding that autosomal dominant polycystic liver disease can develop in individuals carrying mutations in the GIIβ gene (14–17).

Glucosidase II has been characterized to a lesser extent in Saccharomyces cerevisiae. The GLS2 gene encodes a GIIα homologue (Gls2p), and a Δgls2 null mutant is unable to process the G2 form of glycoproteins (8, 18). Here we report that a previously uncharacterized open reading frame (ORF), YDR221w, encodes the yeast homologue of GIIβ. This ORF, which we now refer to as GTB1 (glucosidase two β-subunit), expresses a soluble 96–102 kDa glycoprotein that co-immunoprecipitates in a complex together with Gls2p. In Δgtb1 null mutant cells, we found the stability and localization of Gls2p to be unaltered; thus yeast Gbt1p is not required for retention of Gls2p in the ER. Surprisingly, we found that trimming of G2 to G1 was unaffected in Δgtb1 cells, demonstrating that Gls2p is sufficient for this reaction. In striking contrast, we found that the Δgtb1 cells accumulated monoglucosylated forms of N-linked glycoproteins. These results indicate that Gtb1p is specifically required for the final glucose-trimming event during normal glycoprotein processing. We propose a model in which the β-subunit is specifically required to present monoglucosylated substrates to the catalytic domain of the α-subunit.

**EXPERIMENTAL PROCEDURES**

Yeast Strains, Plasmids, Media, and Growth Conditions—Yeast strains were grown at 30 °C with rotation in YP medium (1% yeast
The Function of ER Glucosidase II β-Subunit

FIGURE 1. ER processing of N-linked core oligosaccharides. The core glycan (G3) has 14 saccharides: 3 glucoses (triangles), 9 mannoses (circles), and 2 N-acetylglucosamines (pentagons). The mannoses are organized into three branches, with the A branch capped by glucoses and a pentamannosyl branch subdivided into branches B and C. The glucose residues are removed sequentially by the action of glucosidases I and II. The monoglucosylated (G1) form specifically interacts with the lectins calnexin and calreticulin during the folding of glycoproteins. After the removal of this final glucose by glucosidase II, reglucosylation by the folding sensor UDP-glucose:glycoprotein glucosyltransferase (GT) can occur if the substrate has not reached its native conformation. This cycle can continue until this is achieved or until the unfolded substrate is recognized by the slow acting mannosidase.

TABLE 1

S. cerevisiae strains

| Strain | Genotype | Source |
|--------|----------|--------|
| BY4741 | MATα, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 | Ref. 19 |
| BY4742 | MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0 | Ref. 19 |
| Haploid a | As BY4741, Δorf-kanMX4 | Ref. 20 |
| BWY616 | As BY4742 except gtb1::HA-kanMX4 | This study |
| BWY618 | As BY4741 except gtb1::HA-kanMX4 | This study |
| BWY619 | MATα, his3Δ1, leu2Δ0, ura3Δ0, gtb1::Myc-kanMX4 | This study |
| BWY620 | MATα, his3Δ1, leu2Δ0, lys2Δ0, gtb1::Myc-kanMX4 | This study |
| BWY625 | MATα, his3Δ1, leu2Δ0, ura3Δ0, gls2::myc-kanMX4, Δgls2 | This study |
| BWY629 | MATα, his3Δ1, leu2Δ0, ura3Δ0, Δgls2, Δgls2 | This study |
| BWY630 | MATα, his3Δ1, leu2Δ0, ura3Δ0, Δgls2, Δgls2, Δgls2 | This study |
| BWY635 | MATα, his3Δ1, leu2Δ0, ura3Δ0, Δgls2, Δgls2, Δgls2, Δgls2 | This study |
| BWY636 | MATα, his3Δ1, leu2Δ0, ura3Δ0, Δgls2, Δgls2, Δgls2, Δgls2 | This study |

Yeast Cell and Medium Extract Preparation—Yeast whole cell extracts were prepared from 5.0 A600 units of exponentially growing cultures by suspension in SDS-PAGE sample buffer containing 5% β-mercaptoethanol and 0.5-mm glass beads, followed by disruption at 6.5 M/s for 30 s (Hybird Ribioler) and incubation for 5 min at 95 °C. Growth medium protein extracts were also prepared from exponentially growing cultures. Typically, 10.0 A600 nm units of culture were subjected to centrifugation at 20,000 × g for 10 min, the supernatant removed to a fresh tube, and the centrifugation repeated again. Trichloroacetic acid was added to the resulting cell-free medium to a final concentration of 15%, with incubation on ice for 30 min. Precipitated material was pel-
lelled by centrifugation at 20,000 × g for 20 min. The pellet was washed twice with ice-cold acetone and then resuspended in 1% SDS, 62 mM Tris, pH 6.8, and 1 mM EDTA at 95 °C.

For endoglycosidase H digestion, whole cell or growth medium extracts prepared in 1% SDS, 62 mM Tris, pH 6.8, and 1 mM EDTA were diluted 10-fold into 50 mM sodium citrate buffer, pH 5.5, containing 0.5 mM 4-(2-aminoethyl)-benzenesulfonylfluoride HCl and 10 mM dithiothreitol. Mock or endoglycosidase H (2 milliunits/μg of microsomes, prepared as previously described (25) were harvested at 17,000 × g for 20 min. The supernatant fractions were subjected to trichloroacetic acid precipitation and resuspended in SDS-polyacrylamide gel sample buffer at 95 °C.

Antibodies and Immunoblot Analysis—Antibodies raised against Lhs1p and Kar2p (22), DPAP B (23), and ppCPY and Sec63p (24) have been previously described. Monoclonal antibodies against the Myc (9E10) and HA (12CA5) epitopes were obtained from Sigma. After migration on SDS-polyacrylamide gels, proteins were electroblotted to polyvinylidene difluoride membranes (Immobilon), and antibody incubations were performed as previously described (25). Immunodetection was carried out using enhanced chemiluminescence (PerkinElmer Life Sciences) with horseradish peroxidase-conjugated IgGs (Sigma).

Membrane Extraction—The membrane association of Gtb1p-HA and Gls2p-Myc were analyzed by the extraction of microsomes with reagents that discriminate between integral, peripheral, and soluble proteins. For each extraction, 2.5 A280 nm units of microsomes prepared as described above were resuspended in 100 μl of solubilization buffer containing 5% Triton X-100, 50 mM 4-(2-aminoethyl)-benzenesulfonylfluoride HCl, and 12% threitol. Mock or endoglycosidase H (2 milliunits/μl of 0.1 M Na2CO3 for 10 min and then precipitated in 5% formaldehyde at room temperature. The cells were washed three times with 1 ml of immunoprecipitation buffer. Antigens acid-precipitated as described above and resuspended in SDS-polyacrylamide gel sample buffer at 95 °C.

Native Immunoprecipitation—For native immunoprecipitation, 2.5 A280 nm units of microsomes, prepared as described above were resuspended in 100 μl of 0.1 M Na2CO3 (pH 11.0). Extractions were incubated on ice for 30 min before centrifugation at 100,000 × g for 30 min. The supernatant fractions were subjected to trichloroacetic acid precipitation, and all fractions were heated in SDS-polyacrylamide gel sample buffer at 95 °C.

Native Immunoprecipitation—For native immunoprecipitation, 2.5 A280 nm units of microsomes, prepared as previously described (25), were harvested at 17,000 × g for 15 min at 4 °C and resuspended with vortexing in 250 μl of solubilization buffer (20 mM Tris-HCl, pH 7.4, 5 mM magnesium(OAc)2, 10 μg/ml leupeptin, 5 μg/ml chymostatin/peptatin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl-fluoride HCl, and 12% glycerol) containing 1% Triton X-100 and 200 mM NaCl. The mixture was incubated on ice for 30 min prior to centrifugation at 10,000 × g for 10 min. For anti-HA precipitation, 25 μl of anti-HA affinity matrix (clone 3F10, Roche Applied Science) washed in solubilization buffer was added to the supernatant followed by incubation at 4 °C for 2 h. Antibodies were dissociated from the beads by the addition of SDS-PAGE sample buffer containing 5% β-mercaptoethanol and heating at 95 °C for 5 min.

Radiolabeling and Denaturing Immunoprecipitation—For [35S]methionine labeling, cells were grown in minimal medium, and cell extracts were prepared as described above. 5.0 A490 nm equivalents of cell extract were added to saturating amounts of antiserum followed by rotation for 2 h at room temperature. Immunocomplexes were collected on protein A-Sepharose CL4B beads (Sigma) at room temperature for 2 h. The beads were pelleted in a microcentrifuge and washed three times with 1 ml of immunoprecipitation buffer. Antibodies were dissociated from beads by the addition of SDS-PAGE sample buffer containing 5% β-mercaptoethanol and heating at 95 °C for 5 min. Radiolabeled samples were processed by 7.5% SDS-PAGE and visualized by phosphorimaging (Fuji FLA 3000) before exposure to x-ray film (Kodak). For Lhs1p and DPAP B immunoprecipitations, 5.0 A490 nm equivalents were loaded per lane and 2.0 equivalents for CPY.

RESULTS

Gtb1p Encodes the Yeast Homologue of GIIβ—There is a single uncharacterized yeast open reading frame, YDR221w, sharing significant (21%) sequence identity with human GIIβ (11) (Fig. 2). ORF, which we now refer to as Gtb1p, would encode a 702-residue polypeptide with a predicted N-terminal signal sequence and six consensus sites for N-glycosylation. However, it lacks a C-terminal ER retrieval motif (-XDELCOOH), which is a general feature of other known GIIβ sequences. The highest degree of sequence similarity is found in the N-terminal region corresponding to residues Gin64–Cys84 of the S. cerevisiae (27) protein (Fig. 2). The equivalent region of the human protein has been shown to be involved in binding GIIα and has significant similarity to a calcium-dependent EF-hand (28). Another conserved region, Asn100–Cys148, has homology with C-type lectin domains (Fig. 2), suggesting a role in calcium-dependent carbohydrate recognition. In addition, there is another region toward the C terminus of the molecule with 27% identity to the mannose-6-phosphate receptor homology domain, including three completely conserved residues that have been implicated in mannose binding (Fig. 2) (29).

Gtb1p Is Required for N-Glycan Processing in Vivo—To examine a possible role for Gtb1p in N-glycan trimming, we analyzed the biogenesis of glycoproteins in null mutant cells. Wild-type, Δgtb1, and Δgls2 cells were labeled for 4 min with [35S]methionine/cysteine in minimal medium at 30 °C. Cell extracts were subjected to immunoprecipitation with antisera directed against three different glycoproteins, namely Lhs1p, CPY, and DPAP B. As expected, Δgls2 mutant cells accumulated glycoproteins that migrated more slowly than those from wild-type cells due to their failure to remove two glucose residues from N-glycans (Fig. 3A, lanes 1 and 5). Glycoproteins from Δgtb1 mutant cells also migrated more slowly than those from wild-type cells but faster than those from the Δgls2 mutant (Fig. 3A, lane 3). This indicated a partial defect in glucose trimming in the Δgtb1 mutant but not the complete block in glucosidase II function seen with the Δgls2 mutant. Treatment with

Analysis of N-Glycans—N-glycans labeled with [3H]mannose for 4 min were prepared from yeast strains and analyzed as described by Jakob et al. (18). 20 A490 nm equivalents of [3H]mannose-labeled N-glycans were run on a Supelcosil LC-ND2 (240 × 4.6 mm) column in acetoni-trile/water (70:30, v/v), at a flow rate of 1 ml/min using an Akta purifier (Amersham Biosciences). Samples (1.0 ml) were collected and analyzed by scintillation counting.

Immunofluorescence—BMY625 cells transformed with pAC54 (SEC61-GFP) were prepared for immunofluorescence analysis essentially as described by Craven et al. (26). Briefly, cultures grown in minimal medium to A490 nm of 0.4 with selection for pAC54 were harvested and fixed in 5% formaldehyde at room temperature. The cells were spheroplasted by treatment with yeast lytic enzyme (ICN), and anti-Myc antibody (1:400; Sigma) staining was carried out for 1 h before visualization with Cy3-conjugated antibody (1:400; Sigma). Stained cells were incubated with 0.1 mg/ml 4,6-diamidino-2-phenylindole for 5 min. Images were captured with a Zeiss Axioshot microscope at 2500× magnification. Plasmid pAC54 expresses the N-terminal 475 residues of Sec61p fused to GFP. A 2.5-kb HindIII fragment containing the SEC61 gene with a unique BamHI site, as described in the construction of a plasmid expressing the N-terminal 475 residues of Sec61p fused to invertase (27), was cloned into HindIII-digested pRS425 (2 μm, LEU2). The GFP gene was derived from pS65T as an EcoRI fragment cloned into pUC118 and then ligated as a BamHI fragment into the SEC61 vector to create pAC54. This plasmid complements the lethal Δsec61 mutation (data not shown).
The Function of ER Glucosidase II β-Subunit

FIGURE 2. Sequence alignment of glucosidase II β-subunit homologues. The primary protein sequence predicted from the S. cerevisiae (Sc) gene GTB1 (YDR221w) was aligned with the Glb2p sequences from Homo sapiens (Hs) and Schizosaccharomyces pombe (Sp). Alignments were performed using the ClustalX program, with black shading representing 100% amino acid conservation and gray representing 67% conservation. The boxed regions indicate conserved regions with specific motifs. Residues GlcI6-Cys357 of the S. cerevisiae sequence shares the highest degree of homology and corresponds to a putative EF-hand. The other boxed regions correspond to a C-type lectin domain (Sc, residues Asn100–Cys147) and a mannose-6-phosphate receptor homology domain (Sc, residues Ser359–Glu369). Three conserved residues present in this domain, which are implicated in mannose binding, are indicated by filled circles. Overall, the human primary sequence shares 21 and 22% identity with the S. cerevisiae and S. pombe sequences, respectively.

Our interpretation of the trimming defect in Δgtb1 cells has thus far considered only the trimming of glucose residues. However, a change in the rate of mannosidase I trimming must also be considered. This is particularly relevant when one considers the possibility that the gel migration assays above might not discriminate between GlcMan, and Glc2Man glycoforms. To address this issue, we used the Δmns1 mutation to block mannose trimming, thus ensuring that all N-glycans retain a full complement of nine mannose residues (18). The Δmns1 allele was combined with either Δgtb1 or Δgtb2 and then glycoproteins analyzed as done previously. We observed a small but reproducible increase in the relative molecular weight of Lhs1p from Δmns1 cells compared with wild type, consistent with the expected defect in mannose trimming (Fig. 3C). The Δgls2/Δmns1 double mutant accumulates GlcMan, glycoforms (18), resulting in a substantially reduced gel mobility compared with the glucose-trimmed Man, (M9) control from Δmns1 (Fig. 3C, compare lanes 2 and 4). Crucially, Lhs1p from the Δgtb1/Δmns1 double mutant has an intermediate mobility, from which we must conclude that it represents monoglucosylated glycans. Similar patterns of migration were observed for other glycoproteins tested, namely DPAP B and CPY (not shown). These findings were further confirmed by HPLC analysis of isolated oligosaccharides prepared from total glycoprotein extracts (Fig. 3D). In this analysis, column retention time increases with the size of the N-glycan structure (18). The [3H]mannose-labeled N-glycans derived from Δgtb1/Δmns1 cells were found to be retained for a shorter time compared with those from Δgls2/Δmns1 cells (Fig. 3D). From these results, we can conclude that Δgtb1 mutant cells accumulate monoglucosylated N-linked glycans.

tunicamycin led to the accumulation of unglycosylated species of the same size in all of the strains, thus confirming that the decreased gel mobility of glycoproteins in the mutants was because of a defect in N-glycan processing. The most obvious explanation for the phenotype of Δgtb1 mutant cells is that the middle glucose is trimmed normally but that there is a defect in the trimming of the final glucose residue.

The trimming of glucose residues was analyzed further using a genetic approach to manipulate the glucosylation state of CPY. As previously reported, the transfer of the incomplete cores onto mutant cells is that the middle glucose is trimmed normally but that there is a defect in the trimming of the final glucose residue.

We have used the Δalg8 mutation to analyze the role of the third glucose in CPY (not shown). This is evidently in the two faster migrating forms of CPY detected in Δalg8 cells compared with wild type (Fig. 3B, lanes 1 and 2). As expected, all glycoforms of CPY were larger in Δalg8 cells than in the wild type, indicating the accumulation of monoglucosylated glycans (Fig. 3B, lane 3). Significantly, the forms of CPY detected in the Δgtb1/Δalg8 strain (Fig. 3B, lane 4) were identical in size to the Δalg8/Δalg8 strain, indicating that both Glc2p and Gtb1p are required to trim the final glucose in N-linked glycans.

Our interpretation of the trimming defect in Δgtb1 cells has thus far considered only the trimming of glucose residues. However, a change in the rate of mannosidase I trimming must also be considered. This is particularly relevant when one considers the possibility that the gel migration assays above might not discriminate between GlcMan, and Glc2Man glycoforms. To address this issue, we used the Δmns1 mutation to block mannose trimming, thus ensuring that all N-glycans retain a full complement of nine mannose residues (18). The Δmns1 allele was combined with either Δgtb1 or Δgtb2 and then glycoproteins analyzed as done previously. We observed a small but reproducible increase in the relative molecular weight of Lhs1p from Δmns1 cells compared with wild type, consistent with the expected defect in mannose trimming (Fig. 3C). The Δgls2/Δmns1 double mutant accumulates GlcMan, glycoforms (18), resulting in a substantially reduced gel mobility compared with the glucose-trimmed Man, (M9) control from Δmns1 (Fig. 3C, compare lanes 2 and 4). Crucially, Lhs1p from the Δgtb1/Δmns1 double mutant has an intermediate mobility, from which we must conclude that it represents monoglucosylated glycans. Similar patterns of migration were observed for other glycoproteins tested, namely DPAP B and CPY (not shown). These findings were further confirmed by HPLC analysis of isolated oligosaccharides prepared from total glycoprotein extracts (Fig. 3D). In this analysis, column retention time increases with the size of the N-glycan structure (18). The [3H]mannose-labeled N-glycans derived from Δgtb1/Δmns1 cells were found to be retained for a shorter time compared with those from Δgls2/Δmns1 cells (Fig. 3D). From these results, we can conclude that Δgtb1 mutant cells accumulate monoglucosylated N-linked glycans.
In these experiments, short pulse labeling times were used; therefore, we next examined the fate of labeled proteins following an extended chase period. Wild-type and Δgtb1 cells were pulse-labeled and samples analyzed during a 40-min chase period (Fig. 3E). It was found that untrimmed Lhs1p did not chase to the completely trimmed form observed in wild-type cells. These data suggest a complete block in glucose trimming.

It has been reported that more than one glycan must be added to polypeptides to promote efficient glucose trimming by mammalian glucosidase II (13). We therefore tested whether this might also be the case in yeast. To do this, we examined the processing of a Pho8-Ura3p fusion protein that is targeted to the ER by the Pho8p signal anchor domain and that has a single N-glycan acceptor site. A low copy plasmid expressing this protein was transformed into wild-type, Δgtb1, and Δgls2 strains and then the fusion protein analyzed after a short pulse labeling as before (Fig. 3F). The protein expressed in wild-type cells migrated more rapidly than that from either of the mutants, demonstrating that a monoglycosylated protein is trimmed efficiently by glucosidase II in the yeast ER.

Expression of Gtb1p and Gls2p—We next examined the expression of both Gls2p and Gtb1p by engineering epitope-tagged genomic alleles. In the absence of any obvious C-terminal ER retrieval sequence, three contiguous copies of the hemagglutinin epitope were added after the

FIGURE 3. Gtb1p is required for the completion of glycoprotein glucose trimming. A, wild-type (WT) and deletion mutant strains were grown in minimal medium at 30 °C and labeled with [35S]methionine/cysteine for 4 min. Tunicamycin was added to 10 mg/ml for 1 h before labeling. Immunoprecipitations were carried out on cell extracts using DPAP B-, CPY-, or Lhs1p-specific antisera. B, wild-type and Δalg8 single and double mutant strains were grown and radiolabeled as described for A, and cell extracts were subjected to immunoprecipitation with CPY-specific antiserum. The bands indicated by stars in lane 2 (Δalg8) correspond to CPY species containing (slowest to fastest migrating) 3, 4, and 2 glucose-trimmed N-glycans, respectively. The bands indicated by stars in lane 3 (Δalg8/Δgls2) correspond to species with the same number of glycans but each larger due to the failure to trim the final glucose, a pattern also evident in lane 4 (Δalg8/Δgtb1). C, single and double mutant strains were grown and radiolabeled as described for A, and cell extracts were immunoprecipitated with Lhs1p-specific antiserum. D, N-glycans prepared from Δalg1/Δmns1 (empty circles) and Δalg2/Δmns1 (filled circles) after a 4-min labeling with [3H]mannose were examined by HPLC (see “Experimental Procedures”). The positions of N-glycan signals are indicated. The signal at 7 min corresponds to monosaccharide and is probably due to the presence of free [3H]mannose carried over from the initial labeling. E, wild-type (BY4742) and Δgtb1 strains were grown in minimal medium at 30 °C and labeled with [35S]methionine/cysteine for 4 min. A cold chase was initiated by the addition of 2 mM each methionine and cysteine, and 5.0 A600 nm equivalents of cells were removed at 10-min time points. Extracts were prepared and subjected to immunoprecipitation with Lhs1p-specific antiserum. F, trimming of a protein containing a single N-linked glycan. Wild-type, Δalg1, and Δgls2 cells transformed with a low copy plasmid expressing a 42-kDa Pho8-Ura3p protein containing a single N-glycan acceptor site were grown at 30 °C in minimal medium with plasmid selection and labeled with [35S]methionine/cysteine for 4 min. The fusion protein was immunoprecipitated from cell extracts with Myc-specific antibodies, and 2.0 A600 nm equivalents were analyzed by 10% SDS-PAGE. The expression of a small amount of non-glycosylated protein is indicated by an asterisk.
Once again, the epitope was added at the extreme C terminus.
The Function of ER Glucosidase II β-Subunit

FIGURE 6. Glis2p does not require Gtb1p for its stable expression and its ER retention. A, analysis of Glis2p secretion into the growth medium. Wild-type or Δgtb1 cultures were harvested as indicated, and then whole cell protein extracts (lanes 1, 2, 5, and 6) and trichloroacetic acid-precipitated growth medium (lanes 3, 4, 7, and 8) were subjected to either endoglycosidase H digestion or mock treatment, as indicated. 1.0 A600 nm equivalent of whole cell extracts and 5.0 A600 nm equivalents of growth media were analyzed by 7.5% SDS-PAGE and immunoblotting with either anti-Myc (Fig. 3A,6-diamidino-2-phenylindole staining. Cy3 (anti-Myc), GFP, and 4',6-diamidino-2-phenylindole (DAPI) fluorescence are shown in separate panels. C, the stability of Gls2p was examined by pulse-chase analysis of strains BWY619 (gls2-myc) and BWY625 (Δgtb1/ gts-myc). Cells were labeled with 135S)methionine/cysteine for 10 min with a chase initiated by the addition of methionine and cysteine to a concentration of 2 mM. Four A600 nm equivalents of cells were harvested at each of the indicated time points and cell extracts prepared and subjected to anti-Myc denaturing immunoprecipitation. Immunoprecipitates were analyzed by 7.5% SDS-PAGE. D, The decay of Gls2p-Myc as visualized in B above was quantified and plotted against time. WT, wild-type.

noprecipitation with an anti-HA matrix. Immunoprecipitates were then analyzed by SDS-PAGE and immunoblotting with either anti-Myc (9E10) or anti-HA (12CA5) antibodies. As expected, Gtb1p-HA was immunoprecipitated from extracts derived from Gtb1p-HA- and Gtb1p-HA/Gls2p-Myc-expressing strains but not from the control strain expressing tagged Gls2p-Myc alone (Fig. 5C, upper panel). Immunoblotting with 9E10 antibodies revealed that Gls2p-Myc co-immunoprecipitated from extracts containing Gtb1p-HA but not from control extracts (Fig. 5C, lower panel, lane 4). In the reciprocal experiment, Gtb1p-HA was also co-immunoprecipitated from BWY620 membranes with 9E10 antibodies (data not shown). These results demonstrated that Gtb1p-HA and Gls2p-Myc form a stable protein complex.

Gls2p Localization Is Unaffected in Δgtb1 Cells—It has been suggested that GIIβ may contribute to the stability and/or ER retention of GIIs in mammalian cells (11, 12). We therefore further examined the expression of Gls2p in Δgtb1 cells. Defects in the retention of other ER luminal proteins results in their transport to the Golgi and subsequent secretion to the medium (34). Therefore, total cell and growth media extracts were prepared from BWY619 (gls2-myc) and BWY625 (Δgtb1/ gts-myc) and analyzed by immunoblotting. Because yeast glycoproteins may become extensively modified in transit through the Golgi (35), we also treated samples with EndoH to collapse any heterogeneous species. First, the level of Gls2p-Myc detected in cell extracts was not reduced in the absence of Gtb1p (Fig. 6A compare lanes 1 and 2 with 5 and 6), and we found no evidence of Gls2p-Myc in the culture medium from either strain (Fig. 6A). Moreover, EndoH treatment of whole cell extracts produced no evidence of any significant pool of Golgi-modified Gls2p-Myc in either wild-type or Δgtb1 cells (Fig. 6A, compare lanes 1 and 2 and lanes 5 and 6). Finally, the localization of Gls2p-Myc was confirmed by immunofluorescence. The Myc-tagged protein exhibited strong perinuclear staining with further staining at or close to the cell periphery (Fig. 6B). This same pattern was also seen with the direct fluorescence of Sec61-GFP in the same cells (Fig. 6B). These results demonstrated that Gls2p-Myc remains ER-localized independently of Gtb1p. A misfolded protein in the ER lumen would be expected to become subject to ER-associated degradation (36). We therefore examined whether Gls2p-Myc might be more rapidly degraded in the absence of Gtb1p. Our data indicated no significant difference in the turnover of Gls2p-Myc in Δgtb1 mutant cells (Fig. 6C, compare lanes 1 and 2 and lanes 5 and 6). Taken together, these data demonstrate that Gtb1p is not required for either the stability or ER retention of Gls2p-Myc. All of these data are consistent with the efficient trimming of G2 glycans observed in Δgtb1 cells (Fig. 3A), which provides compelling evidence of a functional Gls2p.
The Function of ER Glucosidase II β-Subunit

DISCUSSION

In this paper, we have reported the characterization of yeast Gtb1p, which we show to be required for the processing of G1 N-linked glycans in the ER. Gtb1p is a soluble glycoprotein localized in the ER lumen, where it interacts with Gls2p in a manner reminiscent of the heterodimeric structure of mammalian Glucosidase II (11, 12). Interestingly, neither Gls2p nor Gtb1p contain any recognizable ER retention motifs, and yet both are evidently ER-localized. It therefore follows that their localization must involve either a novel retention pathway or, perhaps more likely, an interaction with some factor that is itself retained. This contrasts with the mammalian system where GlIIβ contains an HDEL motif and where ectopic expression of a form lacking this motif leads to the co-secretion of a small percentage of GlIα (11). Of course, it may be that the yeast and mammalian proteins share a conserved retention mechanism but that the mammalian protein is augmented with an HDEL. In this case, the observed co-secretion of ectopically expressed glucosidase subunits might reflect a role for HDEL when the primary pathway becomes saturated.

Gtb1p and mammalian GlIIβ share a conserved mannose lectin (mannose-6-phosphate receptor homology) domain (see Fig. 2) (29), and it has been shown that the pentamannosyl branch present in N-glycans stimulates glucosidase II activity in vitro (38). This has led to a recent model in which the binding of GlIIβ to N-glycans would promote trans-activation of GlIα to initiate G2 processing (13). However, our data demonstrate that Gls2p is sufficient to process G2; therefore, we propose that Gtb1p contributes a second binding activity that is essential to promote G1 processing by Gls2p. This is consistent with kinetic data that indicate that the heterodimeric mammalian glucosidase II has a single active site but two distinct substrate binding affinities (11, 39). In short pulse labeling studies, we saw no evidence of any delay in G2 processing in Δgtb1 cells but could not exclude some subtle effect of Gtb1p on this reaction. In striking contrast, the G1 processing event is completely dependent upon Gtb1p, suggesting that the principal role of the β-subunit is to promote recognition and processing of monoglucosylated substrates.

Of course, monoglucosylated glycans are also the substrates for specific recognition of glycoprotein intermediates by calnexin. Evidence from in vivo studies indicates that G2 processing is rapid but that processing of the resulting G1 occurs more slowly (18, 37), and it has been noted that this would provide a kinetic window within which proteins may enter the calnexin cycle (13). S. cerevisiae contains a calnexin chaperone activity, but its role in vivo remains unclear (40, 41). It also expresses an ER protein, Kre5p (42), which is homologous to UDP-glucose:glycoprotein glucosyltransferase, but no reglucosylation activity has yet been detected either in vitro (43) or in vivo (31, 44). It is therefore unclear whether yeast has a calnexin cycle similar to that of higher cells. However, the differential kinetics of G2 and G1 processing are common to both yeast and mammals, suggesting that this is a fundamental feature of glycoprotein biogenesis in the ER. The role of this kinetic delay in G1 processing in yeast is uncertain, but our data demonstrate that Gtb1p is required to drive proteins out of the G1 state. Were this activity conserved in mammalian GlIβ, then it might be expected to play an important role in the timing of calnexin cycles. In this speculative model, GlIβ would compete with calnexin for the lectin-dependent binding of G1 substrates. Binding by GlIβ would promote GlIα-dependent processing and subsequent release of M9 forms that would either exit the calnexin cycle or re-enter following their reglucosylation. It will be of great interest to determine whether the G1-specific function of Gtb1p is conserved in mammalian GlIβ.

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6332 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 281 • NUMBER 10 • MARCH 10, 2006
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