Characterization of Yeast Yea4p, a Uridine Diphosphate-N-acetylglucosamine Transporter Localized in the Endoplasmic Reticulum and Required for Chitin Synthesis*

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Chitin is an essential cell wall component, synthesis of which is regulated throughout the cell cycle in the yeast Saccharomyces cerevisiae. We cloned an S. cerevisiae gene, YEA4, whose product is homologous to the Kluyveromyces lactis uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) transporter. An epitope-tagged Yea4p localized mainly in the 10,000 × g pellet (P2), suggesting endoplasmic reticulum (ER) localization. Membrane vesicles from the P2 fraction showed an 8-fold higher UDP-GlcNAc transport activity in cells harboring a multicopy YEA4 plasmid than in cells harboring vector alone. The activity distribution is identical with the protein distribution in P2, whether the gene is overexpressed or not, suggesting its native localization in P2. Immunolocalization of epitope-tagged Yea4p further revealed ER localization. The increase in transport activity due to the YEA4 overexpression is specific for UDP-GlcNAc, but not for UDP-galactose and GDP-mannose. yea4-disrupted cells showed a reduced rate of UDP-GlcNAc transport, contained less chitin, and were larger and rounder in shape than the wild type cells. Our results indicate that YEA4 encodes an ER-localized UDP-GlcNAc transporter that is required for cell wall chitin synthesis in S. cerevisiae.

The cell wall of Saccharomyces cerevisiae is composed of three types of polysaccharides: mannoprotein, β-glucan, and chitin. Chitin, a linear homopolymer of β-1,4-linked N-acetylglucosamine (GlcNAc) residues, is a relatively minor component, representing only 1–2% of the dry weight of the wall of vegetative cells (1). The biosynthesis of mannoprotein polysaccharides is well established; it initiates in the ER and continues in the Golgi with the transfer of additional mannose units (2). β-Glucan synthesis is believed to take place along the secretory pathway in the ER and Golgi compartments and in the plasma membrane (3–5). In contrast, chitin synthesis is a complex process and not yet well understood (6–8), although the enzymatic synthesis of chitin has been studied extensively (9). Chitin synthesis requires the activities of several different chitin synthases and many different genes for the control of these enzymes (10).

S. cerevisiae possesses three chitin synthases. Chitin synthases I and II (CSI and CSII), the products of the CHS1 and CHS2, are closely related proteins and require partial proteolysis for their activity in vitro. In contrast, chitin synthase III (CSIII), the product of CHS3 (recently renamed from CAL1/CSD2/DIT101/KT12; Ref. 3), is active without proteolytic treatment and is presumed to be the catalytic subunit of a complex containing the CHS4 (CAL2/CSD4) and CHS5 (CAL3) gene products, which are believed to play regulatory roles (10–12). The three chitin synthases have unique functions. CSI and CSII make only a small portion (<10%) of the total chitin, whereas CSIII is responsible for the remainder (>90%). Hydroxylation analysis of the Chs1p, Chs2p, and Chs3p sequences reveals the presence of putative multimembrane-spanning domains in each polypeptide (11, 13, 14). This is consistent with the idea that chitin synthases polymerize and extrude chitin chains across the plasma membrane using an intracellular source of the UDP-GlcNAc. Previous reports on chitin synthesis and the distribution of chitin synthases demonstrated the existence of chitosomes that are distinct from the plasma membrane, vacuole, and mature secretory vesicles (15, 16). It is still not clear, however, whether the chitosome is the sole reservoir of chitin synthases (6). Previous reports on chitin synthase assays suggest the importance of growth phase on the regulation of chitin synthesis, where three synthase activities can be detected in the cell lysates from the exponentially growing cells, and only CSI is detectable in lysates from stationary phase cells. These data suggest that the synthesis and/or turnover of CSII and CSIII are cell cycle-regulated (17, 18).

In this report we describe the identification and characterization of a UDP-GlcNAc transporter that is required for chitin synthesis in S. cerevisiae. This study was motivated by a glycoengineering research project aimed at manipulating the protein glycosylation system of S. cerevisiae to produce mammalian-type oligosaccharides. We previously reported the production of human-compatible high mannose type Manα3Manβ2GlcNAc2 sugar chains (19) and the expression of rat UDP-GlcNAc:α-3-N-acetylglucosaminyltransferase I (20). As a next step, we wished to evaluate the role of a UDP-GlcNAc transporter in our system toward in vivo GlcNAc addition as we had previously done for a UDP-Gal transporter toward in vivo galactose addition (21, 22). The cloning of a Golgi UDP-GlcNAc transporter gene from Kluyveromyces lactis (23) revealed the
existence of a homologue in *S. cerevisiae* with 40% identity and 70% similarity between their predicted amino acid sequences. The presence of such a gene in *S. cerevisiae* is unexpected because the sugar chains produced by this yeast lack terminal GlcNAc residues.

Here we report that the *S. cerevisiae* ORF named *YEA4* (GenBank™ accession no. U18530) is a functional gene that encodes a UDP-GlcNAc transporter localized in the ER. We have both deleted and overexpressed the gene to analyze its in vivo function. Measurements of sugar nucleotide transport activity in membrane vesicles prepared from wild type, null mutant, and overexpressed cells confirm its specificity for UDP-GlcNAc. FITC-conjugated wheat germ agglutinin (WGA) and Calcofluor white (CFW) staining showed reduced amounts of cell surface chitin in cells deleted for *YEA4*. These data support the notion that Yea4p is involved in cell wall chitin synthesis through its UDP-GlcNAc transporter activity localized in the ER membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—Materials were obtained as follows: UDP-N-acetylglucosamine (UDP-GlcNAc), UDP-glucose (UDP-Glc), UDP-mannose (UDP-Man), UDP-glucose (UDP-Glc), chitinase (Serratia marcescens), and cytolyticase (Helix pomatia) were from Sigma. UDP-[3H]GlcNAc (45 Ci/mmol), UDP-[3H]GlcNAc (200 Ci/mmol), GDP-[3H]Man (251 Ci/mmol), and UDP-[3H]Glc (17.5 Ci/mmol) were from NEN Life Science Products. UDP-[3H]Gal (7.9 Ci/mmol) was obtained from American Pharmacia Biotech. Zymolyase-100T (Kirin Brewery Co.) was obtained from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan) and Complete protease inhibitor mixture tablets from Roche Molecular Biochemicals. A BCA protein assay kit was obtained from Pierce. Filters and filtration apparatus were from Millipore (Millipore Corp., Bedford, MA). GDP was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). p-Dimethylaminobenzaldehyde and Clear-sol scintillation mixture were from Nacalai Tesque, Inc. (Kyoto, Japan). All other reagents were of the highest purity commercially available. Anti-influenza hemagglutinin epitope monoclonal antibody (anti-HA mAb) was obtained from Berkeley Antibody Co. (Richmond, CA). Alkaline phosphatase-conjugated goat anti-rabbit and goat anti-mouse IgG were purchased from Cappel (Organon Teknika Corp. West Chester, PA).

**Strains, Media, and Genetic Methods**—The Saccharomyces cerevisiae strains used in this study are listed in Table I. Strains are described by Chiba et al. (9–11) and the ORF (GenBank™ accession no. U18530) was amplified by PCR, as described by Chiba et al. (9–11). The oligonucleotide primers were: 5′-GGGATCCTAGGATGCAAGGGGAGGTAAGC-3′ and 5′-GGGCGCGGCTTTACTTTCCTTATCGCGG-3′ (single and double underlines indicate *SacI* and *NolI* sites, respectively). PCR was carried out using 2.0 μm primers and 10 ng of genomic DNA of *S. cerevisiae* strain YPH500 as a template in a 50-μl reaction mixture containing Pwo polymerase (Roche Molecular Biochemicals). The reactions were performed with 30 cycles of 95 °C for 20 s, 54 °C for 1 min, and 72 °C for 1 min.

**Sugar Nucleotide Transport Assay**—Sugar nucleotide transport was assayed as described previously (21) with some modifications. Briefly, cells were grown at 30 °C in an appropriate medium to an *A*~*oo*~ of 3.0–3.5. The cells were washed with 10 m NaCl and resuspended in the spheroplast buffer (1.4 m sorbitol, 50 mM potassium phosphate, pH 7.2, and antiprotease mixture) and transferred to a 50-ml Dounce homogenizer (Wheaton Scientific, Millville, NJ) on ice and lysed with 20 strokes of a Teflon pestle. Low speed centrifugation at 1,000 × *g* for 10 min yielded a large pellet (P1) containing unlysed cells and cell wall debris. The post centrifugation supernatant (P2) was carefully collected and then centrifuged at 10,000 × *g* for 15 min at 4 °C (Hitachi, Himac SFR20B, Japan), which yielded the P2 pellet and the S2 supernatant. The S2 fraction was centrifuged at 100,000 × *g* (Beckman ultracentrifuge L-80 Optima, in 70 Ti rotors) and the S3 supernatant was used.

**Topology and Integrity of the Vesicles**—The topology and integrity of the subcellular membrane vesicles was determined by measuring GDPase activity (5). The assay was done essentially as described by Roy et al. (21). Briefly, incubation mixture in a final volume of 0.1 ml contained enzyme (20 μg of P3 fraction; see “Subcellular Fractionation”, CaCl2 (1 μmol), with or without Triton X-100 (100 μg), GDP (0.2 μmol) and imidazole buffer, pH 7.6 (20 μmol). Incubation was for 5 min at 30 °C. The reaction was stopped by adding 10 μl of 10% (w/v) sodium dodecyl sulfate (SDS). Released inorganic phosphate was determined by the Ames method (26). The absorbance was measured at 820 nm, and the amount of inorganic phosphate released was calculated from a calibration curve using KH2PO4 as a standard. Latency of GDPase was calculated according to Castro et al. (5).

**Subcellular Fractionation**—Subcellular fractionation was done as described previously (21) with some modifications. Briefly, cells were grown at 30 °C in an appropriate medium to an *A*~*oo*~ of 3.0–3.5. The cells were washed with 10 m NaCl and resuspended in the spheroplast buffer (1.4 m sorbitol, 50 mM potassium phosphate, pH 7.2, and antiprotease mixture) and transferred to a 50-ml Dounce homogenizer (Wheaton Scientific, Millville, NJ) on ice and lysed with 20 strokes of a Teflon pestle. Low speed centrifugation at 1,000 × *g* × 10 min yielded a large pellet (P1) containing unlysed cells and cell wall debris. The post centrifugation supernatant (P2) was carefully collected and then centrifuged at 10,000 × *g* for 15 min at 4 °C (Hitachi, Himac SFR20B, Japan), which yielded the P2 pellet and the S2 supernatant. The S2 fraction was centrifuged at 100,000 × *g* (Beckman ultracentrifuge L-80 Optima, in 70 Ti rotors) and the S3 supernatant was used.

**Cell Wall Preparation and Western Blotting**—Cell walls were prepared essentially as described by Yoko-o et al. (24). Overexpression of the *YEA4* Gene—The *YEA4* ORF (GenBank™ accession no. U18530) was amplified by PCR, as described by Chiba et al. (9–11). The oligonucleotide primers were: 5′-CCGATCTATGGGATGCAAGGGGAGGTAAGC-3′ and 5′-GGGCGCGGCTTTACTTTCCTTATCGCGG-3′ (underlines indicate *SacI* and *NolI* sites, respectively). PCR was carried out using 2.0 μm primers and 10 ng of genomic DNA of *S. cerevisiae* strain YPH500 as a template in a 50-μl reaction mixture containing Pwo polymerase (Roche Molecular Biochemicals). The reactions were performed with 30 cycles of: 95 °C for 20 s, 54 °C for 1 min, and 72 °C for 1 min.

**Gel Electrophoresis—SDS-Polyacrylamide gel electrophoresis (PAGE) was conducted under reducing conditions, as described previously (27).**

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**Sugar Nucleotide Transport Assay**—Sugar nucleotide transport was assayed as described previously (21) with some modifications. Different subcellular fractions (P2 and P3, 500 μg of protein) were incubated in 100 μl of reaction mixture (20 mM Tris-Cl, pH 7.5, 0.25 μM sucrose, 5.0 mM MgCl2, 1.0 mM MnCl2, 10 mM 2-mercaptoethanol) with radiolabeled sugar nucleotides (as indicated in the figure legends). After incubation at 37 °C for 5 min, the samples were diluted with 3.0 ml of ice-cold stop buffer (20 mM Tris-Cl, pH 7.5, 0.25 μM sucrose, 150 mM KCl, 1.0 mM MgCl2) and placed on ice. Diluted samples were then applied onto a filtration apparatus (HA filters, 24-mm diameter, 0.45-μm pore size). The filters were washed with another 10 ml of ice-cold stop buffer. The filters were air-dried and placed in 15-ml counting vials, and 2.0 ml of ethylene glycol methyl ether was added. The vials were allowed to stand
at room temperature with occasional shaking until the filters dissolved (about 30 min). Ten milliliters of scintillation mixture (Clear-sol) were added, and the samples were counted in a liquid scintillation counter (LS1701; Beckman, Fullerton, CA). The amount of radioactivity bound nonspecifically to the outside of the vesicles was determined by a time 0 assay for each sample.

**Indirect Immunofluorescence Microscopy**—Preparation of cells for immunofluorescence was essentially as described by Pringle et al. (30). Briefly, cells were grown in YPD to mid-logarithmic phase (A<sub>600</sub> = 0.5–1.0) and fixed by addition of 37% (v/v) formaldehyde to 5% (v/v). The cultures were gently shaken at 25 °C for 20 min before centrifugation, and the fixed cells were washed twice with SP (100 mM potassium phosphate, pH 7.5, 1.0 M sorbitol), resuspended in SP-2ME (SP, 0.2% 2-mercaptoethanol, 15 mg/ml bovine serum albumin, 0.1% Tween 20, and 0.02% NaN<sub>3</sub>). Mouse anti-HA mAb (a generous gift from Stephan te Heesen) was used at a 1:500 dilution. Alexa 488 goat anti-mouse and Alexa 568 goat anti-rabbit (Molecular Probe, Inc., OR) IgGs were used at 1:1000 dilution. Rabbit anti-Wbp1p (a generous gift from Sean Munro) was affinity-purified by pre-absorbing with total lysate prepared from Δanp1::LEU2 cells and used at a 1:1000 dilution. Rabbit anti-Wbp1p (a generous gift from Stephan te Heesen) was used at a 1:500 dilution. Alexa 488 goat anti-mouse and Alexa 568 goat anti-rabbit (Molecular Probe, Inc., OR) IgGs were used at 1:250 dilution as secondary antibodies. DNA was stained with 25 ng/ml 4',6-diamidino-2-phenylindole (in water) and examined with an Olympus BX-FLA fluorescence microscope (Olympus, Tokyo, Japan). Images were captured with a MicroMAX CCD camera system (Roper).

**Plate Assay for Drug Sensitivities**—Drug sensitivities were tested on YPD plates supplemented with Calcofluor white (20 μg/ml) and 5–10 ng/ml 4',6-diamidino-2-phenylindole (in water) and examined with an Olympus BX-FLA fluorescence microscope (Olympus, Tokyo, Japan). Images were captured with a MicroMAX CCD camera system (Roper).

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**Chitin Measurement**—For the quantitative measurement of chitin, the amount of GlcNAC released by chitinase was measured by a colorimetric method (18). Washed cells (350–400 mg wet weight) were suspended in 4.0 ml of 6% KOH and heated at 80°C for 90 min to remove the mannan layer of the cell wall. After the alkali treatment, 400 μl of glacial acetic acid was added; then, insoluble material was isolated by centrifugation, washed twice with water, and resuspended in 1.0 ml of 100 mM sodium phosphate buffer (pH 6.3). Chitinase from Serratia marcescens (Sigma) was added (0.4 unit), and the tubes were incubated at 37°C for 1 h. A 100-μl aliquot of each sample was removed and assayed for N-acetylglucosamine by measuring A<sub>540</sub>, (31).

**RESULTS**

**Homology between S. cerevisiae Yea4p and K. lactis Mnn2–2p, a UDP-GlcNAc Transporter**—According to current dogma, there is no physiological need for S. cerevisiae cells to possess a UDP-GlcNAc transporter; S. cerevisiae cells lack the terminal GlcNAc residues in their sugar chains (2). However, we have recently reported the presence of UDP-Gal transport activity in S. cerevisiae (21), an unexpected finding given that S. cerevisiae cells do not add galactose to their N- and O-linked oligosaccharides (2). This finding stimulated us to search for an S. cerevisiae protein homologous to known UDP-GlcNAc transporters. We found that the predicted YEA4 gene product shows 40–60% amino acid similarity to the K. lactis UDP-GlcNAc transporter, encoded by the K. lactis MNN2–2 gene (Fig. 1). To test whether the YEA4 gene product has UDP-GlcNAc transport activity, we overexpressed the gene and assayed the effects on cellular UDP-GlcNAc transport activity.

**Expression and Subcellular Fractionation of Yea4–3xHAp in S. cerevisiae**—The S. cerevisiae YEA4 gene potentially encodes a UDP-GlcNAc transporter homologue. We overexpressed the gene in yeast to analyze its protein function. An expression vector carrying YEA4 under the control of the CUP1 promoter (pYEA4) and the vector alone (pYEX-BX) were introduced into YPH500 cells (Table I). Three copies of the influenza HA epitope were inserted at a position corresponding to the carboxyl terminus of Yea4p for protein surveillance (Fig. 2A).

To investigate the intracellular localization of Yea4–3xHAp, a subcellular fractionation experiment was performed according to the procedure described previously (21). Different subcellular fractions, P2 (10,000 × g pellet) and P3 (100,000 × g pellet), were analyzed by Western blot. The results indicate that Yea4–3xHAp is mainly (85%) localized in the P2 fraction together with a minor (15%) localization in the P3 fraction (Fig. 2B). Using antibodies against marker proteins for different subcellular organelles (α-Dpm1p for ER membranes and α-Anp1p for Golgi membranes), we confirmed that Yea4–3xHAp is accumulated mainly in the ER enriched fraction (P2 pellet), but not in the Golgi enriched fraction (P3 pellet) (Fig. 2B).

**Localization of Yea4–3xHAp by Indirect Immunofluorescence**—To complement the biochemical studies described above, we performed an indirect immunofluorescence experi-
The UDP-GlcNAc Transporter in S. cerevisiae

TABLE I
S. cerevisiae strains

| Strain     | Genotype                        | Reference |
|------------|---------------------------------|-----------|
| YPH 500   | MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-1Δ1 | 45        |
| SRY1      | MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-1Δ1 (pYEXBX) | This study |
| SRY2      | MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-1Δ1 (pYEXYA4) | This study |
| SRY3      | MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-1Δ yea4::His3MX6 | This study |
| SRY4      | MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-1Δ yea4::His3MX6 (pYEXYA4) | This study |

Parentheses indicate plasmid-bearing strains.

Fig. 2. Detection of HA-tagged Yea4p and its subcellular distribution in S. cerevisiae cells. A, whole cell lysates were prepared from yeast cells (YPH500) carrying the pYEX-BX vector alone (SRY1), cells carrying the expression vector with HA-tagged YEA4 (SRY2) (lanes 2 and 3). Cells were grown in the uracil dropout medium without Cu2+ (lane 2) or in presence of 1.0 mM Cu2+ (lane 3). Western blot analysis was performed with 50 μg of proteins prepared from total cell lysates and using monoclonal antibody against HA epitope, as described under “Experimental Procedures.” B, cells carrying the Yea4–3xHAp-expressing plasmid (SRY2) were converted into spheroplasts, lysed, and subjected to subcellular fractionation to yield P1, S1, P2, S2, P3, and S3 (see “Experimental Procedures”). An equivalent amount (~150 μg of protein; all S fractions were concentrated by nonfluorescence staining of Yea4p-3xHAp was specific for the antibody -HA, -Anp1p, and -Dpm1p antibodies, respectively.

Fig. 3. Subcellular localization of Yea4–3xHAp in S. cerevisiae cells by indirect immunofluorescence. Yeast cells were analyzed by indirect immunofluorescence. Cells expressing Yea4–3xHAp (SRY2) were prepared for immunofluorescence, and antibody staining was performed as described under “Experimental Procedures.” A, cells containing epitope (HA)-tagged YEA4 plasmid (SRY2), probed with monoclonal anti-HA (α-HA). B, cells containing HA-tagged YEA4 plasmid (SRY2), probed with rabbit α-Wbp1p (antibody was used as an ER marker). C, cells containing vector alone (SRY1), probed with monoclonal anti-HA (α-HA, vector alone). D, cells containing HA-tagged YEA4 plasmid (SRY2), probed with rabbit α-Anp1p (antibody was used as a Golgi marker). All magnifications, ×100.

mAb (Fig. 3C). The Yea4–3xHAp-expressing cells displayed a hallmark pattern of ER localization (Fig. 3A), distinctly different from a punctate staining, which is typical of Golgi staining (Fig. 3D). 4’,6-Diamidino-2-phenylindole staining of nuclei also supported the ER localization of YEA4 gene product with Yea4–3xHAp localized to the nuclear periphery (data not shown).

Transport of Sugar Nucleotides into Membrane Vesicles—We next wished to determine whether the ER-localized Yea4p functions as a UDP-GlcNAc transporter. For these experiments we chose a biochemical approach, assaying in vitro UDP-GlcNAc transport activities in P2 and P3 membrane vesicles. The intactness and topology of these vesicles were confirmed by measuring luminal marker enzyme activities in the presence or absence of Triton X-100, according to Castro et al. (5). This control indicated that at least 95% of the vesicles were intact, maintaining the same topology as in vivo (data not shown).

To investigate the in vivo role of YEA4, the gene was disrupted by the PCR-based gene deletion method (see “Experimental Procedures”). The sugar nucleotide transport activities were measured by P2 and P3 membrane vesicles prepared from...
wild type, yea4 null mutant (Δyea4), and Yea4–3xHAp-overexpressing cells. The transport activity was standardized on the basis of (i) protein concentration (Fig. 4A), (ii) incubation time (Fig. 4B), (iii) substrate concentration (Fig. 4C), and (iv) incubation temperature (data not shown). In Yea4–3xHAp-expressing cells (Fig. 5A), most of the UDP-GlcNAc transport activity (85% of total) was detected in the P2 fraction, and only a minor amount (15%) was detected in the P3 fraction. UDP-GlcNAc transport activity was 8.3-fold higher in the P2 membrane vesicles prepared from Yea4–3xHAp-expressing wild type cells than in the same vesicles derived from wild type cells harboring vector alone. The overall distribution of UDP-GlcNAc transport activity between P2 and P3 fractions was approximately the same in the Yea4–3xHAp-overexpressing and vector-only cells (P2:P3 ratio; 5.2-fold versus 4.1-fold), respectively, as shown in Fig. 5A. These results strongly suggest that the Yea4–3xHAp localization in the ER is not due to the overexpression of misfolded proteins, but rather due to the reflection of native Yea4p localization. As controls, we measured the activity of two other nucleotide sugar transporters in Yea4–3xHAp-overexpressing and vector only cells. UDP-Gal transport activity remained the same in P2 and P3 membrane vesicles (Fig. 5B), as did GDP-Man transport (120 pmol/mg/5 min) in P3 membrane vesicles.

In the case of membrane vesicles prepared from the Δyea4 cells, the UDP-GlcNAc transport activity was reduced compared with that of wild type cells (vector alone) in both P2 and P3 fractions (Fig. 5A), while the transport of other sugar nucleotides remained unchanged as mentioned above. These data indicate that the Yea4p functions only in UDP-GlcNAc transport activity, and not in the transport of other sugar nucleotides. The rate of UDP-GlcNAc transport activity in the P2 fraction was dependent on the incubation temperature (the rate of transport at 4 °C is about 15% of that obtained at 30 °C), and inhibited in the presence of Triton X-100 (data not shown).

The Vₘₐₓ value of UDP-GlcNAc is 88 pmol/mg/5 min, and apparent Kₘ is about 6.0 μM (see Fig. 4C).

The YEA4 Gene Is Required for Chitin Synthesis—Under the microscope, the Δyea4 cells are larger and rounder than wild type cells (Fig. 6, D and H). The Δyea4 and wild type cells did not show any apparent differences in staining with FITC-conjugated concanavalin A, a mannose-specific lectin (Fig. 6, A and E), consistent with the biochemical results that Δyea4 cells are normal for GDP-mannose transport activity. Cell surface staining with FITC-conjugated WGA, a GlcNAc-specific lectin for yeast, revealed less staining in Δyea4 cells than in wild type cells (Fig. 6, C and G), indicating less chitin content at the cell surface of the Δyea4 cells. A similar difference was found when both cells were stained with CFW (Fig. 6, B and F), a fluorescent dye that binds to chitin microfibrils (12). These data indicate that Yea4p is required for chitin synthesis in S. cerevisiae.

UDP-GlcNAc Transport Activity by Yea4p Is Involved in Chitin Synthesis in Vivo—Sensitivity to the fluorescent dye CFW (10 μg/ml), vanadate (2 mM), and the antibiotic neomycin (5 mM) was examined. The results show that Δyea4 cells are resistant to all the drugs relative to wild type cells (Fig. 7), suggesting reduced chitin content and an abnormal cell wall in the Δyea4 cells. We also compared wild type cells, Δyea4 cells, and Δyea4 cells harboring a YEA4-3xHA-containing plasmid by the staining with either CFW or FITC-conjugated WGA. The staining defect in the Δyea4 cells was complemented by the YEA4 plasmid (Fig. 7, D and E).

We also assayed the UDP-GlcNAc transport activity in Δyea4 cells expressing Yea4–3xHAp. The overexpression of YEA4-3xHA in the Δyea4 strain partially recovered the transport activity compared with the wild type strain with the same expression plasmid (Fig. 5A).

Finally, we biochemically determined cellular chitin content and found that it was reduced in Δyea4 cells (~65%) relative to the wild type (Table II). The overexpression of YEA4-3xHA in the Δyea4 strain caused a significant recovery of chitin content (to ~83%) compared with the wild type cells with the same expression plasmid (Table II). These data support a role for the YEA4 gene in chitin synthesis.

DISCUSSION

In our efforts to synthesize hybrid and complex-type sugar chains from high mannose type sugar chains (19), we are interested in GlcNAc incorporation into sugar chains by expressing N-acetylglucosaminyltransferase (GnT) in S. cerevisiae (20). For that purpose, it is necessary to have a UDP-GlcNAc transporter to translocate UDP-GlcNAc from cytosol to lumen in S. cerevisiae. During our search for a homologue of K. lactis...
UDP-GlcNAc transporter in *S. cerevisiae*, we found a homologous gene YEA4, showing 40% identity and 70% similarity of amino acid sequences (Fig. 1). We therefore tested the possibility that its gene product is UDP-GlcNAc transporter. Here we demonstrate that in *S. cerevisiae* UDP-GlcNAc is transported into the ER lumen. This transport is saturable and shows the apparent $K_m$ values comparable to those measured for the *K. lactis* Golgi-localized UDP-GlcNAc transporter (23) and for the rat liver ER-localized UDP-GlcNAc transporter (34). Overexpression of YEA4 leads to a dramatic increase in UDP-GlcNAc transport activity, which is associated mainly with the ER-enriched pellet fraction (P2) (Fig. 5A). The overall distribution ratio of UDP-GlcNAc transport activity between P2 and P3 fractions remain unchanged in wild type and Δyea4 cells (Fig. 5A). These results indicate that the overexpression of YEA4-3xHA fusion gene does not affect intracellular localization of Yea4p. Indirect immunofluorescence studies and cell fractionation experiments demonstrate that Yea4 protein is localized in the ER.

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To assess the possible biological role of this UDP-GlcNAc transporter in the ER membranes, we constructed and analyzed a yeast strain deleted for YEA4. The null mutant cells showed less UDP-GlcNAc transport activity in both P2 and P3 fractions compared with the wild type, but still contained some remaining activity (Fig. 5A). There are two possibilities; one is that another UDP-GlcNAc transporter may exist, and the other is that another sugar nucleotide transporter may have a broad substrate specificity capable of transporting UDP-GlcNAc. The deletion strain showed greatly reduced, but not a complete lack of, chitin content at the cell surface, as measured by staining with CFW and WGA-FITC and by biochemical quantitation. This is consistent with the transport results (Fig. 5A), suggesting the presence of additional functional genes for UDP-GlcNAc transport. In our homology search, YPL244c gene shows 18.6% identity with YEA4 gene. We have checked the transport activity by either overexpressing or deleting the YPL244c gene. However, we have found no effect of this gene on UDP-GlcNAc transport in both cases. Thus, at present we have not identified the genes responsible for the UDP-GlcNAc transport activity remaining in Δyea4 cells. The estimated gene may not have any apparent homologies with the known UDP-GlcNAc

2 M. Kainuma, unpublished data.
transporters, such as YE44 or MNN2–2. The deletion strain also showed a Calcofluor white-resistant phenotype (Fig. 7A), which is typical characteristics of chitin-deficient mutants (12). Even though the process of chitin synthesis is not yet completely understood in S. cerevisiae, the present hypothesis on chitin synthesis does not refer any requirements of UDP-GlcNAc transporter in the ER during its biosynthetic process (2, 10).

Null mutant cells carrying the YE44 plasmid recovered partially the staining with CFW and FITC-WGA compared with wild type cells (Fig. 7, D and E), and the transport activity also recovered partially when compared with the level of YE44-overexpressing wild type cells (Fig. 5A). It is noteworthy that YE44-3xHA-expressing null mutant cells (SRY4) fail to recover full activity compared with that of YE44-overexpressing wild type cells (SRY2) (Fig. 5A). A similar observation was reported for GDP-Man transporter encoded by VRG4 in S. cerevisiae, indicating a partial recovery of transport activity in vrg4–2 mutant cells by the transformation of a single copy of VRG4 (35). Even though both cases are different in the presence or absence of native forms of transporter protein, these results may suggest that the sugar nucleotide transporter forms a dimer or oligomer, as reported for the mammalian Golgi-localized adenosine 3′-phosphate 5′-phosphosulfate transporter (36). In any case, the complementation of chitin-specific cell surface staining and cell surface chitin content, together with that of UDP-GlcNAc transport activity, in Δyea4 cells by YE44-3xHA further supports the involvement of Yea4p in cell wall chitin synthesis.

In S. cerevisiae, chitin plays an important role in cell division. A ring of chitin is detected at the base of the emerging bud; then, after a nuclear migration and cytokinesis, this ring is filled in to form a disc or primary septum that separates mother and daughter cells (37). Three chitin synthase activities (CSI, CSII, and CSIII) have been identified in S. cerevisiae (10, 17, 18, 37). All three enzymes are membrane-associated and catalyze the synthesis of chitin chains from UDP-GlcNAc. It has been reported that Δyea4 cells are viable, but show a decreased mating efficiency compared with the isogenic wild type cells (38). The null mutants showed the additive phenotype, indicating that the diploid cells lacking both YEA4 and CHS3 were not able to complete sporulation (38). Chitin synthase 3 encoded by CHS3 is a major, if not the only, chitinase activity that is measured in yeast membranes without proteolytic activation (17, 39). The result suggests some genetic interaction between YEA4 and CHS3; however, a variety of mutations affecting the synthesis of the vegetative cell wall components cause a defect in mating and sporulation (40). CHS3 expression is elevated during sporulation (41), while the other component Chs4p/Csd4p is required for chitin synthesis in vegetative cells. However, a Chs4p/Csd4p homologue exists which is typical characteristics of chitin-deficient mutants (12). Our study demonstrates that the UDP-Glc-NAc transporter is involved in the process of chitin synthesis in S. cerevisiae (42). In a newly proposed mechanism, the export of Chs3p from endoplasmic reticulum is regulated by Cha7p. It has been concluded that in vivo activity of Chs3p depends on the level of Cha7p, although the exact role of Cha7p in the export of Chs3p is still not clear. Additional study will be necessary to address the relationship between YEA4 and other genes involved in chitin and cell wall synthesis, for instance, by constructing a series of double mutants to analyze their cell wall-related phenotypes.

The cytoplasm is the sole site of sugar nucleotide synthesis, and sugar nucleotides must be transported into various organelles in which they are utilized as a donor substrate for sugar chain synthesis. To date a number of sugar nucleotide transport activities have been reported in S. cerevisiae, specific for GDP-Man (43), UDP-Gal (21), and UDP-glucose (UDP-Glc) (5). Among these, the transport of GDP-Man has been well characterized (43, 44). A recent study strongly suggests that a Golgi GDP-Man transporter is encoded by VRG4, which is the sole gene so far identified for a sugar nucleotide transporter in S. cerevisiae (35). Our study demonstrates that the UDP-GlcNAc transporter, which is encoded by YEA4 and is localized in the ER, is involved in cell wall chitin synthesis of S. cerevisiae. Castro et al. (5) suggested the possibility that the ER-localized UDP-Glc transporter may be involved in cell wall β-1,6-glucan synthesis in S. cerevisiae, but there is no direct experimental evidence to support the hypothesis. Our study presents the first direct evidence that the sugar nucleotide transporter is involved in cell wall polysaccharide synthesis in eukaryotic cells.

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**Table II**

| Strain | Chitin content in the yeast cells | Ratio |
|--------|----------------------------------|-------|
|        | µg/mg wet cells | %       |
| SRY1 (vector alone) | 0.72 | 72 |
| SRY3 (null) | 0.47 | 47 |
| SRY4 (overexpressed (null)) | 0.60 | 60 |
| S. pombe (wild type) | 0.20 | 20 |

* The amounts of chitin are normalized to the wet weights of the cells. Bulawa (11) reported the chitin content as ~1.3 µg/mg wet cells for S. cerevisiae (wild type) cells.

**Fig. 7.** Drug sensitivities of Δyea4 and wild type cells. Cells of congenic strains, wild type (YPH 500) and Δyea4 (SRY3) were spotted on YPD plates containing 20 µg/ml CFW (A), 2.0 mM vanadate (B), and 5.0 mM neomycin (C). D and E, null mutant (Δyea4) harboring YE44-3xHA plasmid (SRY4). D, cells were stained with CFW. E, cells were stained with WGA-FITC.

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Characterization of Yeast Yea4p, a Uridine Diphosphate-N-acetylglucosamine Transporter Localized in the Endoplasmic Reticulum and Required for Chitin Synthesis

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