Nucleoside Diphosphatase and Glycosyltransferase Activities Can Localize to Different Subcellular Compartments in Schizosaccharomyces pombe*

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Nucleoside diphosphates generated by glycosyltransferases in the fungal, plant, and mammalian cell secretory pathways are converted into monophosphates by nucleoside diphosphatase enzymes in the ER compartment to relieve inhibition of the transferring enzymes and provide substrates for antiport transport systems by which the entrance of nucleotide sugars from the cytosol into the secretory pathway lumen is coupled to the exit of nucleoside monophosphates. Analysis of the yeast Schizosaccharomyces pombe genome revealed that it encodes two enzymes with potential nucleoside diphosphatase activity, Spgda1p and Spynd1p. Characterization of the overexpressed enzymes showed that Spgda1p is a GDPase/UDPase, whereas Spynd1p is an apyrase because it hydrolyzes both nucleoside tri and diphosphates. Subcellular fractionation showed that both activities localize to the Golgi. Individual disruption of their encoding genes did not affect cell viability, but disruption of both genes was synthetically lethal. Disruption of Spgda1p did not affect Golgi N- or O-glycosylation, whereas disruption of Spynd1p affected Golgi N-mannosylation but not O-mannosylation. Although no nucleoside diphosphatase activity was detected in the endoplasmic reticulum (ER), N-glycosylation mediated by the UDP-Glc:glycoprotein glucosyltransferase (GT) was not severely impaired in mutants because first, no ER accumulation of misfolded glycoproteins occurred as revealed by the absence of induction of BiP mRNA, and second, in vivo GT-dependent glycosylation monitored by incorporation of labeled Glc into glycoproteins showed a partial (35–50%) decrease in Spgda1p but was not affected in Spynd1 mutants. Results show that, contrary to what has been assumed to date for eukaryotic cells, in S. pombe nucleoside diphosphatases and glycosyltransferase activities can localize to different subcellular compartments. It is tentatively suggested that ER–Golgi vesicle transport might be involved in nucleoside diphosphate hydrolysis.

Almost all nucleotide sugar-dependent glycosyltransferases in the secretory pathway generate nucleoside diphosphates that are converted into monophosphates to relieve inhibition of the transferring enzymes and provide substrates for antiport transport systems by which the entrance of nucleotide sugars from the cytosol into the lumen of the secretory pathway is coupled to the exit of nucleoside monophosphates (1). Several secretory pathway nucleoside diphosphatases have been described already. There are two enzymes displaying such enzymatic activity in Saccharomyces cerevisiae, a GDPase/UDPase and an apyrase (denominated Gda1p and Ynd1p, respectively) (2–4). Apyrases differ from nucleoside diphosphatases, as the former are able to degrade not only nucleoside diphosphates but also triphosphates. Both enzymatic activities are localized to the Golgi apparatus. Irrespective of their cellular origin, all enzymes able to hydrolyze nucleoside diphosphates (nucleoside diphosphatases proper and apyrases) share four highly similar sequences that have been called apyrase conserved regions. Analysis of the S. cerevisiae genome showed that Gda1p and Ynd1p were the only enzymes displaying those sequences (4). Three nucleoside diphosphatases have been described to date in the mammalian cell secretory pathway, i.e. a Golgi apyrase and two (soluble and membrane bound) endoplasmic reticulum (ER) GDPase/UDPases (5–7). The ER soluble enzyme is functional with Ca$^{2+}$, Mg$^{2+}$, or Mn$^{2+}$ whereas the insoluble enzyme strictly requires the first cation for activity.

The initial in vitro characterization of nucleoside monophosphatases as the metabolites involved in the antiport transport of nucleotide sugars (8) received firm support when it was shown that ablation of the GDA1 or YND1 genes in S. cerevisiae affected both N- and O-protein glycosylation and glycolipid formation (3, 9). In the case of GDA1 disruption, it was shown that the effects observed were a consequence of a reduced entrance rate of GDP-Man into the Golgi lumen (10). Although no evidence was provided, it was assumed that mammalian cell ER nucleoside diphosphatases were required for hydrolysis of UDP generated by the UDP-Glc:glycoprotein glucosyltransferase (GT), an enzyme involved in the quality control of glycoprotein folding (11). In addition to this ubiquitous enzyme (S. cerevisiae is the only eukaryotic cell known so far to be devoid of GT; Refs. 12 and 13), the other nucleotide sugar-dependent glycosyltransferases already described in the ER lumen are the glucuronomethyltransferases that also generate UDP but only occur in certain high eukaryote tissues (14). UDP-Glc- and UDP-GlcpUA-specific transporters have already been described in the rat liver cell ER (15, 16). In the case of UDP-Glc, it was determined that the exit of UMP was coupled to entrance of the nucleotide sugar (15).
The presence of nucleoside diphosphatase and nucleotide sugar-dependent glycosyltransferase activities in the mammalian cell ER and Golgi compartments and the exclusive presence of the former activity in the S. cerevisiae Golgi, that is, in the only secretory pathway compartment in which nucleoside diphosphatases are known to occur in this yeast, supported the notion that the presence of glycosyltransferase-generated nucleoside diphosphates in a subcellular compartment probably implied the presence of an enzyme able to hydrolyze them in the same compartment. To further study the influence of ER nucleoside diphosphatase on GT-mediated glycoprotein folding, we chose Schizosaccharomyces pombe as model system for the ease by which this microorganism can be genetically manipulated and the well documented presence in it of an ER GT involved the quality control of glycoprotein folding (11). On the characterization of S. pombe nucleoside diphosphatases we made the unexpected observation that this yeast, like S. cerevisiae, only expresses two Golgi-located nucleoside diphosphatase activities, thus demonstrating that, contrary to what has been assumed so far, nucleoside diphosphatase and glycosyltransferase activities can localize to different subcellular compartments.

EXPERIMENTAL PROCEDURES

Materials—[14C]Glucose (301 Ci/mmol) was from New England Nuclear. Nucleoside tri, di, and monophosphates, protease inhibitors, supplements for culture media, lysing enzyme, 1-deoxynojirimycin, mannan, and endo-p-N-acetylgalosaminidase H (Endo H) were from Sigma. Restriction enzymes and other enzymes used for DNA procedures were from New England Biolabs. Vector pGEMT-Easy was from Promega. The Pwo polymerase and digoxigenin (DIG DNA labeling and detection kit) were from Roche Applied Science. Zymolyase 100T from Seikagaku Kogyo Co.

Strains and Culture Media—Cloning procedures were performed in Escherichia coli DH10a. The S. pombe strains were Sp61 (h−, ade1, ade6-M210, leu1-32, ura4-D18), Adp (h+, ade6-M216, leu1-32, ura4-D18), and Adm (the same as Adp but h−). The rich medium contained 0.5% yeast extract (Difco), 3% glucose, and 75 mg/liter adenine. The minimal medium was as described (17), supplemented with adenine (75 mg/liter), uracil (75 mg/liter), and leucine (250 mg/liter). Malt extract medium was used for conjugations (18). The S. cerevisiae strain was G2-7 (Mata, ura3-52, lys2, ade2, his3, trpl, leu2, gda1::LEU2), kindly provided by C. Abeijon (Department of Molecular Cell Biology, Boston University). S. cerevisiae cells were grown in rich medium (1% yeast extract, 2% bactopeptone (Difco), and 2% glucose) or in selective medium (0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, supplemented with 30 mg/liter adenine, tryptophan, histidine, and lysine).

N-Glycan Labeling—For assessing the Golgi extension, cells in the exponential growth phase were harvested, extensively washed with 1% yeast nitrogen base (Difco), resuspended in the same medium, and endo-p-N-glycans, see Ref. 12.

Characterization of Spyd1 Mutants—Colony PCR reactions were performed on water-resuspended cells using a primer homologous to a putative gene marker (GenBank accession number NP_588201) (1719 bp) was 5′-ACGCGGATCCATG-GTTGGAAATGTTGA-3′ and 5′-TCCGGAGATTCTAAAGGAGG-GTG-3′ (primer c11E10a-EcoRI). The restriction enzymes employed were BamHI and EcoRI. Primers used for colony analysis were 5′-CTGTCGCCGTCTCTGATTGG-3′ (from the vector) and 5′-AATAATG-TGACCGCTTTGCG-3′ (c11E10a internal primer 1′). Disruption of Spyd1Δ—A 1196-bp internal fragment was synthesized using genomic DNA as the template and primers 5′-TGAATGAT-CAGTTGCTTGTTGCC-3′ and 824A1. The fragment was cloned in the pGEMT-Easy vector and sequenced. The ura4 gene marker (GenBank accession number M36504) was introduced in position 738 of the 1196-bp fragment in the vector (HindIII site). Digestion with NolI liberated a 2960-bp fragment containing the ura4 gene flankned by 738 bp (5′) and 436 bp (3′) of Spyd1Δ. The fragment was transfected into S. pombe Sp61 cells, and transformants were selected in minimal medium supplemented with adenine and leucine. The mutant genotype was h−, ade1, ade6-M210, leu1-32, ura4-D18, Spyd1Δ::ura4Δ. 5′-GATGCTGGTTCC-3′ primer 5′-AAATTTCATATGATAGAGCCTGGCT-3′ was used to amplify the Spyd1Δ gene as probes. Both probes were labeled with digoxigenin. The expected fragments of 1274- and 3038-bp for wild type and mutant cells were obtained.

Characterization of Spyd1 Mutants—Colony PCR reactions were performed on water-resuspended cells using a primer homologous to a portion of Spyd1Δ outside the fragment used for gene disruption (primer c11E10a- EcoRI) and a primer specific for ura4Δ (5′-TGCTC-CTCAACACATTACC-3′). Positive colonies displaying the expected 1309-bp band were submitted to Southern blotting analysis. For this purpose, genomic DNA was digested with Mspl and run on 1.2% agarose gels. The mutant genotypes were h−, ade6-M216, leu1-32, ura4-D18, Spyd1Δ::ura4Δ and h−, ade6-M210, leu1-32, ura4D-18, Spyd1Δ::ura4Δ.

Characterization of alg6 Mutants—alg6 mutation construction was performed as described previously for the Spyd1 strain (which is h−) but with strains Adp and Sp61 (20). The construction was characterized by Southern blotting analysis as described previously (20). The mutant genotypes were h−, ade1-h−, ade6-M210, leu1-32, ura4-D18, Spyd1Δ::ura4Δ and h−, ade6-M216, leu1-32, ura4-D18, Spyd1Δ::ura4Δ.
Similarity indexes of nucleoside diphosphatases were obtained by pairing alignment using the Lippman-Pearson method (Align, DNASTar program).

| Species          | Spgda1p (GDPase/UDPase) | Spynd1p (apyrase) |
|------------------|-------------------------|------------------|
| S. pombe        | 25                      | 18.1             |
| S. cerevisiae Gda1p | 49.4                   | 40.7             |
| S. cerevisiae Ynd1p (apyrase) | 24                   | 40.7             |
| Mammalian ER GDPase/UDPase (soluble) | 41.3       | 28.4             |
| Mammalian ER GDPase/UDPase (membrane bound) | 31.0           | 22.8             |
| Mammalian Golgi apyrase | 24.2               | 23.1             |

**Fig. 1. Characterization of Spgda1p and Spynd1p enzymatic activities.**

**Spgda1** and **Spynd1** were expressed in *S. cerevisiae* Δgda1 mutants. Nucleoside tri, di, and monophosphatase activities were determined in microsomal fractions. For further details see “Experimental Procedures.”

**Fig. 2. Cation dependence of expressed Spgda1p and Spynd1p.**

For further details see “Experimental Procedures.”

**TABLE I**

Percent similarity of nucleoside diphosphatase sequences

% similarity of nucleoside diphosphatase sequences were obtained by pairwise alignment using the Lippman-Pearson method (Align, DNAStar program).

**Fig. 2. Cation dependence of expressed Spgda1p and Spynd1p.**

For further details see “Experimental Procedures.”

**GACGTTTAC-3′** and **5′-CGAATGTTGGTAGTGATGT-3′**, which amplify 800- and 2564-bp bands for the wild type gene and the *alg6::ura4* insert, respectively. For the gene **Spgda1**, the primers used were UDPaseM1 and 824a1, which yield 1104- and 2868-bp bands for the wild type gene and the Spgda1::ura4 insertion, respectively. For the gene **Spynd1**, the primers used were c11E10s and c11E10a-EcoRI, which yield 1492- and 2825-bp fragments for the wild type and the Spynd1::ura4 insertion, respectively. For mating type determination, the primers MT1 (5′-AGAGAGAGAGTAGTAGTAAG-3′), MP (5′-ACGGTATCAGTTGCTGATGT-3′), and MM (5′-TAGCTCTAGTAGCCCTAGGTTGC-3′) were used. They amplify the 728-bp bands in *h*+ strains (MT1/MM) and the 987-bp bands in *h−* strains (MT1/MP), respectively. Germinated spores were grown in rich medium without adenine for determination of the adenine genotype.

**Failed Attempts of Spgda1/Spynd1 Double Mutant Construction—**

Strains *h−*, *ade1*, *ade6-M210*, leu1-32, *ura4-D18*, Spgda1::ura4, and *h−*, *ade6-M216*, leu1-32, *ura4-D18*, Spynd1::ura4 were conjugated and sporulated. The colonies yielded by 24 tetrads were analyzed by colony PCR using above indicated primers that yield differential length fragments for Spgda1*, Spgda1::ura4*, Spynd1*, and Spynd1::ura4* as well as the ability of individual spores to grow in the absence of uracil (the original strains were *ura4−*, and the *ura4−* gene was used to disrupt both Spgda1* and Spynd1*). 

**Enzymatic Assays—**

*S. pombe* and *S. cerevisiae* microsomes were prepared as described previously (12). Nucleoside phosphatase activities were essentially assayed as described with slight modifications (21). Briefly, between 10 and 50 µg of membrane proteins were incubated in a total volume of 100 µl in 0.2 M imidazole buffer, pH 7.2, 0.1% digitonin, 10 mM CaCl₂, 2 mM the corresponding nucleoside phosphate, and incubated for 5–10 min at 30 °C. Reactions were stopped on the addition of 100 µl of 10% SDS and 100 µl of water. Liberated phosphate was assayed as described (22), employing 15-min incubations at 45 °C. Phosphates present in microsomes and reagents or liberated during incubations at 45 °C were estimated for each tube with blank in which the SDS was added before the membrane fractions. Glucosidase II was assayed using [*3H]*GlcMan;GlcNAc, (23) or p-nitrophenylglucoside (24) as substrates, depending on whether microsomes or sucrose gradient fractions were the enzyme source. Galectosyltransferase was
assayed in a total volume of 50 μl containing 0.1 M HEPES buffer, pH 7.2, 1.2 mM MnCl₂, 0.2% Triton X-100, UDP-[¹⁴C]Gal (300 Ci/mol), 2 mg of S. cerevisiae mannan, and 10 μl of gradient fraction. After 60 min at 30 °C, a label soluble in 10% trichloroacetic acid but insoluble in 66% methanol containing 0.1 M LiCl was quantified. Values obtained on incubations in which mannan was added after stopping the reactions were subtracted. OT was assayed with denatured thyroglobulin as the acceptor substrate as described previously (25).

Subcellular Fractionation—Subcellular fractionation was performed as described (26, 27) with some modifications. Mutant cells (∼4 g) grown in rich medium to an OD₆₀₀ of 2 were harvested, washed with 10 mM sodium azide, and resuspended in 50 mM Tris-Cl buffer, pH 7.5, 1.4 M sorbitol, 10 mM sodium azide, and 40 mM 2-mercaptoethanol. Lysing enzyme and zymolyase 100T were then added to a final concentration of 0.5 mg/ml each. Conversion into spheroplasts was performed at 37 °C and was monitored by the decrease in absorbance at 600 nm after a one-hundredth dilution in water. The reaction was stopped by the addition of 1 mM EDTA after ∼75% of cells had been converted into

spheroplasts. The latter were purified by a 12-min centrifugation through a 1.8 M sorbitol cushion. The spheroplasts were resuspended in a hypotonic buffer (15 mM triethanolamine-acetate buffer, pH 7.2, 0.3 M sorbitol, and 1 mM EDTA containing 10 μM leupeptin, 1 μM pepstatin, 1 μM phenylmethylsulfonyl fluoride, 1 μM tosylphenylanilanyl chloromethyl ketone, 1 μM E-64, and 0.5 mM tosyl-lysine chloromethyl ketone), lysed by several passages through a serological pipette, and homogenized with 15 strokes in a Wheaton B (Wheaton Scientific) homogenizer. Non-lysed cells were removed by centrifugation (450 × g for 3 min). Each of two 1.5-ml aliquots of the post-nuclear supernatant (S₄₀₀) was poured onto a gradient having 1 ml of 14, 18, 22, 26, 30, 34, 38, 40, 42, 46, and 50% sucrose concentrations (for the Spgdal mutant the 42% sucrose fraction was omitted) in 15 mM triethanolamine-acetate buffer, pH 7.2, 1 mM EDTA, and 3 mM MgCl₂. Tubes were centrifuged for 2.5 h at 185,000 × g in a swinging bucket rotor. Fractions were collected from the top (the first fraction had 1.5 ml, and successive ones had 1 ml). Materials in the first fractions and pellets were discarded. Sucrose concentrations were determined with a refractometer, and fractions having similar concentrations in both gradients were pooled, 5-fold diluted with 20 mM imidazole buffer, pH 7.5, 0.25 M sucrose, and 1 mM EDTA, and centrifuged for 1 h at 100,000 × g. The pellets were resuspended in 0.2 ml of the last buffer containing the above indicated protease inhibitor concentrations. Supernatants (S₄₀₀) were also centrifuged for 1 h at 100,000 × g and resuspended as the gradient fraction pellets to determine total enzymatic activities. The latter as well as the protein concentrations were determined in all fractions.

RESULTS

Analysis of S. pombe Genome—BLAST analysis of proteins encoded in the recently completed S. pombe genome, using as probes sequences of S. cerevisiae Gda1p or Ynd1p, mammalian soluble ER GDPase/UDPase, mammalian membrane-bound ER Ca²⁺-dependent GDPase/UDPase, human Golgi apyrase (respectively GenBank™ accession numbers NP_010872, AAF17573, CAB45533, CAC85467 and AAC17217), or the protein portions encompassing the four so-called apyrase conserved regions in the three first enzymes, showed that the fission yeast genome codes for the two proteins with putative nucleoside diphosphatase activity (GenBank™ accession numbers NP_593447 and NP_588201) were the same as that of S. cerevisiae. The first sequence corresponds to a 556-amino acid protein with a molecular mass of 61.6 kDa. The protein displays a signal peptide, one potential N-glycosylation consensus sequence, the four canonical apyrase conserved regions, and, as with S. cerevisiae Gda1p, no other potential transmembrane region besides the signal peptide. The second sequence corresponds to a 572-amino acid protein with a molecular mass of 64.6 kDa. This protein does not display a signal peptide but does have a potential transmembrane region close to the C terminus, thus suggesting a type I membrane protein. These same features are shared by S. cerevisiae Ynd1p. The second putative S. pombe nucleoside diphosphatase displays the four apyrase conserved regions and three potential N-glycosylation
consensus sequences. Proteins coded by sequences of NP_593447 and NP_588201 will be referred to as Spgda1p and Spynd1p, respectively, because of their respective similarity to the *S. cerevisiae* enzymes. Percent similarities of the above mentioned proteins are shown in Table I.

Characterization of Putative *S. pombe* Nucleoside Diphosphatase Activities—Genes coding for Spgda1p and Spynd1p were transfected and overexpressed in *S. cerevisiae* mutants. Figs. 1, A and B and 2, A and B show the nucleoside diphosphate specificity and cation requirements of Spgda1p and Spynd1p. The first enzyme is essentially a GDPase/UDPase, whereas the second one is an apyrase as it hydrolyzes not only nucleoside diphosphates but also triphosphates. In addition, no marked specificity toward the base was observed in Spynd1p. Both enzymes required a bivalent cation for activity (Ca²⁺, Mg²⁺, or Mn²⁺). Results show that Spgda1p and Spynd1p closely resemble *S. cerevisiae* Gda1p and Ynd1p, respectively.

Disruption of Spgda1p- and Spynd1p-encoding Genes—Genes were disrupted as indicated under “Experimental Pro-
The patterns of glycans released from whole cell glycoproteins by Endo H are shown in Figs. 5, A–C. Whereas the ablation of Spgda1<R sup 1< /sup > did not affect Golgi extension, that of Spynd1<R sup 1< /sup > diminished formation of the larger compounds. Percentages of label in specific ER N-glycans (Man<sub>9</sub>GlcNAc<sub>2</sub> and Man<sub>8</sub>GlcNAc<sub>2</sub>) were 34, 33, and 53% in wild type, Spgda<sub>1</sub>, and Spynd<sub>1</sub> cells, respectively, the rest corresponding to Golgi-elongated glycans. Evidently, more Man<sub>9</sub>GlcNAc<sub>2</sub> and Man<sub>8</sub>GlcNAc<sub>2</sub> species traversed the Golgi without being elongated in Spynd<sub>1</sub> cells than in the other two strains. Because different aliquot volumes of samples were run on Fig. 5, A–C, total amounts of N-glycans synthesized in different strains may not be estimated from total label values in runs shown in those figures, although for each sample a valid calculation of percentages of ER- and Golgi specific N-glycans can be performed. The fact that ablation of the main Golgi GDPase/UDPase activity-encoding gene did not affect Golgi elongation, whereas absence of the minor activity had a limited but detectable effect, is puzzling but not surprising. For instance, disruption of GDA1 in S. cerevisiae hardly affected invertase Golgi N-glycosylation, but it had profound effects in the elongation of carboxypeptidase Y N-glycans and almost totally inhibited formation of mannosylated ceramides (9). The differential effects observed in S. pombe might be related to possible different locations of Spgda1p and Spynd1p within Golgi cisternae.

Golgi O-glycosylation in this yeast also involves the addition of Man and Gal residues, but to the Man unit added to proteins in the ER (30). Wild type and mutant cells were incubated with labeled Glc and chased with the unlabeled monosaccharide, and O-linked glycans were released from whole cell glycoproteins by a mild alkaline treatment. The patterns of oligosaccharides obtained are depicted in Fig. 6, A–C. Ablation of either one of both nucleoside diphosphatase genes did not affect Golgi O-extension of glycans. The null or very limited effects of disruption of only one of both nucleoside diphosphatase genes on Golgi N- and O-glycosylation agree with their similar subcellular localizations.

Effect of Spgda1<sup>−</sup> and Spynd1<sup>−</sup> Disruption on ER Nucleotide Sugar-dependent N-Glycosylation—As mentioned above, GT-mediated N-glycosylation is the only nucleotide sugar-dependent reaction described to date in S. pombe ER lumen. This reaction is not essential for cell viability under normal growth conditions (31). Moreover, complete or severe reduction in monoglucosylated glycan formation caused by disruption of glucosidase II subunit α- or β- or GT-encoding genes did not affect cell growth or morphology. Nevertheless, hindering the formation of monoglucosylated glycans led to ER accumulation of misfolded glycoproteins, as revealed by the induction of BiP-encoding mRNA. Such induction is particularly easy to assess in S. pombe because, whereas under normal conditions only one messenger is formed, the accumulation of misfolded proteins leads to the synthesis of two BiP mRNAs (32). As shown by Northern blotting analysis (Fig. 7, lanes 1–3), only one BiP mRNA was synthesized in wild type, Spgda<sub>1</sub>, and Spynd<sub>1</sub> mutant cells, thus indicating that the formation of monoglucosylated glycoproteins was not severely impaired upon disruption of either one of the nucleoside diphosphatase-encoding genes. On the contrary, a gpt<sup>1</sup> mutant cell (GT null) yielded the expected two-band pattern (Fig. 7, lane 4).

To confirm that GT-dependent glucosylation was indeed not severely impaired in Spgda<sub>1</sub> and Spynd<sub>1</sub> cells, double mutants Spgda<sub>1</sub>gpt<sup>6</sup> and Spynd<sub>1</sub>gpt<sup>6</sup> were constructed. GPT<sup>6</sup> codes for the dolichol-P-Glc-dependent glucosyltransferase that is responsible for the addition of the first Glc unit to Man<sub>3</sub>GlcNAc<sub>2</sub>-P-P-dolichol. Alg<sup>6</sup> mutants, therefore, transfer Man<sub>3</sub>GlcNAc<sub>2</sub> to protein, and the formation of monoglucosylated glycans in them is...
exclusively mediated by GT activity. The above mentioned double as well as single \textit{alg6} mutant cells were incubated for 15 min with $^{14}$C[Glc for 15 min as indicated under “Experimental Procedures” in the presence of 5 mM 1-deoxynojirimycin. Endo H- liberated N-glycans were run on paper chromatography with solvent A (A–C). All material except for that running as the Man$_n$GlcNAc standard was submitted to strong acid hydrolysis and run on paper chromatography with solvent C (D–E). \textit{GM} stands for the label ratio in material running as Glc and Man standards. Standards are 1, Man; 2, Glc; 3, Gal; 8, Man$_8$GlcNAc; 9, Man$_9$GlcNAc; 10, Man$_{10}$GlcNAc; and 11, Glc$_1$Man$_9$GlcNAc.

Fig. 8. ER N-glycan synthesis in wild type and mutant cells. Cells were pulsed with $^{14}$C[Glc for 15 min as indicated under “Experimental Procedures” in the presence of 5 mM 1-deoxynojirimycin and 5 mM dithiothreitol. Endo H- liberated N-glycans were run on paper chromatography with solvent A (A–C). All material except for that running as the Man$_n$GlcNAc standard was submitted to strong acid hydrolysis and run on paper chromatography with solvent C (C and D). \textit{GM} stands for the label ratio in material running as Glc and Man standards. Standards are M, Man; G, Glc; 8, Man$_8$GlcNAc; 9, Man$_9$GlcNAc; and 11, Glc$_1$Man$_9$GlcNAc.

exclusively mediated by GT activity. The above mentioned double as well as single \textit{alg6} mutant cells were incubated for 15 min with 5 mM $^{14}$C[Glc in the presence of deoxynojirimycin, a glucosidase II inhibitor. Under these short incubation and low Glc concentration conditions, there is minimal Golgi enlargement of N-glycans. Compounds migrating as Man$_n$GlcNAc, Man$_n$GlcNAc, Glc$_1$Man$_n$GlcNAc, and Man$_n$GlcNAc appeared in the chromatogram, but patterns of N-oligosaccharides obtained from whole cell glycoproteins were very similar for the three cell types (Fig. 8, A–C). Strong acid hydrolysis of the compounds formed yielded labeled Man, Glc, and Gal units, but whereas the Glc/Man ratio was identical in compounds formed in \textit{alg6} and \textit{Spynd1}/\textit{alg6} cells, glycans synthesized in \textit{Spgda1}/\textit{alg6} mutants yielded a lower ratio, thus indicating a partially impaired GT-mediated glucosylation in those cells (Fig. 8, D–F). As almost all of the Man units in the compounds formed had been transferred to proteins from a dolichol-P-P derivative, \textit{i.e.} through a pathway not involving luminal nucleotide sugar-dependent glycosyltransferases, nucleotide sugar transporters, and nucleoside diphosphatases, the Glc/Man ratio provides a reliable indication of GT-mediated glucosylation levels in intact simple (\textit{alg6}) and double (\textit{Spgda1}/\textit{alg6} and \textit{Spynd1}/\textit{alg6}) mutant cells.

To confirm that GT-mediated glucosylation was indeed diminished in \textit{Spgda1} cells, the cells were incubated as above, but in the presence of dithiothreitol. This reagent prevents proper folding of most glycoproteins by hindering disulfide bond formation and, therefore, their ER exit to the Golgi also (33). As depicted in Fig. 9, A and B, a lower proportion of Glc$_1$Man$_9$GlcNAc was formed in \textit{Spgda1}/\textit{alg6} than in \textit{alg6} cells. Strong acid hydrolysis of compounds formed showed, as expected, a lower Glc/Man ratio in glycans synthesized in \textit{Spgda1}/\textit{alg6} cells (Fig. 9, C and D). It may be concluded, there-
fore, that whereas absence of Spynd1p did not affect GT-mediated glucosylation, deficiency of Spgda1p partially (35–50%) reduced such reaction in vivo.

DISCUSSION

Like S. cerevisiae, S. pombe expresses two enzymes capable of nucleoside diphosphate d Gly-mediated glucosylation in the fission yeast genome did not reveal sequences coding for additional enzymes catalyzing similar reactions. Like S. cerevisiae Gta1p and Ynd1p, both S. pombe enzymes localized to the Golgi, as shown by sucrose gradient centrifugation of post-nuclear supernatants. Furthermore, this technique confirmed the absence of a third ER nucleoside diphosphatase. The Golgi localization of both Spgda1p and Spynd1p agreed with the occurrence of Golgi N- and O-glycosylation reactions in cells in which either one of the encoding genes had been disrupted. Results obtained showed that one enzyme took the place of the other in the hydrolysis of nucleoside diphosphates required for relieving glycosyltransferase inhibition and providing nucleoside monophosphates for the ant port mechanism responsible for nucleotide sugar entry into the Golgi lumen. Only a decrease in the formation of the larger Golgi N-glycans was observed in Spynd1 mutants. The redundant function of S. cerevisiae Gta1p and Ynda1p in the hydrolysis of nucleoside diphosphates generated by Golgi N- and O-glycosylation reactions has been observed previously (3).

The most puzzling result was that, contrary to what happens in mammalian cells, there was no nucleoside diphosphatase activity in S. pombe ER. The fission yeast, the same as all other eukaryotic cells with the (to date) only known exception of S. cerevisiae (12, 13), expresses an ER enzyme (GT) that is involved in the quality control of glycoprotein folding and generates UDP. As mentioned above, this enzyme together with liver glucuronosyltransferase are the only fully described nucleotide sugar-dependent glycosyltransferases occurring in the ER lumen. GT is a soluble protein that localizes to the ER and the ER-Golgi intermediate compartment. Like the other components of the quality control mechanism (glucosidase II, calnexin, and calreticulin), it displays an ER retrieval sequence at its C terminus (11). It may be assumed, therefore, that it is continuously transported between the ER and cis-Golgi cisterneae by COP I and COPII vesicles. In fact, not only GT but also glucosidase II and calreticulin have been detected in the mammalian cell ER-Golgi intermediate compartment by quantitative immunogold electron microscopy (34).

How is GT-generated UDP hydrolyzed in S. pombe? Ablation of either Spgda1 or Spynd1 did not elicit induction of BiP mRNA, as happened in mutants in which the formation of monoglucosylated N-glycans was severely or totally impaired (gls2a, gls2b, and gpt1 mutants, respectively, devoid of glucosidase II α or β subunits or GT; Ref. 19). Moreover, an in vivo assay of GT-mediated glycoprotein glucosylation revealed that the reaction was only partially affected in Spgda1 and not affected at all in Spynd1 mutants. A possibility could be that, in the particular case of the S. pombe ER UDP-Glc transporter, UDP and not UMP is the metabolite involved in the nucleotide sugar transport and, therefore, no UDP hydrolysis would be required. This possibility seems highly unlikely, however, because no such ant port mechanism has ever been described for nucleotide sugar entry into the secretory pathway in any of the cells (fungal, plant, or mammalian) studied to date, and it would not account for the effect of Spgda1 disruption on GT-mediated glucosylation. Concerning the exit of nucleoside diphosphates from the ER, our results show that probably ADP and not AMP is the still unidentified ant port in the entrance of ATP into the ER lumen (1, 35), because ablation of the only gene coding for an ADPase activity in the S. pombe secretory pathway (Spynd1p) not only did not affect cell viability but also did not elicit induction of BiP mRNA. It would have been expected that, if AMP was the ant port, a lower rate of ATP entrance into the ER lumen caused by nucleoside diphosphatase deficiency would certainly affect molecular chaperone-assisted protein folding.

A more attractive possibility is that vesicular transport between the ER and cis Golgi cisterneae might not only transport the macromolecular components of the quality control mechanism (S. pombe lacks calreticulin but expresses calnexin; Ref. 36) but also metabolites like UDP in the anterograde movement to be hydrolyzed by Spgda1p and/or Spynd1p and UMP in the retrograde transport. This possibility is supported by the partial decrease in the glucosylation of folding intermediates observed in Spgda1 mutants. The involvement of vesicular ER-Golgi traffic in UDP disposal could be tested if the conditional mutants affected in such traffic were available. Unfortunately, although those mutants have been extensively studied in S. cerevisiae, no similar S. pombe mutants have been characterized to date. Future work will be directed to their production and characterization to be used as tools for testing the postulated role of ER-Golgi transport in GT-generated UDP hydrolysis. An additional experiment to be performed would be to assay in vivo GT-mediated glucosylation in Spgda1 or Spynd1 mutant cells expressing the other nucleoside diphosphatase under the control of a repressible promoter. If as proposed, Spgda1p and Spynd1p were indeed involved in GT-generated UDP hydrolysis, then no GT activity would be detected under conditions of total repression. However, the best of such promoters available for S. pombe (inmt, for no message in thiamine) is apparently leaky, as we have been unable to completely shut off GT expression when encoded in plasmids containing that promoter transfected into GT null mutants. As the amount of nucleoside diphosphatase allowing ER glucosylation of folding intermediates is presently unknown, ambiguous results would be obtained.

Finally, a third possibility to account for the observed lack of UDPase activity in S. pombe ER lumen could be the entrance of UDP-Glc into the Golgi by a "classical" transporter followed by vesicular retrograde transport of the nucleotide sugar to the ER lumen and anterograde transport of GT-generated UDP to the Golgi to be hydrolyzed by Spgda1p and/or Spynd1p. This last possibility seems highly unlikely, as we have detected substantial labeling of endogenous glycoproteins on the incubation of isolated intact microsomal vesicles with [14C]Glc, i.e. under conditions in which ER-Golgi transport is most probably not functional due to the absence of energy sources (ATP, GTP) and cytosolic proteins. Results presented show, therefore, that contrary to what has been assumed to date, nucleoside diphosphatase and glycosyltransferase activities do not necessarily localize to the same subcellular compartments in all eukaryotic cells. It is tentatively suggested that vesicle ER-Golgi transport might be involved in the hydrolysis of ER-generated nucleoside diphosphates.

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