Functional Expression of the Murine Golgi CMP-Sialic Acid Transporter in Saccharomyces cerevisiae

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We have functionally expressed the murine Golgi putative CMP-sialic acid transporter in Saccharomyces cerevisiae. Using a galactose-inducible expression system, S. cerevisiae vesicles were able to transport CMP-sialic acid. Transport was dependent on galactose induction and was temperature-dependent and saturable with an apparent $K_m$ of 2.9 $\mu$m. Transport was inhibited by CMP, and upon vesicle disruption with Triton X-100 parameters were very similar to the previously described CMP-sialic acid transport characteristics observed with mammalian Golgi vesicles. CMP-sialic acid transport induction was specific as no transport of UDP-galactose was observed even though the latter putative CMP-sialic acid transporter has a high degree of amino acid sequence identity with the CMP-sialic acid transporter. Together, the above results demonstrate that the previously described cDNA encoding the putative CMP-sialic acid transporter encodes the transporter protein per se and suggests that this heterologous expression system may be used for further structural and functional studies of other Golgi membrane transporter proteins.

Transporters for nucleotide sugars, nucleotide sulfate, and ATP of the Golgi apparatus membrane are required for the translocation of these solutes from the cytosol into the lumen of this organelle (1, 2). Within this compartment these nucleotide derivatives and ATP are substrates for glycosylation, sulfation, and phosphorylation of secretory and membrane-bound proteins as well as lipids (1, 2). Studies in vitro and in vivo have shown these transporters to be antiporers with the corresponding nucleoside monophosphates (1, 2). These transport activities have been detected in Golgi vesicles from mammals (1, 2), yeast (1, 2), protozoa (3), and plants (4). Mutants defective in transport activities of CMP-sialic acid (1, 2), UDP-galactose (1, 2), UDP-N-acetylglucosamine (1, 2), and GDP-mannose (3, 5) have been described in these organisms and have a block of glycosylation of proteins and lipids in vivo. These phenotypes have been used as selection for expression cloning of nucleic acids, which encode proteins that correct the phenotype of the yeast UDP-GlcNac transporter (6), the murine CMP-sialic acid transporter (7), the Leishmania donovani GDP-mannose transporter (5), and the human UDP-galactose transporter (8). So far in every instance, a highly hydrophobic multitransmembrane spanning domain protein was found to correct the mutant phenotype, suggesting that the protein is the corresponding Golgi membrane nucleotide sugar transporter. In some studies, the protein was localized to the Golgi apparatus (3, 7), while in other cases Golgi vesicles from the transfected mutant cells were shown to have recovered the ability to transport the nucleotide sugar, which the corresponding mutant cell line vesicle lacked (3, 6).

We have expressed the murine Golgi apparatus membrane putative CMP-sialic acid transporter in Saccharomyces cerevisiae for the following reasons. (a) Because yeast cells do not synthesize sialylglycoconjugates and do not have such transporters, expression of transport activity would provide strong evidence that the putative CMP-sialic acid transporter is, indeed, the transporter protein per se and not a protein regulating the transporting the nucleotide sugar, which the corresponding mutant cell line vesicle lacked (3, 6).

MATERIALS AND METHODS

Construction of pPB11—The cDNA for the CMP-sialic acid transport tagged in the C terminus with 33 nucleotides coding for the hemagglutinin (HA) epitope (1.1 kilobase pairs) was obtained by digesting pME8-HA (7) with NcoI, filled in with Klenow DNA polymerase, and then digested with XhoI and isolated from agarose using Spin-X filters (Costar), followed by phenol/chloroform extraction and ethanol precipitation. pYES2 (Invitrogen, Inc.) was digested with SacI, filled in with Klenow DNA polymerase, and then digested with XhoI and ligated to the 1.1-kilobase pair fragment. Following transformation of DH5α Escherichia coli, plasmid DNA obtained from transformants was analyzed by restriction digestion. One of the plasmids, pPB11, was used for further studies.

Expression of the CMP-Sialic Acid Transporter in S. cerevisiae—S. cerevisiae INVSSc1 (MATα, his3-d1, Leu2, trp1-289, ura3–52) (Invitrogen, Inc.) cells were made competent for electroporation by extensive washing in ice-cold sterile water, resuspended in 10% glycerol, and electroporated with 1 $\mu$g of pYES2 or pPB11 using a Bio-Rad gene pulser (2.5 kV, 25 microfarads, 200 ohms). Cells were incubated for 1 hour at 30 °C in YPD medium before plating in SD agar plates containing 30 mg/liter 1-leucine, 2 mg/liter 1-histidine, and 20 mg/liter 1-tryptophan. Transformants grown at 30 °C for 2 days were isolated and grown in liquid selective medium (0.67% Bacto-yeast nitrogen base, 2% dextrose, 600 of 2.0) at 30 °C for 4 days. Samples were taken uninduced, and the remaining cells were spun at 2,000 × g for 10 min to remove the medium. Cells were then resuspended in the same volume of selective medium in which raffinose had been replaced by 2% galactose and allowed to grow at 30 °C for the times indicated in each case.

Western Blots—To determine expression of the CMP-sialic acid transporter protein, samples of uninduced and induced cells were taken at different times (A$_{600}$ of 1.0 each), and total extracts were prepared as described previously (9). Total extracts were fractionated in 12% SDS-polyacrylamide gel electrophoresis gels, electrotransferred to polyvi-
nylindone difluoride membranes, and following blocking with 3% gelatin, 1% milk, 0.05% Tween 20, incubated with monoclonal anti-HA (1:1,000; Babco, Inc.). Detection was performed using horseradish peroxidase-conjugated mouse IgG (Promega) followed by chemiluminescence using Lumiglo (Kirkegaard & Perry Laboratories).

**Nucleotide Sugar Transport Assay—**Preparation of Golgi-enriched vesicles from induced and uninduced cells was as described previously, and transport assays were performed as described previously (10, 11).

**RESULTS**

To express the mammalian putative CMP-sialic acid transporter in *S. cerevisiae*, a yeast expression vector with an inducible promoter was chosen, since this would allow the separation of the growth phase under uninduced conditions from the expression phase in the presence of the inducer. This approach may also circumvent the possibility that high levels of expression of this heterologous protein may be toxic or growth inhibitory. For these reasons, pYES2, a 2-μm derived plasmid containing the strong galactose 1 promoter was chosen.

The putative CMP-sialic acid transporter cDNA, tagged with an HA epitope to facilitate the detection of the expressed protein, was cloned into pYES2 giving rise to pPB11. *S. cerevisiae* INVSc1 was transformed with pYES2 (vector alone) and pPB11. Total cell extracts were prepared from the transformants grown under uninduced conditions (4% raffinose) and found in mammalian Golgi vesicles. Fig. 2 shows that transport was saturable within an apparent *Kₘ* of 2.9 μM.

**DISCUSSION**

The following criteria provide strong evidence that we have expressed the murine Golgi membrane CMP-sialic acid transporter protein in *S. cerevisiae*. (a) A protein of apparent mobility of 30 kDa was expressed in *S. cerevisiae* only as a result of induction of the galactose 1 promoter. Induction of cells with plasmids without the cDNA did not result in expression of the above protein. The murine putative CMP-sialic acid transporter expressed in yeast cells has the same mobility as COS-1 cells that were transfected with pME8HA and expressed. (b) Upon isolation of a Golgi-enriched fraction from *S. cerevisiae* and measurements of CMP-sialic acid transport into these vesicles, the following characteristics of this transport activity were obtained. (i) Transport of CMP-sialic acid was temperature-dependent with the value at 30 °C being 50-fold that at 0 °C. (ii) Transport was dependent on time and protein concentration and was saturable with an apparent *Kₘ* of 2.9 μM, a value very similar to that previously described for rat liver Golgi membrane CMP-sialic acid transporter (1). (iii) Transport was inhibited by 20 μM of CMP in a manner analogous to the previously described transport of CMP-sialic acid into rat liver Golgi-derived vesicles (1); (iv) transport was specific for CMP-sialic acid, and *S. cerevisiae* Golgi-enriched vesicles from induced and uninduced cultures were unable to transport UDP-galactose. (v) Treatment of vesicles with Triton X-100 abolished transport. (vi) Following transport of CMP-sialic acid into the Golgi lumen, the concentration of solutes derived from this nucleotide sugar was concentrated 8–10-fold in relation to the concentration in the reaction medium. Together, these results provide strong evidence that the protein encoded by the plasmid pME8-HA that corrected the phenotype of the Chinese hamster ovary Lec2 mutant of mammalian cells (7) does indeed encode the Golgi membrane CMP-sialic acid transporter protein. The likelihood of the protein being an activator of the Golgi membrane CMP-sialic acid transporter protein is remote. This heterologous expression also opens the possibility of expressing and purifying large amounts of the CMP-sialic acid transporter in other yeast and reconstituting this protein into proteoliposomes (12) as we have also done previously with the transporters for UDP-galactose (13), UDP-glucuronic acid (13), and adenosine 3'-phosphate,5'-phosphosulfate (12, 14). Although reconstitution is a powerful direct proof that the protein is the transporter *per se* this approach has such problems that insertion of all the transporter protein molecules into liposomes from uninduced cells was not significantly different at both temperatures. Transport of CMP-sialic acid in the induced cells was dependent on vesicle integrity because Triton X-100 caused virtually complete absence. Transport of CMP-sialic acid was also inhibited by 5'-CMP as previously reported (12).

To determine the specificity of the CMP-sialic acid transport measurements including the substrate specificity of the CMP-sialic acid transporter *per se*, transport of UDP-galactose into the same vesicles as described above was also measured. *S. cerevisiae* do not have galactose in their mannans and do not transport UDP-galactose into the Golgi lumen. As shown in Table I, the putative UDP-galactose transport signal was not dependent on temperature or vesicle integrity, both very important controls for true transport into vesicles. In addition, transport of CMP-sialic acid was not affected by the presence of 15 μM UDP-galactose in the incubation medium (Table I) demonstrating that UDP-galactose was not competing with CMP-sialic acid for entering into the vesicles.

Finally, it was important to determine whether transport of CMP-sialic acid into yeast vesicles was saturable, as previously found in mammalian Golgi vesicles. Fig. 2 shows that transport was saturable within an apparent *Kₘ* of 2.9 μM.

**FIG. 1.** Expression of HA-tagged CMP-sialic acid transporter by yeasts transformants and COS-1 transfectants. Extracts of transformants carrying pYES2 (– insert) or pPB11 (+ insert) grown in raffinose (uninduced (U)) and shifted to galactose for 4 and 8 h (4G and 8G) and extracts from pME8-HA-transformed COS-1 cells (C, 1 μg of protein; C', 5 μg of protein) were analyzed by immunoblotting using a monoclonal anti-HA antibody, as described under “Materials and Methods.”

Table I shows that vesicles from induced cells transport CMP-sialic acid in a temperature-dependent manner with a 50-fold higher rate at 30 °C than at 0 °C; in contrast transport into vesicles derived from uninduced cells was not significantly different at both temperatures. Transport of CMP-sialic acid in the induced cells was dependent on vesicle integrity because Triton X-100 caused virtually complete absence. Transport of CMP-sialic acid was also inhibited by 5'-CMP as previously reported (12).

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Transport of CMP-sialic acid and UDP-galactose into S. cerevisiae vesicles from uninduced and galactose-induced cells

A P3 vesicle fraction (1 mg of protein) was incubated for 3 min. Transport was measured as described under “Materials and Methods.” [Sm], concentration of solutes in the incubation medium; St, total solutes in the vesicle pellet; So, solutes outside vesicles in the pellet; Si, solutes within vesicles in the pellet; [Si], concentration of solutes within vesicles in the pellet. The concentration of CMP-sialic acid in the incubation medium was 0.8 μM, and that of UDP-galactose was 1 μM. Results are the average of two independent determinations.

| Cells          | Substrate          | Additions       | Temperature | St     | So     | Si     | [Si] | [Si]/[Sm] |
|----------------|--------------------|-----------------|-------------|--------|--------|--------|------|----------|
| Uninduced      | CMP-sialic acid    |                 | 30 °C       | 0.4    | 0.5    | 0      | 0    | 0        |
| Uninduced      | CMP-sialic acid    | 0.05% Triton X-100 | 30 °C      | 0.5    | 0.5    | 0.2    | 0.2  | 0.2      |
| Uninduced      | UDP-galactose      |                 | 30 °C       | 0.6    | 0.6    | 0      | 0    | 0        |
| Induced        | CMP-sialic acid    |                 | 30 °C       | 0.6    | 0.5    | 0.1    | 0.1  | 0.1      |
| Induced        | CMP-sialic acid    | 20 μM 5’ CMP    | 30 °C       | 0.7    | 0.5    | 0.2    | 0.3  | 0.4      |
| Induced        | CMP-sialic acid    | 15 μM UDP-galactose | 30 °C     | 1.9    | 0.5    | 1.4    | 2    | 2.5      |
| Induced        | UDP-galactose      |                 | 30 °C       | 1.9    | 0.6    | 1.3    | 1.8  | 1.8      |
| Induced        | UDP-galactose      | 0.05% Triton X-100 | 30 °C     | 2.3    | 0.6    | 1.7    | 2.3  | 2.3      |

The apparent concentration within the lumen of S. cerevisiae vesicles of CMP-sialic acid, relative to its concentration in the reaction medium, deserves some attention. Assuming this observation is correct, where does the energy for concentration within the lumen come from? In mammals, studies in vitro have strongly suggested that the CMP-sialic acid transporter is an antiporter with CMP, the reaction product following sialylation, as described (11). However, there are many exceptions to this in mammals and yeast (19, 20). When the intact full-length human β-1,4-galactosyltransferase or α-2,3-sialyltransferase were expressed in S. cerevisiae, the active enzymes appeared to be retained in the endoplasmic reticulum (21). While the former studies (15, 16) suggest that mammalian Golgi-targeting domains can be used in S. cerevisiae, the latter do not (21).

The apparent concentration within the lumen of S. cerevisiae vesicles of CMP-sialic acid, relative to its concentration in the reaction medium, deserves some attention. Assuming this observation is correct, where does the energy for concentration within the lumen come from? In mammals, studies in vitro have strongly suggested that the CMP-sialic acid transporter is an antiporter with CMP, the reaction product following sialylation of proteins and lipids (1, 12). CMP-sialic acid is unique as a nucleotide sugar in that it does not need a nucleoside diphosphate in the Golgi lumen to generate the nucleoside monophosphate as antiporter solute (1). In S. cerevisiae, where CMP-sialic acid is not a donor in sialylation, the mechanism for CMP-sialic acid concentration in the vesicle lumen remains to be determined.

Is there an amino acid sequence motif for targeting these multitransmembrane proteins to the Golgi membrane in S. cerevisiae and mammals? To date, answers to this question are not available and the topography of such proteins is not known in the Golgi membrane. The expression system described here should be helpful in providing answers to these questions as well as determining the nucleotide sugar and nucleoside monophosphate binding sites in these proteins.

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