

Functional expression of the CMP-sialic acid transporter in *Escherichia coli* and its identification as a simple mobile carrier

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The architectural conservation of nucleotide sugar transport proteins (NSTs) enabled the theoretical prediction of putative NSTs in diverse gene databases. In the human genome, 17 NST sequences have been identified but only six have been unequivocally characterized with respect to their transport specificities. Defining transport characteristics of recombinant NSTs has become a major challenge because true zero background systems are widely absent. Production of recombinant NSTs in heterologous systems has developed multifunctionality for some NSTs leading to a novel level of complexity in the field. Assuming that (1) the specificity of NSTs is determined at the primary sequence level and (2) the proteins are autonomously functional units, final definition of the substrate specificity will depend on the use of isolated transport proteins. Herein, we describe the first report of the functional expression of mouse CMP-sialic acid transporter (CST) in *Escherichia coli* and thus provide significant progress towards the production of transporter proteins in quantities suitable for functional and structural analyses. Recovery of the active NST from inclusion bodies was achieved after solubilization with 8 M urea and stepwise renaturation. After reconstitution into phospholipid vesicles, the recombinant protein demonstrated specific transport for CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac) with no transport of UDP-sugars. Kinetic studies carried out with CMP-Neu5Ac and established CMP-Neu5Ac antagonist's evaluated natural conformation of the reconstituted protein and clearly demonstrate that the transporter acts as a simple mobile carrier.

Key words: CMP-sialic acid transporter/*Escherichia coli* heterologous expression/nucleotide sugar transporter/simple mobile carrier

Introduction

Activation of sugars to sugar nucleotides precedes their entry into the glycoconjugate production pathways, which, in eukaryotic cells, mainly reside in the endoplasmic reticulum (ER) and Golgi apparatus. Sugar activation does not only provide the energy for the catabolic reactions, it is also the nucleotide sugar that is selectively transported over the compartmental membranes. Although in the ER only a minor fraction enters the ER lumen as nucleotide sugar (the major shuttle system for sugars in the ER is dolichol phosphate; for review see Hirschberg *et al.*, 1998), transport into the Golgi is exclusively mediated *via* nucleotide sugar transport proteins (NSTs). In depth, characterization at the biochemical level has been ongoing for over three decades (Hirschberg and Snider, 1987; Hirschberg *et al.*, 1998); however, molecular cloning of the first representatives did not occur until 1996.

Using complementation cloning in mutants that are defective in the transport activity of a number of sugar nucleotides, NSTs have been isolated from yeast (Abejón *et al.*, 1996a; Tabuchi *et al.*, 1997), mammals (Eckhardt *et al.*, 1996; Ishida *et al.*, 1996; Guillen *et al.*, 1998), and protozoa (Descoteaux *et al.*, 1995; Ma *et al.*, 1997). The molecular cloning revealed a family of architecturally conserved type III membrane proteins with 8–10 *trans* membrane domains (Berninsone and Hirschberg, 2000; Gerardy-Schahn *et al.*, 2001; Martinez-Duncker *et al.*, 2003). Interestingly, this architectural conservation parallels functional conservation, enabling expression and complementation in heterologous systems. Based on this, the canine UDP-*N*-acetylglucosamine (UDP-GlcNAc) transporter was identified by complementation cloning in *Kluyveromyces lactis* (Guillen *et al.*, 1998). Similarly, the lack of GDP-fucose transport in primary fibroblasts isolated from a patient with leukocyte adhesion deficiency syndrome type II (LADII) was complemented with the corresponding *Cenorhabditis elegans* cDNA (Luehn *et al.*, 2001). The high evolutionary conservation of the GDP-fucose transport protein in addition enabled the identification of the human ortholog (Luehn *et al.*, 2001).

Remarkably, conservation at the primary sequence level is not a common feature of functionally identical transporters. This can be most impressively seen for NSTs that exhibit UDP-GlcNAc transport activity as singular or one of multispecificities (Abejón *et al.*, 1996a; Guillen *et al.*, 1998; Ishida *et al.*, 1999; Roy *et al.*, 2000; Höflich *et al.*, 2004; Suda *et al.*, 2004; Ishida *et al.*, 2005). Moreover, while previous biochemical studies and phenotype analyses of NST-deficient mutants suggested that NST are monospecific proteins (Hirschberg *et al.*, 1998; Berninsone and Hirschberg, 2000), the testing of heterologously expressed recombinant proteins displayed multisubstrate specificity

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for most of the genes (Hong *et al.*, 2000; Berninsone *et al.*, 2001; Muraoka *et al.*, 2001; Segawa *et al.*, 2002). Transport of UDP-glucuronic acid (UDP-GlcA) and UDP-*N*-acetyl-galactosamine (UDP-GalNAc) (Gerardy-Schahn *et al.*, 2001; Martinez-Duncker *et al.*, 2003) have so far only been identified as part of multifunctional proteins. An important question is therefore: Is multisubstrate recognition a true feature of the individual protein or a consequence of the non-natural environment?

The yeast strain *Saccharomyces cerevisiae* is the most commonly used tool in expression studies, because microsomal vesicles isolated from *S. cerevisiae* exhibit potent nucleotide sugar transport activity only for GDP-Man (Abeijon *et al.*, 1989), however, significant activity has also been reported for UDP-galactose (UDP-Gal) (Roy *et al.*, 1998), UDP-glucose (UDP-Glc) (Castro *et al.*, 1999), and UDP-GlcNAc (Roy *et al.*, 2000). First used to confirm the function of the murine CMP-sialic acid transporter (CST) (Berninsone *et al.*, 1997), expression in *S. cerevisiae* is now regarded as a standard system for the evaluation of NST specificity (Sun-Wada *et al.*, 1998; Ishida *et al.*, 1999; Aoki *et al.*, 2001).

A systematic approach has recently begun in our laboratories to evaluate the specificities of identified human NSTs under identical assay conditions. Human NST-genes were cloned into a yeast expression vector, and proteins were expressed under the control of a galactose-inducible promoter. Surprisingly, as is outlined here, we found that the growth of yeast cells on different carbon sources sufficiently changes the transport capabilities of isolated Golgi vesicles. This finding together with the large number of putative NSTs, that exceeds the number of nucleotide sugars requiring transport, highlights the need for alternative zero background assay systems.

Using the mouse CST (Eckhardt *et al.*, 1996) as a model system, a protocol has been elaborated in this study that allows recombinant expression in bacteria. Renaturation of the protein from inclusion bodies and insertion into artificial liposomes gave rise to proteoliposomes with selective CMP-sialic acid (CMP-Sia) transport. Kinetic studies carried out with the reconstituted system closely approximate the data established with Golgi vesicles isolated from mouse and rat liver for both the natural substrate (Carey *et al.*, 1980; Capasso and Hirschberg, 1984c; Milla *et al.*, 1989) and established CMP-Sia transport inhibitors (Capasso and Hirschberg, 1984a; Tiralongo *et al.*, 2000) and demonstrates that the protein acts as a simple mobile carrier. This study not only provides an alternative heterologous expression system for the characterization of putative NSTs, but also represents the first key step towards generating sufficient protein for detailed structure-function investigations.

Results

Influence of the carbon source

With the aim of comparatively evaluating the transport capabilities of known human NSTs under identical assay conditions, the genes encoding the human CST (Ishida *et al.*, 1996), UGT II (Ishida *et al.*, 1996), and UDP-GlcNAc transporter (Ishida *et al.*, 1999) were sub-cloned into the yeast expression vector pYES2/NT-C, which allows protein

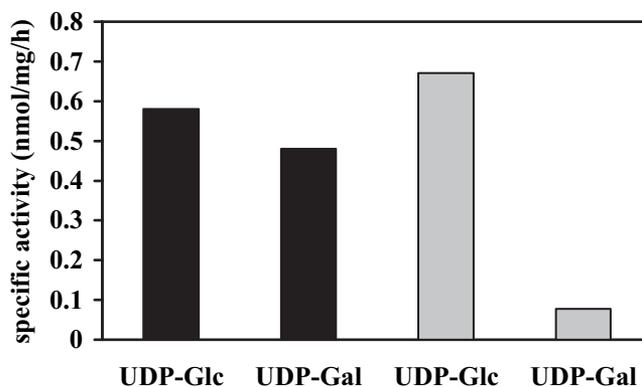


Fig. 1. *Saccharomyces cerevisiae* were grown in media containing either galactose (black bars) or glucose (grey bars) as the carbon source. Golgi vesicles were isolated as described in *Materials and methods* and used to measure the uptake of UDP-Gal and UDP-Glc. Growth of yeast cells in galactose-containing media dramatically alters the transport capabilities of isolated Golgi vesicles, whereas cells grown on glucose-containing media are unable to transport UDP-Gal.

expression under the control of a galactose inducible promoter. Golgi-rich fractions isolated from transformed cells were used to test transport activity with five different nucleotide sugars. Unexpectedly, all isolated Golgi fractions including the Golgi fraction isolated from mock transformed yeast cells demonstrated a significant increase in UDP-Gal transport (data not shown). To further analyze this phenomenon, native yeast cells were grown in media containing either galactose or glucose as the carbon source and isolated Golgi vesicles were tested for UDP-Gal and UDP-Glc transport activities. As shown in Figure 1, the Golgi vesicles isolated from yeast cells grown in glucose are unable to transport UDP-Gal, however, Golgi vesicles from cells cultivated in galactose-containing media transport UDP-Gal with efficiency similar to the endogenous UDP-Glc transport. These results clearly underline the difficulties associated with characterizing recombinant NST specificities using the yeast system, particularly as shown here when multifunctionality, and consequently an additional level of complexity, can be induced. An alternative, true zero background assay system would circumvent this problem and provide a unique tool for investigating if multisubstrate specificity is a true feature of individual NSTs or is an artefact of expression in a non-natural system. With this in mind, we outline here the expression of the mouse CST in *Escherichia coli*, a system lacking any endogenous NST activity.

The expression of mouse CST in E. coli

The expression of the mouse CST in *E. coli* was tightly controlled by the isopropyl- β -D-thiogalactopyranoside-(IPTG) inducible *trpllac* promoter and was not observed in *E. coli* transformed with pTrcME8HA (Figure 2, lanes 1 and 2) unless IPTG was added (lane 3). The recombinant HA-tagged protein was quantitatively sequestered in the inclusion body pellet (lane 3).

Interestingly, the apparent molecular masses of the immunoreactive proteins are lower than the 39 kDa predicted for the mouse CST (Eckhardt *et al.*, 1996). Similar observations

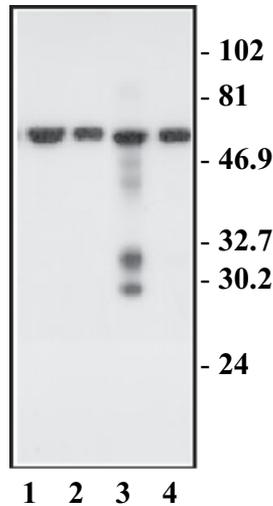


Fig. 2. The expression of mouse CST in *Escherichia coli*. Two μg of insoluble and soluble protein fractions, isolated from uninduced pTrcME8HA transformed *E. coli* cells (lanes 1 and 2), and insoluble and soluble protein fractions, isolated from IPTG-induced *E. coli* cells (lanes 3 and 4) were fractionated on 12% SDS–polyacrylamide gels and electrotransferred to polyvinylidene fluoride membranes. The anti-HA monoclonal antibody 12CA5 (Roche Applied Science, Castle Hill, Australia) and HRP-conjugated anti-mouse Ig (Silenus Laboratories, Hawthorn, Australia) were used as the primary and secondary antibodies, respectively. Samples analysed by SDS–PAGE were heated at 37°C for 10 min, instead of boiling, in SDS–PAGE sample buffer containing 4 M urea (Ragan, 1986).

have been made upon expression of the cDNA in either *S. cerevisiae* (Berninsone *et al.*, 1997; Tiralongo *et al.*, 2000) or COS-1 cells (Eckhardt *et al.*, 1999) that resulted in proteins of apparent molecular masses of ~ 31 kDa. These outcomes lead us to predict that the 32.5 kDa protein is the mouse CST. The second protein of ~ 28 kDa is probably a truncated form of the transporter resulting from translation beginning at an alternative ATG codon, because its generation could not be reduced by the addition of protease inhibitors to buffers used in protein isolation and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Furthermore, the transient expression of CST in COS cells has also been shown to result in the co-expression of a 27-kDa protein along with the expected 30-kDa protein (Eckhardt *et al.*, 1999).

Functional reconstitution of the recombinant mouse CST into artificial liposomes

Mouse CST solubilized from inclusion bodies and renatured as described, was reconstituted into proteoliposomes using the freeze-thawing method of Kasahara and Hinkle (1976) and used in nucleotide sugar transport assays. Successful reconstitution into phosphatidylcholine liposomes was determined by size exclusion chromatography on Sepharose CL-4B (data not shown) as described by Milla and Hirschberg (1989). Proteoliposomes generated in this way were found to transport CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac) but not UDP-Glc, UDP-Gal, UDP-GlcNAc, or UDP-GalNAc (Figure 3). This illustrates that the mouse CST expressed in *E. coli* and reconstituted into phosphatidylcholine liposomes retains substrate specificity. CMP-

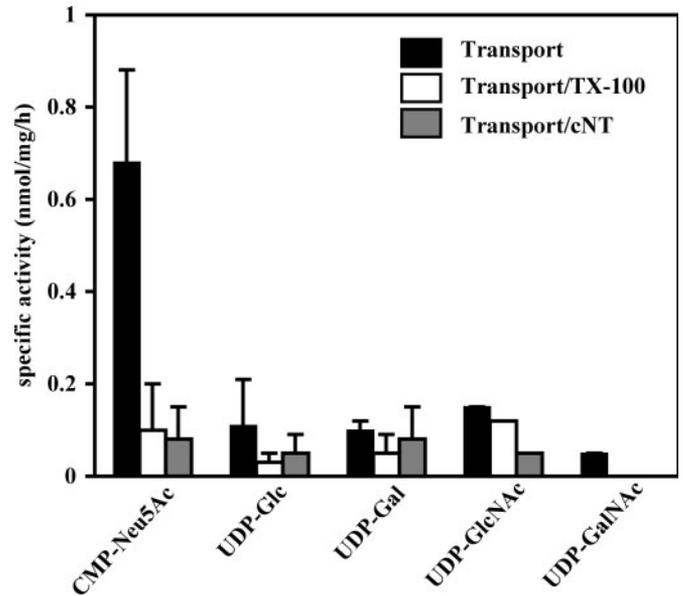


Fig. 3. The transport of CMP-Neu5Ac into proteoliposomes. CMP-Neu5Ac, UDP-Glc, UDP-Gal, UDP-GlcNAc, and UDP-GalNAc transport was measured as described in *Materials and methods*. All transport activities were calculated by subtracting for passive diffusion measured through the addition of $100\ \mu\text{M}$ DIDS to control assays performed in parallel. Triton X-100 (TX-100) and the corresponding nucleotide (cNT) were added to a final concentration of 0.2 % and $100\ \mu\text{M}$, respectively.

Neu5Ac transport was also found to be dependent on proteoliposome integrity with the addition of 0.2% Triton X-100 resulting in a 7-fold reduction in CMP-Neu5Ac transport activity (Figure 3). Transport was inhibited by the addition of $100\ \mu\text{M}$ CMP (Figure 3) but not AMP or UDP and was dependent on temperature with a 3.5-fold higher rate at 37°C (0.68 ± 0.20 nmol/mg/h) than at 0°C (0.20 ± 0.10 nmol/mg/h). As a control, a protein fraction obtained from mock-transformed *E. coli* cells was found not to transport CMP-Neu5Ac when reconstituted into proteoliposomes (data not shown). More detailed analyses showed that the transport of CMP-Neu5Ac was linear for protein concentrations up to ~ 1.5 mg/mL and for time points up to 10 min. Moreover, transport was inhibited by 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) in a manner consistent with the measurement of passive diffusion (data not shown). These results are consistent with the transport characteristics of CMP-Neu5Ac by a specific transporter as previously reported (Carey *et al.*, 1980; Capasso and Hirschberg, 1984b; Milla and Hirschberg, 1989; Berninsone *et al.*, 1997).

The transport of CMP-Neu5Ac into proteoliposomes preloaded with CMP (CMP-trans entry) and CMP-Neu5Ac (equilibrium exchange)

To more fully explore the mechanism of CMP-Neu5Ac transport by the *E. coli*-expressed CST, experiments were designed that would measure the effects of preloading proteoliposomes with CMP and equilibrium concentrations of CMP-Neu5Ac. Proteoliposomes were either preloaded with

Table I. The kinetics of CMP-Neu5Ac transport into proteoliposomes under zero-*trans* entry, CMP-*trans* entry and equilibrium exchange conditions

Experiment ^a	K_m (μM) ^b	V_{\max} (nmol/mg/h) ^b	V_{\max}/K_m ratio ^c
Zero- <i>trans</i> entry	32.9 ± 3.8	21.6 ± 3.6	0.65 ± 0.20
CMP- <i>trans</i> entry ^d	106.0 ± 23.8	56.6 ± 3.7	0.53 ± 0.23
Equilibrium exchange	67.2 ± 17.2	44.1 ± 5.8	0.65 ± 0.28

^aTransport of CMP-Neu5Ac into proteoliposomes was linear for time points up to and including 5 min.

^bMean \pm standard deviation (SD) for at least three separate experiments.

^c V_{\max}/K_m ratios are expressed as arbitrary units.

^dDesignates that proteoliposomes have been preloaded with CMP (present in the *trans* compartment) before measuring CMP-Neu5Ac transport.

a fixed concentration (0.1 mM) of CMP and the entry of radiolabelled CMP-Neu5Ac measured (CMP-*trans* entry) or preloaded with between 5 and 250 μM CMP-Neu5Ac and the entry of radiolabelled substrate, present in equilibrium concentrations, measured (equilibrium exchange).

The transport of CMP-Neu5Ac into the lumen of the rat liver Golgi apparatus (Capasso and Hirschberg, 1984c) and into proteoliposomes (Milla and Hirschberg, 1989) has been shown to be coupled with the exchange of luminal CMP in an antiport mechanism, leading to an increase in the transport rate. Table I shows that the activity of the *E. coli*-expressed mouse CST can be similarly stimulated under CMP-*trans* entry conditions. The maximum rate (V_{\max}) of CMP-Neu5Ac transport was ~ 3 times higher (Figure 4B) than transport into proteoliposomes prepared in the absence of CMP (zero-*trans* entry, Figure 4A). This result is consistent with that seen for the transport of CMP-Neu5Ac into rat liver Golgi proteoliposomes preloaded with CMP (Milla and Hirschberg, 1989; Chiaramonte *et al.*, 2001) and suggests that CMP-Neu5Ac transport into proteoliposomes by recombinant CST is also *via* an antiport mechanism.

The data in Table I also shows that the maximum rate of entry of radiolabelled CMP-Neu5Ac is stimulated under equilibrium exchange conditions (Figure 4C). This phenomenon, known as *trans*-acceleration or stimulation (Lieb, 1982), is defined as the acceleration of the *cis* to *trans* unidirectional flux resulting from the presence of substrate in the *trans* (inside) compartment and is consistent with solute transport by a simple mobile carrier model. Likewise, the stimulation of transport in the presence of the antiport molecule, CMP, at the *trans* side of the membrane further supports the classification of the mouse transporter as a carrier with a binding site that alternates between both sides of the membrane.

The V_{\max}/K_m ratios for all experimental modes employed, including CMP-*trans* entry, were also determined. The ratios presented in Table I are expressed as arbitrary units because turnover numbers could not be calculated. Therefore, the identification of the rate-limiting step in CMP-Neu5Ac transport as being the movement of the substrate-carrier complex across the membrane rather than the dissociation of the substrate, as is predominantly seen in enzyme systems, could not be conclusively resolved. Nevertheless, the

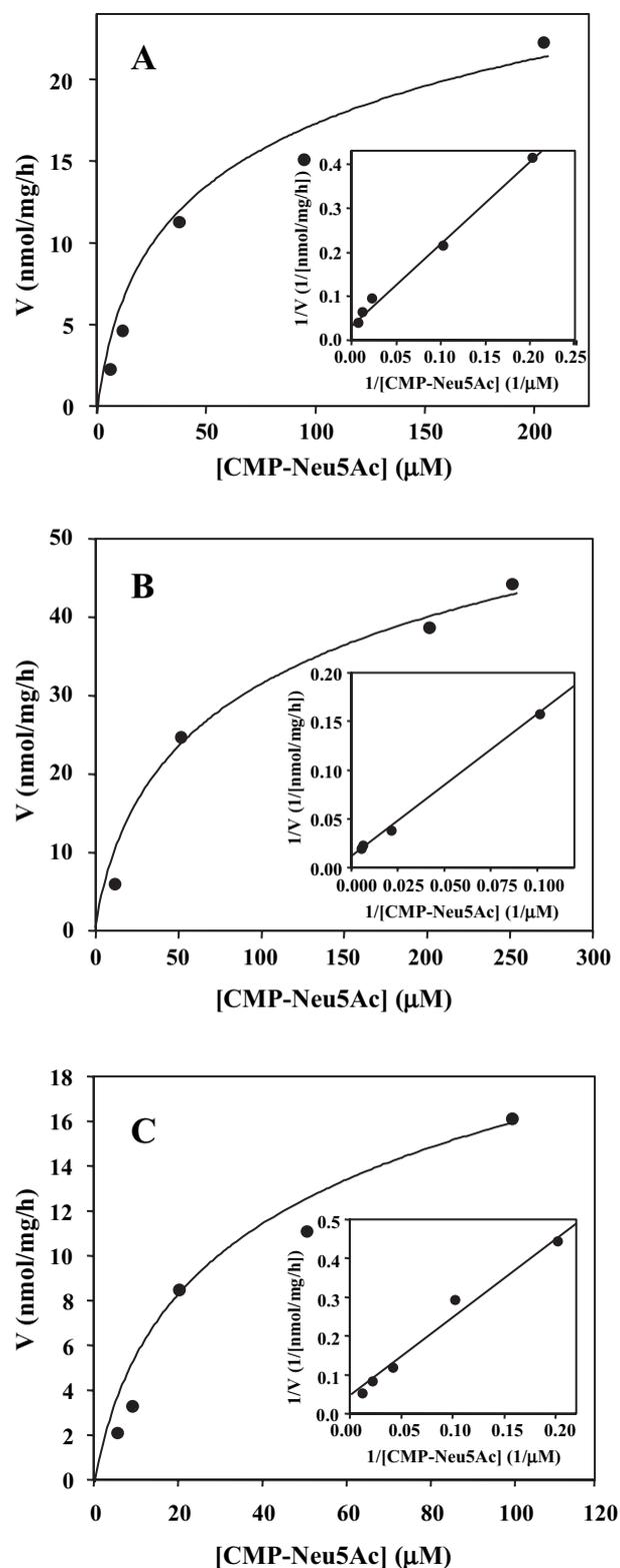


Fig. 4. Panel A, the saturation curve (V versus [CMP-Neu5Ac]) and Lineweaver-Burk plot (1/V versus 1/[CMP-Neu5Ac]; inset) of CMP-Neu5Ac transport under zero-*trans* entry conditions. Panel B, the saturation curve (V versus [CMP-Neu5Ac]) and Lineweaver-Burk plot (1/V versus 1/[CMP-Neu5Ac]; inset) of CMP-Neu5Ac transport into proteoliposomes preloaded with 0.1 mM CMP (CMP-*trans* entry). Panel C, the saturation curve (V versus [CMP-Neu5Ac]) and Lineweaver-Burk plot (1/V versus 1/[CMP-Neu5Ac]; inset) of CMP-Neu5Ac transport into proteoliposomes preloaded with 5–250 μM CMP-Neu5Ac (equilibrium exchange).

observation that V_{\max}/K_m ratios for the zero-*trans* entry and equilibrium exchange experiments was, within error, equal further identifies the facilitated transport of CMP-Neu5Ac by the mouse CST as being compatible with the simple carrier model (Lieb and Stein, 1974; Jarvis *et al.*, 1983; Stein, 1986).

Discussion

The yeast strain *S. cerevisiae* is the most commonly used tool in NST expression studies (Berninsone *et al.*, 1997; Sun-Wada *et al.*, 1998; Ishida *et al.*, 1999; Aoki *et al.*, 2001). Surprisingly, as is outlined here, we found that growth of yeast cells on different carbon sources sufficiently alters the background transport profile of isolated Golgi vesicles. This combined with the growing number of reports demonstrating multifunctionality for recombinant expressed NST (Hong *et al.*, 2000; Berninsone *et al.*, 2001; Muraoka *et al.*, 2001; Segawa *et al.*, 2002, 2005; Suda *et al.*, 2004) is adding a new level of complexity to the field. This recently recognized multisubstrate specificity, however, stands in marked contrast to earlier data obtained using natural ER and Golgi vesicles that demonstrated mono-specificity and a strict pattern of subcellular distribution (Carey *et al.*, 1980; Sommers and Hirschberg, 1982; Capasso and Hirschberg, 1984a; Milla and Hirschberg, 1989; Milla *et al.*, 1992; Hirschberg *et al.*, 1998). Moreover, the recently cloned human ortholog of the *D. melanogaster* *frc*, nematode *SQV-7* and human *UGTrel7* transporters, the *HFRC1* (or *SLC35D2*) transporter, has been shown to possess multisubstrate specificity by Suda *et al.* (2004), however was found to only transport UDP-GlcNAc by Ishida *et al.* (2005). Transport specificity in both instances was determined by heterologous expression in yeast, although multisubstrate specificity was also observed in mammalian cells (Suda *et al.*, 2004).

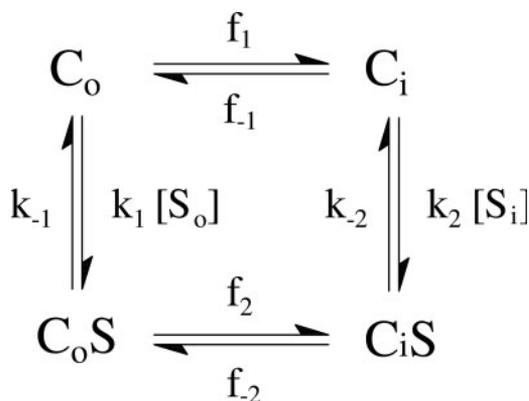
These recent findings, together with the large number of putative NSTs, that exceeds the number of nucleotide sugars requiring transport, highlights the necessity for the development of alternative zero background assay systems that allow the substrate specificities of the family of NSTs to be evaluated.

In this study, we provide the first conclusive evidence that recombinant NSTs can be produced in bacteria. Our report follows the recent purification and reconstitution of active GDP-Man transporter (LPG2) expressed in *Leishmania donovani* (Segawa *et al.*, 2005). This report highlighted the value of characterizing NST activity in a zero background environment, achieved by purification and reconstitution into artificial liposomes. The recombinant GDP-Man transporter was found unequivocally to have multisubstrate specificity (transporting several GDP-sugars) and not require interactions with other Golgi-resident proteins for activity, which may be important in the UDP-Gal transport system (Sprong *et al.*, 2003). Here, we have used the mouse CST (Eckhardt *et al.*, 1996) as a model system to evaluate the potential of recombinant NST expression in bacteria. The CST has been shown in a number of systems, including in yeast (Berninsone *et al.*, 1997), to only transport CMP-Neu5Ac, even though it shares 40–50% sequence identity with the mammalian UDP-Gal and UDP-GlcNAc trans-

porters (Berninsone and Hirschberg, 2000; Gerardy-Schahn *et al.*, 2001). Using this system, a protocol has been developed for recombinant expression of NSTs in bacteria.

The mouse CST expressed in *E. coli* accumulated as inclusion bodies, however, the protein could be solubilized by the use of 8 M urea and reconstituted, following stepwise renaturation, into phosphatidylcholine liposomes resulting in transport-active proteoliposomes. The transport of CMP-Neu5Ac into these proteoliposomes was dependent on membrane integrity, temperature, time, and protein concentration. Importantly, transport was found to be specific for CMP-Neu5Ac. This specific transport of CMP-Neu5Ac was also inhibited by DIDS and CMP and was saturable (K_m : 32.9 μ M). It should be noted that the apparent K_m is slightly higher than that observed for both the corresponding *S. cerevisiae*-expressed CST (2.9 μ M) (Berninsone *et al.*, 1997) and the rat liver Golgi CST (1.4 μ M) (Milla and Hirschberg, 1989). Interestingly, this reduced substrate affinity (~20-fold) is strikingly similar to the K_m difference observed between the reconstituted LPG2 GDP-Man transporter (6.6 μ M) expressed in *L. donovani* (Segawa *et al.*, 2005) and that from isolated microsomes (0.3 μ M) (Ma *et al.*, 1997). The reason for this reduced apparent affinity is as yet unclear. However, given that LPG2 GDP-Man transporter expressed and purified from *L. donovani* has a similar reduced affinity when reconstituted into liposomes (Segawa *et al.*, 2005), it is possible that either the suboptimal size and/or lipid composition of the artificial bi-layer is an issue, or optimal activity of NSTs might require interaction with other Golgi resident or cytosolic proteins that are absent in the protein from bacteria. Both studies used phosphatidylcholine to produce proteoliposomes, although it is more than likely that in the case of the GDP-Man transporter endogenous lipids would have been co-purified and thus also incorporated into the proteoliposomes. This would not be the case in this study. We are currently in the process of optimizing the reconstitution method used to form mouse CST-containing proteoliposomes, not only to improve affinity but also to ultimately incorporate large amounts of transporter into artificial lipid bi-layers for structure function studies. A recent report describing the stimulation of UDP-Gal transport into the ER by co-expression in Lec8 cells of the Golgi UDP-Gal transporter and galactosyltransferase (Sprong *et al.*, 2003) supports the notion that other factors in the glycosylation machinery may be required for optimal NST activity.

Detailed kinetic studies carried out here with the reconstituted mouse CST clearly demonstrate that the transport of CMP-Neu5Ac into proteoliposomes could be stimulated under equilibrium exchange and CMP-*trans* entry conditions. This *trans*-acceleration or stimulation phenomenon is compatible with the *E. coli*-expressed CST being a simple mobile carrier as defined by West (1983) and Stein (1986) (Scheme 1). A similar mechanism for CMP-Neu5Ac transport has also been reported using isolated rat liver Golgi vesicles mechanically loaded with a variety of CMP-Neu5Ac antagonists (Chiaramonte *et al.*, 2001), as well as a number of other nucleotide-substrate and non-nucleotide-substrate transport systems, including the adenosine 3'-phosphate 5'-phosphosulfate translocase (Ozeran *et al.*, 1996) from rat liver Golgi, and the choline (Krupka and Deves, 1980) and



Scheme 1. Where C stands for the carrier protein and S for the substrate. The subscripts o and i stand for outside and inside, respectively. The relative locations of substrate across a membrane can also be referred to in terms of cis and trans, where cis designates the compartment occupied by the reference substrate, whereas trans designates the compartment on the opposite side of the membrane. The rate constants for each reversible step are as shown. The rate constants k designates the substrate association and dissociation steps and the rate constants f designates the translocation of the free carrier and the substrate-carrier complex.

uridine (Cabantchik and Ginsburg, 1977) transporters in red blood cells. Further reports presenting data consistent with *trans*-acceleration in a number of NST systems (Capasso and Hirschberg, 1984c; Milla *et al.*, 1989, 1992), including the recently reported GDP-Man transporter expressed in *L. donovani* (Segawa *et al.*, 2005), clearly substantiates the functionality of CST expressed in *E. coli*.

The biological significance of CST being able to efficiently export the by-product of the sialyltransferase reactions (CMP—a known inhibitor of sialylation) from the Golgi for the import of CMP-Neu5Ac is evident. It is therefore worthwhile considering the underlying mechanism driving this phenomenon more closely. This can be achieved by considering *trans*-acceleration in terms of rate constants. The V_{\max} of transport under zero-*trans* entry conditions is dependent on f_2 and f_{-1} (Scheme 1), the rate constants for the inward translocation of the carrier-substrate (CS) complex and outward movement of the free carrier (C), respectively. While under equilibrium exchange conditions (and CMP-*trans* entry) the V_{\max} depends only on the rate at which the CS complex is translocated, f_2 and f_{-2} . Therefore, the stimulation of CMP-Neu5Ac transport under equilibrium exchange (*trans*-acceleration) and CMP-*trans* entry conditions indicates that the movement of the CS complex from one side of the membrane to the other side is much faster than the translocation of the free carrier. In other words, the binding of the CMP-Neu5Ac or CMP to the free carrier at the *trans* (inside) face would facilitate the return of the carrier site to the *cis* (outside) face, resulting in stimulation. This implies that the binding site alternates between both sides of the membrane, a key feature of the simple mobile carrier (Lieb, 1982; West, 1983; Stein, 1986; Ozeran *et al.*, 1996). A simple mobile carrier must also possess a substrate-specific binding site, and the observed V_{\max}/K_m ratios for experiments performed under zero-*trans* entry and equilibrium exchange conditions must be equal. These criteria for the CST have been fulfilled.

There are many critical points concerning the nature of NST transport raised here and in the recent literature that require discussion. Unanswered questions regarding (1) how transport activities are influenced by membrane components; (2) what adaptor factors that coat ER and Golgi membranes are essential in shuttling proteins between the different compartments; (3) the influence of lipid/protein ratios on the ER and Golgi apparatus; and (4) the ratio between expressed NST and other factors in the glycosylation machinery, such as glycosyltransferases that may form functional units (Sprong *et al.*, 2003). Studies to shed light on these questions would dramatically profit from systems that allow this complexity to be broken down under artificial and controllable assay conditions. This study clearly shows that expression of a functional NST in bacteria is possible. To our knowledge, this represents the first such report, thus providing not only an alternative heterologous expression system for characterizing putative NSTs under zero background conditions, but also the foundation for the production of adequate quantities of functional protein for further downstream evaluation, a process that is currently ongoing in our laboratory.

Materials and methods

Reagents and radiochemicals

All materials, unless otherwise stated, were purchased from Sigma-Aldrich Fine Chemicals (St Louis, MO). CMP-[9-³H]Neu5Ac, UDP-[1-³H]galactose, UDP-[6-³H]glucose and UDP-*N*-[6-³H]acetylglucosamine were purchased from NEN Life Sciences Products (Boston, MA). Protein concentrations were determined using the BCA assay from Pierce Biotechnology (Rockford, IL).

Plasmid

Construction of the bacterial expression vector pTrcME8HA was performed as follows. The coding sequence of pME8 (Eckhardt *et al.*, 1996) was amplified by PCR using the oligonucleotides 5'-GCGGATCCATGGCTCCGGCGAGAG-3' and 5'-GCGGATCCACAC-CAATGATTCTCTCTTT-3' that introduced BamHI restriction sites upstream and downstream of the protein coding sequence. The PCR products were treated with BamHI, purified, and ligated into the BamHI site of the eukaryotic expression vector pEVRF0-HA (kindly supplied by R. Janknecht, The Salk Institute, La Jolla, CA). The coding sequence, including the C-terminal influenza HA epitope (YPYDVPDYASL), was then isolated *via* restriction digestion with BamHI/XbaI and cloned into the respective sites of pTrc99A resulting in the plasmid pTrcME8HA.

Subcellular fractionation of *S. cerevisiae* and in vitro activity assay

S. cerevisiae cells (strain INVSc1; Invitrogen, Karlsruhe, Germany) were transformed with the empty vector pYES2/NT-C using the lithium acetate technique (Ito *et al.*, 1983). Yeast cells were cultured on medium containing 0.67% Bacto-yeast nitrogen base without amino acids but supplemented with L-leucine, L-histidine, L-tryptophan, L-lysine,

adenine and 2% raffinose. Cells were grown to OD₆₀₀ 0.8 and then supplemented with either 2% glucose or galactose and cultured for another 3 h.

Sub-cellular fractionation and *in vitro* transport assay were performed as previously described (Aoki *et al.*, 2001; Segawa *et al.*, 2002) with slight modifications. Cells were harvested by centrifugation at 1500 *g* for 5 min. and washed twice with ice-cold 10 mM NaN₃. The weight of wet cells was measured, and cells were resuspended in zymolyase buffer (3 mL/gram cells; 50 mM KPO₄ (pH 7.5), 1.4 M Sorbitol, 10 mM NaN₃ and 0.3% β-mercaptoethanol) containing 0.6 mg/mL of Zymolyase-100T (ICN Biomedicals, Costa Mesa, CA). The suspension was incubated at 37°C for 20 min. The spheroplasts were collected by centrifugation (1000 *g*, 5 min) and lysed by resuspending in four volumes of lyses buffer (10 mM Hepes-Tris [pH 7.4], 0.8 M sorbitol and 1 mM EDTA) containing a protease inhibitor cocktail (Roche Applied Science). After homogenization with 10 strokes in a Dounce homogenizer, the homogenate was centrifuged (1500 *g*, 5 min) and the resulting supernatant further centrifuged at 10,000 *g* for 10 min. A Golgi rich fraction was subsequently isolated from the post-10,000 *g* supernatant by centrifugation of at 100,000 *g* for 1 h. The pellet was resuspended in lyses buffer (0.8 mL/g cells) and aliquots (100 μL) of the vesicle preparation were snap frozen and stored at -80°C.

For transport assays, equal volumes (50 μL each) of 2 μM radioactive nucleotide sugar (2000–4000 dpm/pmol) in assay buffer (10 mM Tris-HCl [pH 7.0], 0.8 M Sorbitol, 2 mM MgCl₂) and vesicle preparation (equivalent to 75–100 μg of protein) were incubated for 30 s at 30°C. Reactions were stopped by dilution with 1 mL ice-cold assay buffer containing 1 μM of the respective cold nucleotide sugar. Golgi vesicles were collected on nitrocellulose filters (Millipore, Richmond, Australia), subsequently washed three times with assay buffer, dried and measured by liquid scintillation in a LS 5000CE counter (Beckman Coulter, Fullerton, CA).

Expression of mouse CST in *E. coli*

The *E. coli* strain used in expression experiments was DH5α (*endA1*, *hdsR17*(r_K⁺ m_K⁺), *supE44*, *thi-1*, *recA1*, *gyrA*, (NaI^r), *relA1*, Δ(*lacZYA-argF*)_{U169}(*m80lacZAM15*). Overnight cultures of pTrcME8HA-transformed *E. coli* were diluted 1:10 with fresh LB medium containing ampicillin and incubated at 37°C for 45 min with an aliquot (10 mL) of cells being removed just before induction (uninduced cells). Expression was induced by the addition of IPTG to a final concentration of 1 mM and incubated at 37°C. Cells were harvested 4 h post-IPTG induction (induced cells) by centrifugation at 4000 × *g* for 10 min.

Isolation of recombinant mouse CST from *E. coli*

Cell lysis, isolation, and washing of inclusion bodies were performed by a modification of the procedure described in Lin and Cheng (1991). The washed inclusion bodies pellet was solubilized by resuspension in 10 mL of 50 mM Tris-HCl (pH 8.0), 8 M urea, and 5 mM EDTA (deionized) and incubated at room temperature for 1 h. The urea treated suspension was centrifuged at 12,000 × *g* for 30 min and the resulting supernatant added to 100 mL of renaturation buffer A (50 mM Tris-HCl [pH 8.0], 20% [v/v] glycerol,

10 mM MgCl₂, 1 μg/mL leupeptin, 20 μg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride and 25 mM dithiothreitol [DTT]) and gently stirred at 4°C overnight. The suspension was clarified by centrifugation at 13,000 × *g* for 30 min and the supernatant concentrated to ~2 mg/mL in an ultrafiltration stirred cell (Amicon Model 8050, Millipore) fitted with a YM-10 membrane (Amicon MWCO 10 kDa, Millipore). The concentrated supernatant was then dialyzed (dialysis tube type 453105, 25 mm diameter; BioLab, Clayton, Australia) against 100 volumes of renaturation buffer A.

The efficiency of solubilization of the CST from inclusion bodies was monitored by immunoblotting. The effectiveness of transporter renaturation was determined by measuring the transport of CMP-Neu5Ac into proteoliposomes.

Formation of phosphatidylcholine proteoliposomes

Phosphatidylcholine liposomes were prepared for zero-*trans* entry experiments as described in Tiralongo *et al.* (2000). Liposomes preloaded with CMP (CMP-*trans* entry) were prepared by the addition of 0.1 mM CMP to buffer I (15 mM Tris-HCl [pH 7.4], 10 mM MgCl₂ and 1% [v/v] glycerol) before the swelling of the dried phosphatidylcholine. Liposomes were prepared for equilibrium exchange experiments by swelling dried phosphatidylcholine in buffer I containing 5–200 μM CMP-Neu5Ac. The reconstitution of the CST into phosphatidylcholine liposomes was performed as described (Tiralongo *et al.*, 2000).

Sugar nucleotide transport assays into proteoliposomes

CMP-Neu5Ac, UDP-Gal, UDP-Glc, UDP-GlcNAc, and UDP-GalNAc transport into proteoliposomes was measured by a modification of the procedure described in Ozeran *et al.* (1996). Proteoliposomes (0.1 mg) were incubated with 5 μM CMP-Neu5Ac or 2.5 μM of either UDP-Gal, UDP-Glc, UDP-GlcNAc, or UDP-GalNAc containing the corresponding [³H]-labelled sugar nucleotide at a constant specific activity of 100,000–300,000 dpm/nmol in buffer I at 37°C for 5 min in a final reaction volume of 200 μL. Transport was terminated by passing 100 μL of the reaction mixture through a Bio-Spin-30 column (Bio-Rad, Regents Park, Australia) and collecting the void volume. The void volume (~100 μL) was added to 5 mL Eco-Lite scintillation cocktail (ICN Pharmaceuticals, Costa Mesa, CA) and counted in a Tri-Carb 2000CA liquid scintillation analyser (United Technologies Packard, Markham, UK). A negative control for sugar nucleotide transport was afforded by performing parallel assays where 100 μM DIDS was added.

CMP-Sia transport under zero-*trans* entry, CMP-*trans* entry and equilibrium-exchange conditions were performed in 96-well round bottom plates at 4–6 CMP-[³H]Neu5Ac (constant specific activity of 5000–10,000 dpm/nmol) concentrations between 5 and 250 μM at 37°C for an incubation time of 5 min. Each well typically contained 30 μL of buffer I containing CMP-Neu5Ac at a given concentration. To each well, 30 μL of proteoliposomes (0.1–0.15 mg), pre-incubated at 37°C for 5 min, was added. To measure the passive diffusion of CMP-Neu5Ac into the proteoliposomes, assays were also performed in the presence 100 μM DIDS. Proteoliposomes were preincubated with DIDS at 37°C for 5 min before their use. All assays were performed in triplicate.

Transport was terminated by passing 45 μ L of reaction mixture through Multiscreen BV plates (1.2 μ m Durapore; Millipore) loaded with Sephadex G-50 resin (fine; Pharmacia, Rydalmere, Australia). Multiscreen mini-columns were centrifuged at 2000 rpm (910 \times g) for 5 min and the void volume collected. Following collection, 250 μ L of Microscint-40 scintillation cocktail (Canberra Packard, Canberra, Australia) was added to each well and the plates were counted on a TopCount microplate scintillation counter (Canberra Packard). All kinetic data were analysed using the Enzyme Kinetic Program (version 2.0) (Hearne Scientific Software, Melbourne, Australia). This program is adapted from that described by Cleland (1979).

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Abbreviations

CMP-Neu5Ac, CMP-*N*-acetylneuraminic acid; CMP-Sia, CMP-sialic acid; CST, CMP-sialic acid transporter; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; ER, endoplasmic reticulum; HA, hemagglutinin; IPTG, isopropyl- β -D-thiogalactopyranoside; Neu5Ac, *N*-acetylneuraminic acid; NSTs, nucleotide sugar transport proteins; Sia, sialic acid; UDP-Gal, UDP-galactose; UDP-GalNAc, UDP-*N*-acetylgalactosamine; UDP-Glc, UDP-glucose; UDP-GlcNAc, UDP-*N*-acetylglucosamine.

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