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 (Abeijon et al., 1996a; Tabuchi et al., 1997), mammals (Eckhardt et al., 1996; Ishida et al., 1996; Guillem 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 (Bernsnone 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-acetylgalactosamine (UDP-GlcNAc) transporter was identified by complementation cloning in Kleobyromyes lactis (Guillem 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 (Abeijon et al., 1996a; Guillem 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; Bernsnone and Hirschberg, 2000), the testing of heterologously expressed recombinant proteins displayed multisubstrate specificity...
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 stan-
dard 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 labora-
tories 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 pro-
moter. 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 trans-
port, 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 artifi-
cial liposomes gave rise to proteoliposomes with selective
CMP-sialic acid (CMP-Sia) transport. Kinetic studies car-
died 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.

The expression of mouse CST in E. coli

The expression of the mouse CST in E. coli was tightly con-
trolled by the isopropyl-β-D-thiogalactopyranoside-(IPTG)
inducible trplac 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 sequestrated in the inclusion body
pellet (lane 3).

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

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
expression under the control of a galactose inducible pro-
moter. Golgi-rich fractions isolated from transformed cells
were used to test transport activity with five different nucle-
otide sugars. Unexpectedly, all isolated Golgi fractions includ-
ing the Golgi fraction isolated from mock transformed yeast
cells demonstrated a significant increase in UDP-Gal trans-
port (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 cul-
tivated in galactose-containing media transport UDP-Gal
with efficiency similar to the endogenous UDP-Glc trans-
port. These results clearly underline the difficulties associ-
ated with characterizing recombinant NST specificities using
the yeast system, particularly as shown here when multi-
functionality, and consequently an additional level of com-
plexity, 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 expres-
sion 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.

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.

The expression of mouse CST in E. coli

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pellet (lane 3).
CMP-sialic acid transporter expression in *E. coli*

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 led 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-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 μM 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′-diisothiocyanato-stilbene-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).

![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).](image1)

![Fig. 3. The transport of CMP-Neu5Ac into proteoliposomes.](image2)
Table I. The kinetics of CMP-Neu5Ac transport into proteoliposomes under zero-trans entry, CMP-trans entry and equilibrium exchange conditions

<table>
<thead>
<tr>
<th>Experiment¹</th>
<th>$K_m$ (µM)b</th>
<th>$V_{max}$ (nmol/mg/h)b</th>
<th>$V_{max}/K_m$ ratioc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-trans entry</td>
<td>32.9 ± 3.8</td>
<td>21.6 ± 3.6</td>
<td>0.65 ± 0.20</td>
</tr>
<tr>
<td>CMP-trans entryd</td>
<td>106.0 ± 23.8</td>
<td>56.6 ± 3.7</td>
<td>0.53 ± 0.23</td>
</tr>
<tr>
<td>Equilibrium exchange</td>
<td>67.2 ± 17.2</td>
<td>44.1 ± 5.8</td>
<td>0.65 ± 0.28</td>
</tr>
</tbody>
</table>

¹Transport of CMP-Neu5Ac into proteoliposomes was linear for time points up to and including 5 min.
²Mean ± standard deviation (SD) for at least three separate experiments.
³$V_{max}/K_m$ ratios are expressed as arbitrary units.
⁴Designates that proteoliposomes have been preloaded with CMP (present in the trans compartment) before measuring CMP-Neu5Ac transport.

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 $V_{\text{max}}/K_m$ 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 HFR1c (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 transporters (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 μM). 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 μM) (Berninsone et al., 1997) and the rat liver Golgi CST (1.4 μM) (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 μM) expressed in *L. donovani* (Segawa et al., 2005) and that from isolated microsomes (0.3 μM) (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 reconstructed 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...
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 shutting 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′-GGCGATCATGCTCCGGCGAGAG-3′ and 5′-GGCGATCCCAACCAATGATTCTCCTCTTTT-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 pEVRF0HA (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,

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**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 \( V_{\text{max}} \) 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 \( V_{\text{max}} \) 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 \( V_{\text{max}}/K_m \) ratios for experiments performed under zero-*trans* entry and equilibrium exchange conditions must be equal. These criteria for the CST have been fulfilled.
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 NaNO₃. 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 NaNO₃ 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 (10,000 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 37°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, hsdR17(rK–M–K+), supE44, thi-1, recA1, gyrA, (Nalρ), relA1, ΔlacZYA-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 the 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 phosphatidycholine proteoliposomes

Phosphatidycholine 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 phosphatidycholine. Liposomes were prepared for equilibrium exchange experiments by swelling dried phosphatidycholine in buffer I containing 5–200 μM CMP-Neu5Ac. The reconstitution of the CST into phosphatidycholine 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 μL of reaction mixture through Multiscreen BV plates (1.2 μm Durapore; Millipore) loaded with Sephadex G-50 resin (fine; Pharmacia, Rydalmere, Australia). Multiscreen mini-columns were centrifuged at 2000 rpm (910 × g) for 5 min and the void volume collected. Following collection, 250 μL of Microcentrifuge 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′-disothiocyanato-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-acetylgalactosamine.

References


