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Transport of CMP-*N*-glycoloylneuraminic acid into mouse liver Golgi vesicles

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CMP-Neu5Gc has been shown to be transported into mouse liver Golgi vesicles by a specific carrier the characteristics of which were investigated in detail. In the system employed, CMP-Neu5Gc enters the Golgi vesicles within 2 min; transport was saturable with high concentrations of the sugar-nucleotide and was dependent on temperature. A kinetic analysis gave an apparent K_m of 1.3 μM and a maximal transport velocity of 335 pmol/mg protein per min. Almost identical values were obtained with CMP-Neu5Ac, under the same incubation conditions. Furthermore, the uptake of CMP-Neu5Gc was inhibited by CMP-Neu5Ac, a substrate analogue. Conversely, the uptake of CMP-Neu5Ac was inhibited by CMP-Neu5Gc to the same extent, leading to the conclusion that the transport of CMP-Neu5Ac and CMP-Neu5Gc is mediated by the same carrier molecule. This transport system for CMP-Neu5Gc involves both CMP and CMP-Neu5Gc since intravesicular CMP induced the entry of external CMP-Neu5Gc.

Transport; CMP-sialic acid; Golgi vesicle

1. INTRODUCTION

The sialic acids are a family of *N*- and *O*-substituted derivatives of neuraminic acid (5-amino-3,5-di-deoxy-D-glycero-β-D-galacto-non-ulosonic acid), whose two principal members are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycoloylneuraminic acid (Neu5Gc) [1]. They occur most frequently as the terminal sugar residues on the glycan chains of many glycoconjugates. During the formation of glycoconjugate-bound Neu5Ac, the activated sugar nucleotide, CMP-Neu5Ac, which is present in the cytosol, has to gain access to the lumen of the Golgi apparatus where it serves as the sialic acid donor for the sialyltransferases.

Several laboratories have demonstrated the uptake of CMP-Neu5Ac into mouse liver microsomes [2], rat liver Golgi vesicles [3], and plasma-membrane-permeabilized mouse thymocytes [4]. The saturation of this uptake by increasing concentrations of CMP-Neu5Ac, its inhibition by substrate analogues and dependence on temperature indicate that a specific carrier system is involved in the transport of CMP-Neu5Ac into the Golgi lumen. The recent finding that the biosynthesis of Neu5Gc results from the hydroxylation of CMP-Neu5Ac by a soluble, possibly cytosolic enzyme [5] prompts the suggestion that the CMP-Neu5Gc product might also enter the Golgi lumen by means of a similar carrier system.

In the present paper, we characterize the uptake of CMP-[^{14}C]Neu5Gc into Golgi vesicles isolated from mouse liver. The data provide evidence that these membranes contain a high-affinity transport system for CMP-Neu5Gc. A kinetic analysis and

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Abbreviations: CMP-Neu5Ac, cytidine-5'-monophospho-*N*-acetyl-β-D-neuraminic acid; CMP-Neu5Gc, cytidine-5'-monophospho-*N*-glycoloyl-β-D-neuraminic acid; CMP, cytidine monophosphate

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competition experiments are performed to examine whether CMP-Neu5Gc and CMP-Neu5Ac share the same carrier molecule. The effect of luminal CMP on the transport process is also studied.

2. MATERIALS AND METHODS

2.1. *Chemicals and reagents*

All reagents were of analytical grade. CMP-[^{14}C]Neu5Ac (9.69 GBq/mmol) was purchased from Amersham International (Bucks., England). CMP-Neu5Ac, CMP, bovine serum albumin and fetuin were of the highest purity available and were obtained from Sigma (St. Louis, MO).

2.2. *Isolation of Golgi vesicles from mouse liver*

Golgi vesicles, derived from the livers of 4 week-old Swiss mice, were isolated using a modification of the subcellular fractionation procedure originally described by Balch et al. [6]. The mice were killed with chloroform, the liver excised and placed in ice-cold STM (0.25 M sucrose; 10 mM Tris-HCl, pH 7.4; 1 mM $MgCl_2$). All subsequent operations were carried out at 4°C. After passage through a tissue press, the finely divided liver was homogenized with 4 strokes in a 30 ml Potter homogenizer (B. Braun, Melsungen) at low speed (750 rpm) into 3 vols of STM buffer. The crude homogenate was adjusted to 1.4 M sucrose by the addition of ice-cold 10 mM Tris-HCl (pH 7.4) containing 2.3 M sucrose and 1 mM EDTA. 3 ml of the latter buffer was loaded into a Beckman SW 27 tube and overlaid with 3 ml of 10 mM Tris-HCl (pH 7.4) containing 1.6 M sucrose, and then with 10 ml of the 1.4 M sucrose-containing crude homogenate. This was subsequently overlaid with 10 ml of 10 mM Tris-HCl (pH 7.4) containing 1.2 M sucrose and finally with 10 ml of 0.8 M sucrose in 10 mM Tris-HCl, pH 7.4. The gradients were centrifuged for 2.5 h at 25 000 rpm (90 000 × *g*) in a Beckman SW 27 rotor. The turbid band at the 0.8 M/1.2 M sucrose interface was harvested in a minimum volume by syringe puncture, diluted two-fold in 10 mM Tris-HCl, pH 7.4, and centrifuged at 90 000 × *g* for 1 h in the SW 27 rotor. The resulting pellet of Golgi membranes could be used immediately, or more conveniently, frozen and stored at −80°C. The frozen membrane fractions should be thawed shortly before the assay by a short exposure to 37°C and maintained on ice prior to use.

The following marker enzymes were used to confirm the purity and the recovery of isolated Golgi-derived vesicles. They were enriched 143-fold in sialyltransferase specific activity (1.6% yield) [7], 0.96-fold in glucose-6-phosphatase specific activity (0.01% yield) [8], 5-fold in 5′-nucleotide specific activity (0.06% yield) [8], 15-fold in β-hexosaminidase specific activity (0.175% yield) [8].

2.3. *Preparation of radioactive and non-radioactive CMP-Neu5Gc*

Unless stated otherwise, all procedures were carried out at 4°C.

2.3.1. Extraction of soluble protein from mouse liver

Livers from freshly killed Balb/c mice were homogenized in 5 vols (ml/g wt tissue) of ice-cold 50 mM Tris-HCl, pH 7.4, using a Potter-Elvehjem homogeniser and centrifuged at 120 000 × *g* (Beckman 45Ti rotor; 39 000 rpm) for 1 h. The resulting supernatant was either used immediately or stored at −70°C until use.

2.3.2. Enzymatic hydroxylation of CMP-[^{14}C]Neu5Ac by CMP-Neu5Ac hydroxylase from mouse liver (EC 1.14.99.18)

37 kBq CMP-[^{14}C]Neu5Ac (purchased from New England Nuclear, D-6072 Dreieich; spec. act. 10.1 GBq/mmol) was incubated for 3 h at 37°C with 20 ml of the mouse liver supernatant in the presence of 1 mM NADH and 0.5 mM $FeSO_4 \cdot 7H_2O$. The reaction was stopped by cooling on ice and protein was removed by ultrafiltration at 4°C (Sartorius membrane filter; 20 000 Da molecular mass limit). The clear, colourless eluate from ultrafiltration was diluted two-fold with water and applied to a 1.4 × 26 cm column of Dowex 1 × 8 (200–400 mesh; HCO_3^- counterion) equilibrated in 10 mM triethylamine hydrogencarbonate (pH 7.8). The column was washed with 2 vols of equilibration buffer and developed with an 800 ml linear gradient up to 1 M triethylamine hydrogencarbonate (pH 7.8). Fractions containing CMP-[^{14}C]Neu5Gc (emerging between 0.3 M and 0.4 M buffer) were pooled and lyophilised. The residue was resuspended in 1.5 ml of 0.3 M triethylamine hydrogencarbonate (pH 7.8) and gel filtered on a 1.5 × 80 cm column of Sephadex G-10 (superfine) equilibrated in 5 mM Tris-HCl (pH 7.4), collecting 1 ml fractions. Fractions constituting the main peak of radioactivity were pooled, lyophilised and resuspended in 1 ml water.

The final product contained less than 1% CMP-[^{14}C]Neu5Ac as revealed by radio-TLC of the intact and acid-hydrolysed product [5]. Furthermore, no free sialic acid was detected by HPLC analyses using the conditions described by Gross et al. [9] and radiochemical detection.

Non-radioactive CMP-Neu5Gc was synthesised from CTP and Neu5Gc using the CMP-sialate synthetase (EC 2.7.7.43) present in a soluble protein extract of frog liver [10] and purified by the same ion-exchange and gel-filtration procedures described above.

2.4. *Protein determination*

Proteins from cells or Golgi vesicles, solubilized in the presence of SDS were estimated using a modification of the method of Lowry with bovine serum albumin as standard [11].

2.5. *Incubation of Golgi vesicles with CMP-[^{14}C]sialic acid*

Golgi vesicles, which were found to be sealed and of the same membrane topography as in vivo (see section 3) were incubated in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 150 mM KCl, 1 mM $MgCl_2$, 5 mM $MnCl_2$ and varying amounts of radiolabelled CMP-[^{14}C]sialic acid, in a total volume of 100 μl. After incubation, the uptake of CMP-[^{14}C]sialic acid by the Golgi vesicles and the transfer of [^{14}C]sialic acid onto endogenous acceptors were determined by rapid filtration on millipore filters (GS type, 2.5 cm, pore size 0.22 μm), using a modified procedure originally described by Fleischer [12].

The uptake of CMP-[^{14}C]sialic acid was determined by adding 1 ml of ice-cold stop buffer (0.25 M sucrose; 10 mM Tris-HCl, pH 7.4; 1 mM $MgCl_2$) to one half of the incubation medium, followed by rapid filtration. The filter and retained vesicles were washed with 6 × 1 ml of the cold stop buffer, and

the radioactivity associated with the filter was then estimated by liquid scintillation counting.

The transfer of [^{14}C]sialic acid was determined by precipitation of the glycosylated acceptors present in the second half of the incubation medium with 5% phosphotungstic acid in 2 N HCl, followed by filtration through Millipore filters and further washing with 5% (w/v) trichloroacetic acid. The radioactivity associated with the filter was then counted.

We have checked for non-specific adsorption of CMP-[^{14}C]-sialic acid by addition of a 100-fold excess of unlabelled CMP-sialic acid after incubation and prior to filtration on a Millipore filter. It was shown that there was no change in the amount of filter-associated CMP-[^{14}C]sialic acid.

3. RESULTS AND DISCUSSION

3.1. *Latency of sialyltransferase towards CMP-[^{14}C]Neu5Gc*

To be of physiological significance, the assay of CMP-[^{14}C]Neu5Gc uptake had to be performed only with Golgi vesicles shown to be sealed and of the same membrane topography as in vivo [13]. When sialyltransferases were assayed with increasing concentrations of Triton X-100 using labelled CMP-[^{14}C]Neu5Gc and unlabelled asialofetuin as the macromolecular exogenous acceptor [5], an increased incorporation was observed (fig.1), indicating that 91% of the Golgi vesicles were well sealed and of the same topographical orientation as in vivo. Furthermore, this approach provides evidence that CMP-[^{14}C]Neu5Gc has to be capable of crossing intracellular membranes in order to be used by the sialyltransferases present in the lumen of the Golgi vesicles.

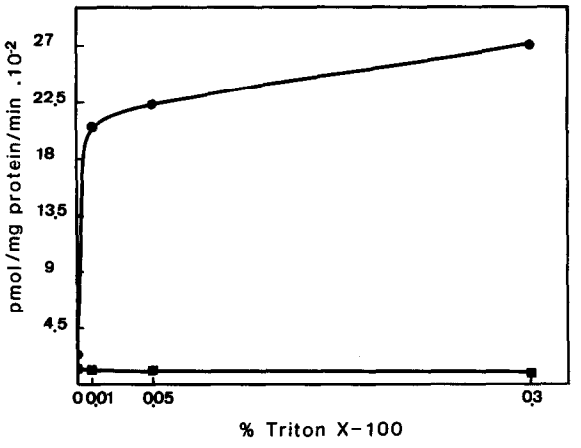

Fig.1. Latency of sialyltransferase with endogenous acceptors and asialofetuin as exogenous acceptor. Golgi vesicles were incubated at 37°C for 4 min with CMP-[^{14}C]Neu5Gc (1 μM, 63 000 dpm) in the presence or absence of asialofetuin (4 mg/ml) as exogenous acceptor, and various concentrations of Triton X-100 ranging from 0 to 0.3%. After incubation, the glycosylated acceptors were precipitated with phosphotungstic acid and washed by filtration with 5% trichloroacetic acid. The radioactivity associated with the filter was then counted. The incorporation onto asialofetuin (●—●) was obtained by the difference between two incubations one lacking exogenous acceptor (■—■).

3.2. *Time course of CMP-[^{14}C]Neu5Gc penetration into mouse liver Golgi vesicles and of Neu5Gc transfer onto endogenous acceptors*

Mouse liver Golgi vesicles were incubated with 1.5 μM CMP-[^{14}C]Neu5Gc. The rapid filtration technique resulted in virtually complete separation of the vesicles and incubation medium within seconds, in contrast to the alternative approach which involves a lengthy period of centrifugation [14]. Using this procedure, the rate of uptake of radiolabelled CMP-Neu5Gc by Golgi vesicles was shown to be linear and rapid for the first 4 min, after which it decreased (fig.2). We also checked for [^{14}C]Neu5Gc transfer onto endogenous acceptors as a function of time (fig.2). Presumably, because of a high sialyltransferase activity and the low concentration of CMP-[^{14}C]Neu5Gc used,

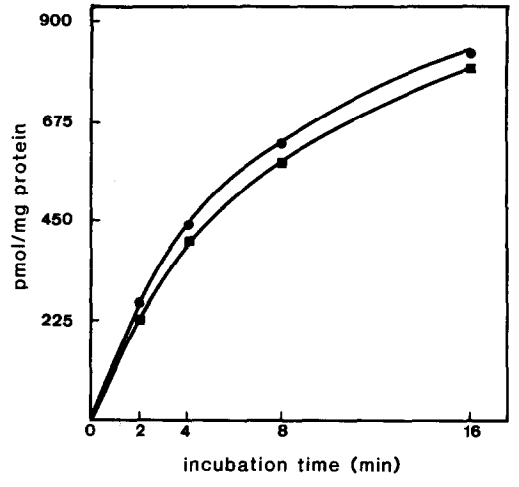

Fig.2. Time course of CMP-[^{14}C]Neu5Gc penetration into mouse liver Golgi vesicles and Neu5Gc transfer onto endogenous acceptors. CMP-[^{14}C]NeuGc (1.5 μM, 94 000 dpm) was incubated with Golgi vesicles in STM buffer (pH 7.4) at 37°C for the times listed in the figures. (●—●) The total vesicle-associated radioactivity and the acid-precipitable radioactivity (■—■) (transport and transfer, respectively) were measured as described in section 2.

there was very little difference between total and acid-precipitable radioactivity.

The transport of CMP-[^{14}C]Neu5Gc was also dependent on temperature: uptake at 4°C being 9% of that at 37°C (data not shown).

3.3. *Concentration dependence of CMP-[^{14}C]-Neu5Gc transport*

The penetration rate of CMP-[^{14}C]Neu5Ac was saturable with increasing concentrations of CMP-[^{14}C]Neu5Ac (0.5–20 μM) in the medium (fig.3a). Initial uptake rates were measured with a 2 min incubation, and the resulting transfer of [^{14}C]Neu5Ac onto endogenous acceptors studied. The points of the double reciprocal plot were fitted by linear regression analysis to a straight line (r = 0.998) which gave an apparent K_m of 1.3 μM and a maximal velocity of 335 pmol/mg protein per min (fig.3a, inset). These values are of the same order of magnitude as those previously reported by Sommers and Hirschberg [14] for transport of CMP-Neu5Ac into rat liver Golgi.

Using exactly the same incubation conditions, we have shown (fig.3b) that the transport of CMP-Neu5Gc is saturable with high concentrations of CMP-[^{14}C]Neu5Gc in the medium, giving rise to an apparent K_m of 1.25 μM and a maximal velocity of 325 pmol/mg protein per min (fig.3b, inset).

The results obtained above on the temperature dependence and substrate concentration dependence of CMP-Neu5Gc transport establish the existence of a carrier mechanism for CMP-Neu5Gc in mouse liver Golgi vesicles.

3.4. *Specificity of CMP-Neu5Gc transport*

To evaluate the specificity of this carrier process, radiolabelled CMP-[^{14}C]Neu5Gc uptake by Golgi vesicles was assayed in the presence of various concentrations of unlabelled CMP-Neu5Ac up to 100-fold greater (500 μM) than that of radiolabelled CMP-[^{14}C]Neu5Gc (5 μM). Uptake of CMP-[^{14}C]Neu5Gc was inhibited by 50% at 8.5 μM CMP-Neu5Ac as shown in fig.4b. The results of similar competitive studies performed using radiolabelled CMP-[^{14}C]Neu5Ac and unlabelled CMP-Neu5Gc under identical incubation conditions are shown in fig.4a. 50% inhibition was obtained at 5.6 μM CMP-Neu5Gc. This behaviour is consistent with the existence of a single carrier molecule present in the mouse liver Golgi vesicles mediating the uptake of both CMP-Neu5Ac and CMP-Neu5Gc substrates. The presence of a *N*-acetyl group in the sugar moiety does not constitute an important recognition feature as it can be replaced by a *N*-glycoloyl group without any change in the uptake rate of the CMP-sialic acid.

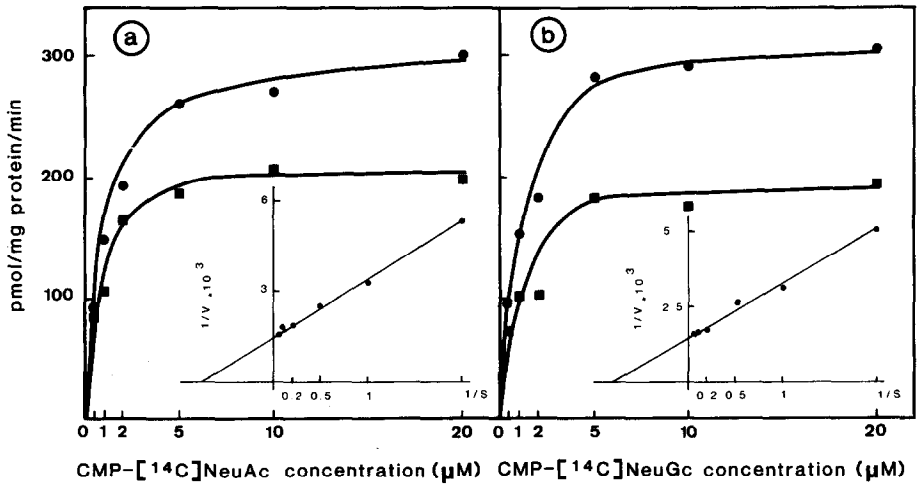

Fig.3. Concentration dependence of CMP-Neu5Gc transport. Golgi vesicles were incubated for 2 min with various concentrations of either CMP-[^{14}C]Neu5Ac (a), or CMP-[^{14}C]Neu5Gc (b). After incubation, the total vesicle-associated radioactivity (●—●, transport) and the acid-precipitable radioactivity (■—■, transfer) were measured as described in section 2. The insets show the Lineweaver-Burk plots.

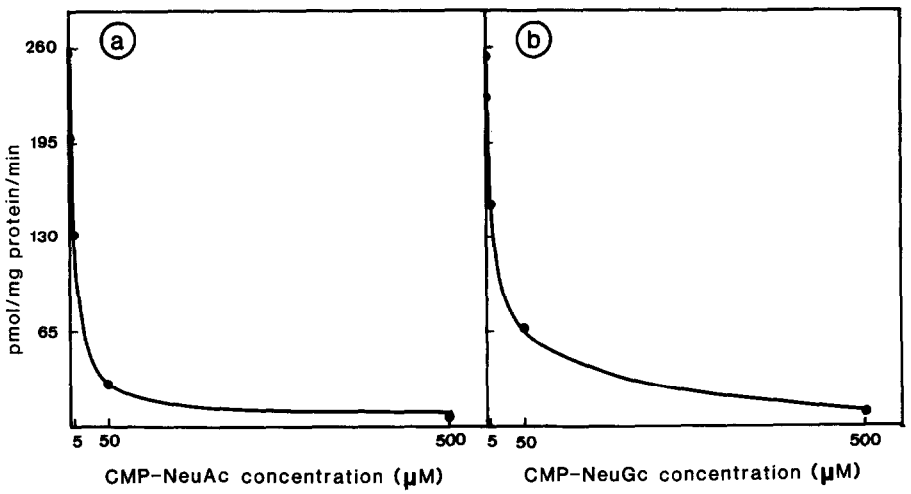

Fig.4. Mutual competition between CMP-Neu5Ac and CMP-Neu5Gc for their uptake by mouse liver Golgi vesicles. Golgi vesicles were incubated 4 min at 37°C with either CMP-[^{14}C]Neu5Ac (5 μM, 277 500 dpm) and various concentrations of unlabelled CMP-Neu5Gc ranging from 0 to 500 μM (a), or with CMP-[^{14}C]Neu5Gc (5 μM, 133 200 dpm) and various concentrations of CMP-Neu5Ac ranging from 0 to 500 μM (b). Total vesicle-associated radioactivity (transport) was measured as described previously in section 2.

3.5. *Effect of intravesicular CMP*

Finally, as was hypothesized by Capasso and Hirschberg [3], we have checked for the existence of an antiport mechanism involving the exchange of CMP with CMP-Neu5Gc. We set out to determine whether the uptake of CMP-Neu5Gc from the cytoplasm was coupled with an exchange of CMP from the lumen by preloading Golgi vesicles with 100 μM CMP for 10 min. Previous experiments showed that Golgi vesicles were able to take up CMP (data not shown) as already described by Fleischer [12,15]. After removing extravesicular CMP by repeated centrifugation and washing, the CMP-preloaded vesicles were then incubated with 2 μM CMP-[^{114}C]Neu5Gc and its uptake measured.

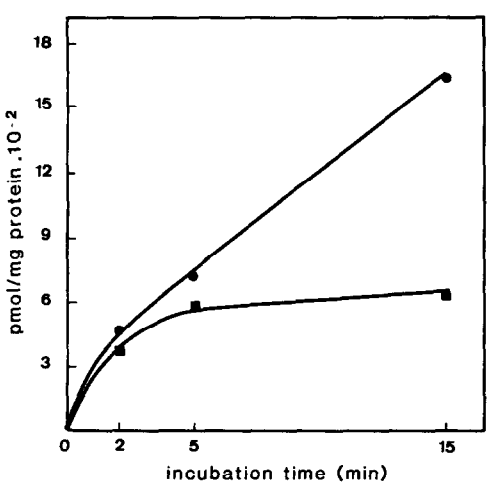

Fig.5. Effect of preloading Golgi vesicles with CMP on the uptake of CMP-[^{14}C]Neu5Gc. Golgi vesicles were preincubated for 10 min at 37°C with 100 μM CMP (●—●) or without CMP (■—■). The extravesicular CMP was removed by centrifugation and further washing with the STM buffer and the Golgi vesicles were incubated for various times shown in the figure with 2 μM CMP-[^{14}C]Neu5Gc at 37°C. The total vesicle-associated radioactivity was measured as described in section 2.

The results in fig.5 show that under these conditions, the total radioactivity associated with the vesicles, corresponding to CMP-Neu5Gc uptake, was enhanced with time in CMP-preloaded vesicles. This stimulating effect of increased lumenal CMP concentrations suggests an exchange mechanism between intravesiclar CMP and extravesicular CMP-Neu5Gc.

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