Dependence of rat liver CMP-*N*-acetylneuraminate:GM_1 sialyltransferase (SAT IV) activity on the ceramide composition of GM_1 ganglioside

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Abstract The dependence of CMP-*N*-acetylneuraminate:GM_1 sialyltransferase (SAT IV) activity of rat liver Golgi apparatus on GM_1 ganglioside ceramide composition was evaluated. SAT IV activity was assayed on GM_1 molecular species carrying homogeneous ceramide moieties containing long chain bases of different length (18 or 20 C atoms) unsaturated or not, linked to 14:0, 16:0, 18:0 or 22:0 fatty acids. The results obtained in the presence of the detergent Triton CF-54, when enzyme and substrate are presumably part of the same supramolecular structure, show that either the long chain base or the fatty acid composition can affect enzyme activity. This feature was not displayed when GM_1 was embedded in dipalmitoylphosphatidylcholine vesicles in the absence of detergent. Under the latter conditions, the enzyme was not sensitive to the lipid composition of GM_1 but to the ganglioside/phospholipid ratio in the vesicles. These results indicate for the first time that SAT IV is affected by the lipid composition of the substrate and strengthen the hypothesis that glycosyltransferases may contribute to control the cellular glycosphingolipid ceramide pattern.

Key words: Sialyltransferase; GM_1; Ceramide

1. Introduction

It is known that gangliosides differ in the chemical composition of their oligosaccharide and lipid moieties. In fact, long chain bases (LCB) and fatty acids, ceramide components, may vary in both length and unsaturation, yielding several molecular species [1,2]. Remarkable differences in the lipid composition are observed in extraneural tissues, including liver [3], and, for a given tissue, also between gangliosides deriving from distinct biosynthetic pathways (e.g. the so-called 'a' and 'b' series) [2]. Ganglioside biosynthesis proceeds through the sequential transfer of monosaccharide units from sugar nucleotide donors to appropriate sphingolipid acceptors, catalyzed by specific glycosyltransferases [4]. Most of these enzymes, and among them rat liver CMP-*N*-acetylneuraminate:GM_1 sialyltransferase (SAT IV), have been localized within the Golgi apparatus and have been shown to be integral membrane proteins [5]. Previous findings suggest that the ganglioside pattern may be regulated, at least in part, at the level of sialyltransferase activities. Accordingly, the differential expression of glycosyltransferases is very likely a major factor determining the distinct cell glycosphingolipid pattern encountered at different stages of growth, development and oncogeny [6,7]. Moreover, it has been shown that glycosyltransferases are able to recognize different molecular species of the substrate, providing a mechanism aiming to maintain the glycolipid ceramide pattern of a given tissue [8]. The present work was carried out in order to assess whether SAT IV differentially affects molecular species of the same substrate, namely GM_1 ganglioside, carrying different, homogeneous, ceramide moieties.

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Abbreviations: DPPC, dipalmitoylphosphatidylcholine; CMP-Neu5Ac, cytidine 5'-monophospho-*N*-acetylneuraminic acid; LCB, long chain base; SAT IV, CMP-*N*-acetylneuraminate:GM_1 sialyltransferase

2. Materials and methods

2.1. Materials

Commercial chemicals were of the highest purity available. Solvents were redistilled before use. HPTLC plates, sucrose for density gradient centrifugation and common chemicals were from Merck (Darmstadt, Germany). Cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac), Triton CF-54, mucin, asialofetuin and fatty acid anhydrides were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dipalmitoylphosphatidylcholine (DPPC) was from Fluka AG (Buchs, Switzerland). CMP-[9-^{3}H]Neu5Ac (21.2 Ci/mmol) was from Du Pont New England Nuclear (NEN) (Boston, MA, USA). NaB^3H_4 (5 Ci/mmol) and UDP-[U-^{14}C]galactose (340 mCi/mmol) were from Amersham International (Amersham, UK).

2.2. Preparation and radiolabeling of ganglioside molecular species

GM_1 ganglioside molecular species were prepared following essentially a procedure already described [9], with minor modifications. Briefly, lyso-GM_1 homogeneous in the saccharide portion but not in the lipid moiety was prepared starting from GM_1 extracted and purified from beef brain (native GM_1) [10]. GM_1 homogeneous in the fatty acid moiety, but not in LCB, was obtained from lyso-GM_1 by reaction with fatty acid anhydrides (14:0, 16:0, 18:0 and 22:0) and purified by silica gel column chromatography. Further separation into GM_1 molecular species having homogeneous LCB (C18:1 or C20:1) moiety was performed by reverse-phase HPLC [9]. Seven GM_1 molecular species were obtained and used for SAT IV activity assays: C18:1–14:0, C18:1–16:0, C18:1–18:0, C18:1–22:0, C18:0–18:0, C20:1–18:0 and C20:0–18:0. GM_1 molecular species C18:1–18:0, C20:1–18:0, radiolabeled at the level of C-3 of the LCB, were prepared as described [11]. The specific radioactivity was 1.3 Ci/mmol. Standard GD_{1a} ganglioside was obtained and radiolabeled as previously described [12]. The final purity of all gangliosides was over 99%.

2.3. Preparation of vesicles containing GM_1

Mixtures of DPPC and GM_1, dried and lyophilized from chloroform/methanol (2:1, v/v) solutions, were resuspended in 154 mM cacodylate buffer, pH 6.5, and vesicles prepared by extrusion (10 times) through 100 nm pore filters (Nucleopore, Pleasanton, CA, USA), using an N_2 pressure-operated extruder (Lipoprep, Ottawa, Canada). Vesicles were used within 1 day from the preparation for SAT IV assay. GM_1 concentrations were calculated assuming that glycolipid molecules were equally partitioned into the outer and inner layer of the vesicles and therefore only 50% of total GM_1 was accessible to the enzyme [13].

2.4. Preparation of rat liver Golgi membranes

Liver Golgi membrane fractions were prepared from male Sprague-Dawley rats (Charles River, Milan, Italy) weighing 150–175 g, as already reported [14].

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2.5. Assay of SAT IV activity on GM_1 ganglioside using CMP-[9-^{3}H]Neu5Ac

SAT IV activity was routinely assayed upon GM_1 ganglioside according to a widely used procedure [15–17], by radiochemical quantification of the product (GD_{1a} ganglioside). Briefly, incubation mixtures contained 0.2% Triton CF-54, 20–30 μg of the Golgi fraction (as protein), 0.025–0.4 mM GM_1 and 0.5 mM CMP-[9-^{3}H]Neu5Ac (100 000 dpm) as sialic acid acceptor and donor, respectively, in a final volume of 50 μl of 154 mM cacodylate buffer, pH 6.5. Incubation was at 37°C for 1 h. Blanks were prepared by omitting GM_1 in the reaction mixture. At the end of the incubation, the reaction mixture was applied on Whatman 3MM paper and developed by descending chromatography in 1% tetraborate, followed by radioactivity counting of the appropriate areas [15].

SAT IV activity was also assayed on GM_1 embedded in DPPC vesicles. In this case, a mixture was prepared in the absence of detergent and contained 0.025–0.25 mM GM_1 in vesicles, 20–30 μg (as proteins) of Golgi fraction (previously sonicated in a bath for a few seconds [18]), in a final volume of 50 μl of 154 mM cacodylate buffer pH 6.5. CMP-[9-^{3}H]Neu5Ac (0.5 mM, 100 000 dpm) was added and the mixture was incubated at 37°C for 1 h. At the end of the incubation the whole reaction mixture was processed as above. Drying of the sample loaded on chromatographic paper before descending chromatography prevented separation of radiolabeled CMP-Neu5Ac from newly synthesized radiolabeled GD_{1a}, and therefore it was avoided. Reaction product characterization was performed as already described [16].

2.6. Assay of SAT IV activity using radiolabeled GM_1

Reaction mixtures almost identical to those described above were prepared with the only modification that radiolabeled GM_1 (100 000 dpm, corresponding to about 38 pmol) was added besides unlabeled GM_1, and that unlabeled CMP-Neu5Ac was used [19]. Blank mixtures were prepared using an heat-inactivated enzyme source. After incubation the samples were submitted to lipid extraction by adding 4 vols. of tetrahydrofuran, vortexing and centrifuging (10 000×*g*, 10 min). Aliquots of the supernatant were chromatographed by HPTLC, (first run: chloroform/methanol/H_2O, 110:40:6; second run: chloroform/methanol/$CaCl_2$ 0.2%, 50:42:11 v/v) and scanned with a radiochromatoscanner [20]. Reaction product characterization was performed as already described [16].

2.7. Other methods

The kinetic parameters K_m and V_{max} were determined from the double-reciprocal plot according to Lineweaver-Burk, by linear regression [21]. Golgi markers enzymes, CMP-Neu5Ac:asialofetuin sialyltransferase and UDP-Gal:asialomucin galactosyltransferase were assayed according to [22] and [23]. Gangliosides were determined as bound Neu5Ac [24], using Neu5Ac as standard; proteins [25] using bovine serum albumin as the standard.

3. Results and discussion

Many studies dealt with the specificity of sialyltransferases towards the oligosaccharide moiety of their glycolipid substrates. In particular, it is known that rat liver CMP-*N*-acetylneuraminate:GM_1 sialyltransferase (SAT IV), an enzyme localized in the Golgi apparatus, is able to catalyze the synthesis of GM_3, GM_{1b}, GD_{1a}, and GT_{1b} gangliosides [26]. In contrast, the specificity of sialyltransferases towards molecular species of the same substrate, carrying different ceramide moieties, has not yet been fully investigated. Remarkably, existing reports indicate that the chemical composition of the ceramide portion can affect the enzyme activity [27,28].

In the present study, for the first time, the GD_{1a}-synthetase activity of SAT IV was studied on a number of GM_1 ganglioside molecular species, differing in either LCB or fatty acid composition, generally representative of the variability normally encountered in vivo [2,3]. The enzyme source used for the study of SAT IV activity was a highly purified Golgi apparatus fraction prepared from rat liver. The assay of Golgi marker enzymes CMP-Neu5Ac:asialofetuin sialyltransferase and UDP-Gal:asialo-mucin galactosyltransferase showed an enrichment over 60-and 75-fold, respectively.

One of the problems encountered in studying glycosyltransferases is introducing exogenous substrates into the membrane where the enzyme is located. Traditionally, this is accomplished by dispersing the substrate with detergents [18,29,30]. For this reason, the enzyme activity was assayed in the presence of Triton CF-54 [15] and labeled CMP-Neu5Ac. Four GM_1 molecular species carrying the same C18:1 LCB moiety and acyl chains of different length were tested. The results are reported in Fig. 1 and in Table 1. The data suggest that the kinetics is not markedly affected by fatty acid length, in the range between 14 and 18 C atoms. A significantly different behavior is displayed upon the molecular species carrying 22:0 acyl chains, the value of the apparent V_{max} and K_m for this latter species (56 and 0.15, respectively) being about 1/3–1/4 with respect to the others.

Subsequently, SAT IV activity was assayed on four GM_1 molecular species carrying the same fatty acyl moiety (18:0) and different LCB. The results are reported in Fig. 1. On increasing the substrate concentration, V increases displaying an apparently classical behavior, from which V_{max} and K_m can be calculated (Table 1). Marked inhibition is recorded with C20 LCB species at substrate concentrations above K_m, as has also been observed for other glycosyltransferases [31,32]. Also, the C18 species demonstrated the inhibition phenomenon, but at higher concentration (about 0.4 mM, data not shown). Due

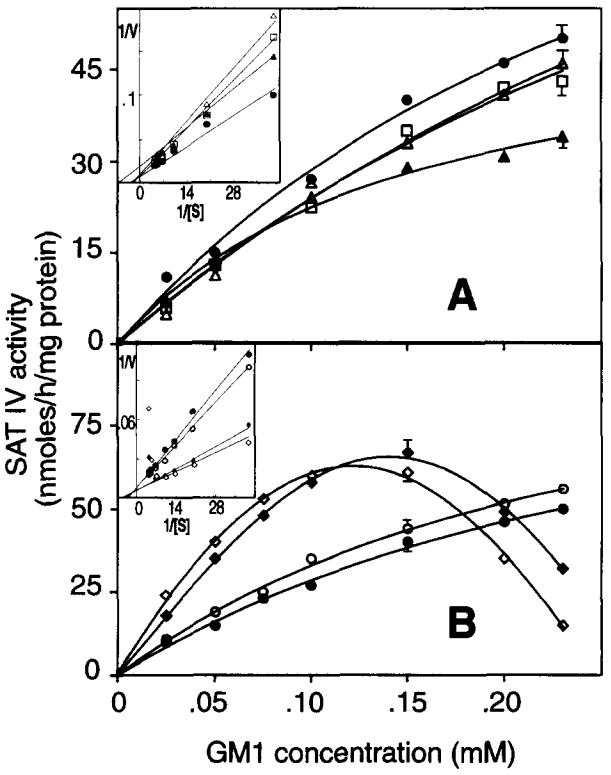

Fig. 1. Substrate dependence of SAT IV activity on GM1 ganglioside ceramide composition. Data shown are the mean of three experiments. Bars indicate the S.D. values. (A) Molecular species carrying C18:1 long chain base and 14:0 (△), 16:0 (□), 18:0 (●) or 22:0 (▲), fatty acid. (Inset) Lineweaver-Burk reciprocal plot of the same data. (B) Molecular species carrying 18:0 fatty acid and C18:0 (○), C18:1 (●), C20:0 (◇) or C20:1 (◆) long chain base. (Inset) Lineweaver-Burk reciprocal plot of the same data.

Table 1
Kinetic parameters of SAT IV activity on GM1 molecular species in the presence of detergent or [a]embedded in phospholipid vesicles, in the absence of detergent

GM1 molecular species (long chain base/fatty acid)	V_{max} (nmol/h per mg protein)	K_m (mM)
C18:1/14:0	123	0.41
C18:1/16:0	136	0.47
C18:1/18:0	120	0.33
C18:1/22:0	56	0.15
C18:0/18:0	130	0.29
C20:1/18:0	191	0.19
C20:0/18:0	196	0.21
C18:1/18:0[a]	10.7	0.11
C20:1/18:0[a]	9.9	0.12

The kinetic parameters are related to GM1 ganglioside. V_{max} and K_m values were calculated from insets of Figs. 1 and 2 by linear regression; the correlation coefficients (c) were $0.999 > c > 0.992$.

to these reasons, the relative activity upon the different molecular species depended on the actual substrate concentration. At low concentration, at which the inhibition was not yet apparent, the longer molecular species C20 LCB were preferentially selected with respect to the shorter C18 ones, irrespective of whether or not unsaturation was present. In contrast, at higher substrate concentration the enzyme had less effect on GM_1 containing C20 LCB, due to the earlier substrate inhibition encountered with these species. These results were also confirmed by the assay performed in the presence of GM_1 molecular species C18:1–18:0, C20:1–18:0, radiolabeled at the level of C-3 of the LCB (data not shown). Taken together, these results suggest that SAT IV activity is affected by the lipid composition of the substrate, GM_1 ganglioside. As has already been discussed [27], and not completely clarified, various reasons could explain this striking feature. One of these hypotheses is that insertion into the same membrane is a prerequisite for the enzyme to recognize molecular species [29,30]. To test this possibility, SAT IV was assayed on labeled GM_1 molecular species carrying the same fatty acid moiety (18:0) and different LCBs (C18:1,C20:1) embedded in phospholipid vesicles, in the absence of detergents. For these experiments an enzyme preparation previously sonicated was used, as suggested [18], in order to expose the catalytic domain of the enzyme. Accordingly, the activity of non-sonicated enzyme preparations was undetectable in the absence of detergents. Moreover, sonication did not alter the enzyme activity, assayed in the presence of detergent. Two observations are worth mentioning, on comparing the results shown in Fig. 2 with those obtained in the presence of the detergent and reported in Fig. 1. First, at a given substrate concentration the activity is much lower and, second, the activity is not apparently affected by the LCB composition of the substrate present in a liposomal dispersion. The same results were obtained using cold GM_1 and labeled CMP-Neu5Ac (data not shown). Therefore, the dependence of enzyme activity upon

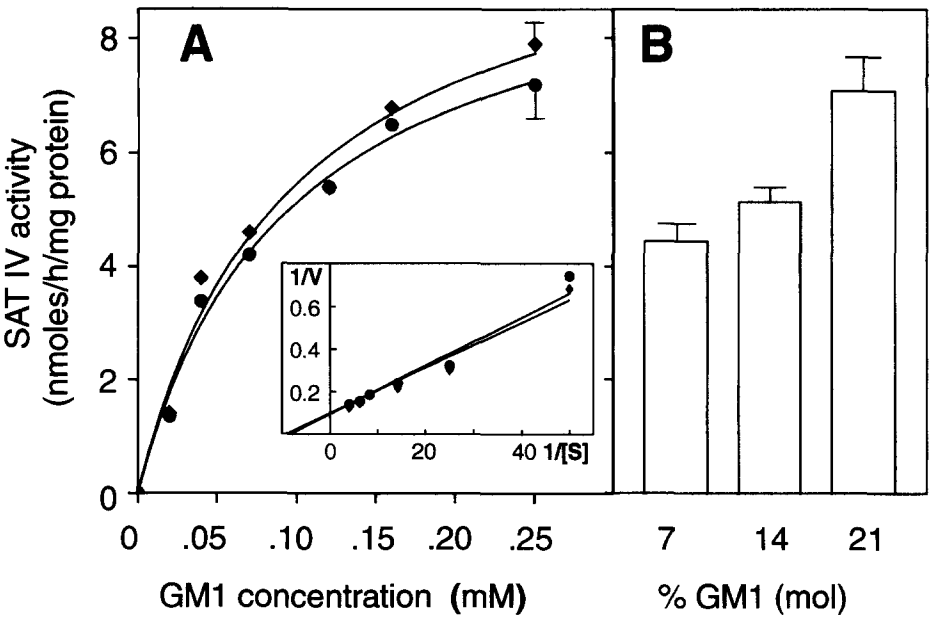

Fig. 2. Substrate dependence of SAT IV activity on GM1 ganglioside molecular species (carrying 18:0 fatty acid) embedded in dipalmitoylphosphatidylcholine vesicles. Data shown are the mean of three experiments. Bars indicate the S.D. values. (A) Dependence on the concentration of species carrying C18:1 long chain base (●) or C20:1 long chain base (♦). Ganglioside/phospholipid molar ratio was 21% for all experiments. (Inset) Lineweaver-Burk reciprocal plot of the same data. (B) SAT IV activity dependence on the % (ganglioside/phospholipid molar ratio) of GM1 species carrying C18:1 long chain base embedded in the vesicles.

GM_1 ceramide is observed only when the enzyme and the substrate are added to the incubation mixture in the presence of detergents, and are presumably part of the same supramolecular structures. These results suggest the possibility of a direct recognition of the acceptor lipid moiety by the transmembrane domain of the enzyme, as hypothesized for CMP-*N*-acetylneuraminate:lactosylceramide sialyltransferase [27]. The dependence of the enzyme activity on the GM_1/phospholipid ratio was also investigated using the C18:1/18:0 species. The results, reported in Fig. 2, show that the substrate density within the vesicles affects SAT IV activity, which increases together with the percentage of GM_1 in the bilayer. These results suggest that the different segregation properties displayed by ganglioside molecular species [33,34] may be important in modulating the enzymatic activity. Moreover, the segregation of gangliosides is known to be affected by the difference between their lipid composition and that of surrounding phospholipids [33]. Thus, the molecular species specificity of SAT IV, as already suggested for CMP-*N*-acetylneuraminate:lactosylceramide sialyltransferase [8] could depend on the phospholipid molecular species composition of the membranous microenvironment of the enzyme.

In conclusion, these results indicate for the first time that SAT IV, as other glycosyltransferases, is sensitive to the lipid moiety composition of the substrate and strengthen the hypothesis of the presence of a ceramide-modulated, glycosyltransferase-driven glycosphingolipid biosynthesis.

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