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Glycosyltransferase complexes improve glycolipid synthesis

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ABSTRACT

The synthesis of gangliosides GM3 and GD3 is carried out by the successive addition of sialic acid residues on lactosylceramide (LacCer) by the Golgi located sialyltransferases Sial-T1 and Sial-T2, respectively. CHO-K1 cells lack Sial-T2 and only express GM3. Here we show that the activity of Sial-T1 was near 2.5-fold higher in homogenates of CHO-K1 cells transfected to express Sial-T2 (CHO-K1$^{Sial\text{-}T2}$) than in untransfected cells. The appearance of Sial-T1 enzyme or gene transcription activators or the stabilization of the Sial-T1 protein were discarded as possible causes of the activation. Sial-T2 lacking the catalytic domain failed to promote Sial-T1 activation. Since Gal-T1, Sial-T1 and Sial-T2 form a multienzyme complex, we propose that transformation of formed GM3 into GD3 and GT3 by Sial-T2 in the complex leaves Sial-T1 unoccupied, enabled for new rounds of LacCer utilization, which results in its apparent activation.

1. Introduction

Glycolipid oligosaccharides are synthesized in the Golgi apparatus by a complex membrane-bound machinery formed by glycosyltransferases, sugar nucleotide transporters, and ceramide bound acceptors [1]. Glycolipid glycosyltransferases form multienzyme complexes [2–6] that may modify the activity of partners of the complex [5,9], or the sub cellular localization of the participating enzymes [7–9]. At least two complexes of ganglioside glycosyltransferases have been described in Chinese Hamster Ovary (CHO)-K1 cells, one formed by Gal-T1, Sial-T1, and Sial-T2 [10] and another by Gal-T2 and GalNAc-T, of more distal Golgi location [2]. In this work we examined if the presence of Sial-T2 in the complex formed by Gal-T1/Sial-T1/Sial-T2 affects the activity of the preceding enzyme (Sial-T1).

2. Materials and methods

2.1. Cell culture and transfection

CHO-K1 cells (ATCC, Manassas, VA) (wt) and clones of CHO-K1 cells that stably expresses full-length chicken SialT2-HA (CHO-K1$^{Sial\text{-}T2}$ cells) [11] or the N-terminal domain of Sial-T2 (CHO-K1^{ST2Ntd} cells) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin.

2.2. Determination of ganglioside glycosyltransferase activities and its synthesized products

Sial-T1 activity was determined essentially as described [12] in homogenates of CHO-K1^{wt} and CHO-K1$^{Sial\text{-}T2}$. Briefly, the incubation system contained, in a final volume of 30 μl, 400 μM LacCer, 100 μM CMP-[^{3}H]NeuAc (250,000 cpm; sp. act. 33 cpm/pmol), 20 mM $MnCl_2$,1 mM $MgCl_2$, 20 μg of Triton CF54/Tween 80 (2:1 w/w), 100 mM sodium cacodylate–HCl buffer (pH 6.5), and cell extract (40 μg of protein). Sial-T2 activity was determined in the same incubation system except that 400 μM GM3, instead of LacCer, was the acceptor. Incubations were performed at 37 °C for 90 min. Under these conditions the incorporation into LacCer was linear with time and protein concentration. Samples without exogenous acceptor were used to correct the incorporation into endogenous acceptors. Reactions were stopped with 1 ml of 5% (w/v) trichloroacetic acid/0.5% phosphotungstic acid, and the radioactivity incorporated into lipids was determined by liquid scintillation counting. For high performance TLC (HPTLC) analysis, lipids were extracted with 1 ml of chloroform: methanol (2:1 v/v), the extract passed through Sephadex-G25 column, the solvent evaporated and glycolipids separated using as solvent chloroform:methanol (4:1 v/v) in a first run up to two-thirds of the plate and chloroform:methanol:0.2% $CaCl_2$ (60:36:8 v/v) in a second run up to the front of the plate. Radioactivity in chromatograms was

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recorded either by phosphorimaging or by exposure to radiographic films. Densitometric quantification of X-ray plates was done using ImageJ software (National Institute of Health, USA).

2.3. Semi-quantitative RT-PCR

Total RNA was purified from CHO-K1^{wt} and CHO-K1$^{Sial\text{-}T2}$ cells using the "*illustra RNAspin® Mini Kit*" (GE Healthcare Biosciences, Pittsburgh, PA, USA), and total mRNA with *"PolyATtract® System 1000"* (Promega Corporation, WI, USA). Total mRNA was used for total cDNA synthesis using oligo dT as primers for the reverse transcription reaction using T7 reverse transcriptase. Fragments for Sial-T1 and β-actin genes were amplified by PCR using specific primers and the synthesized cDNA as template. PCR conditions were set up and the optimal numbers of cycles for each of transcripts were defined. PCR reaction products were analyzed by agarose gel electrophoresis and quantified using the ImageJ software. Raw quantifications were normalized to the level of β-actin transcript.

3. Results

3.1. Sial-T1 activity increases in cells expressing Sial-T2

In preparations from CHO-K1 cell clones that stably express different glycosyltransferases we noticed that in those from clones stably expressing Sial-T2 (CHO-K1$^{Sial\text{-}T2}$ cells) the activity of Sial-T1 towards exogenous LacCer was increased near 2.5-fold with respect to untransfected CHO-K1^{wt} cells (Table 1).

Among several different possibilities to explain this observation it was considered that the synthesis of new glycolipid products due to the stable expression of Sial-T2 could affect positively the membrane microenvironment of Sial-T1. To examine this possibility both CHO-K1^{wt} and CHO-K1$^{Sial\text{-}T2}$ cells were grown for four days in the presence of 1.2 μM of the inhibitor of GlcCer synthase D,L-threo-1-Phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl (P4). Under these culture conditions the synthesis of glycolipids and gangliosides is blocked and cell membranes are essentially devoid of them, as previously shown [12]. Incorporation of sialic acid into endogenous lipid acceptors in P4 treated cells decreased to near background levels, as expected from the overall depletion of sialic acid acceptor glycolipids in Golgi membranes (Table 1). However, the Sial-T1 activity towards exogenous LacCer was still higher (about 1.8-fold) in P4 treated CHO-K1$^{Sial\text{-}T2}$ cells than in P4 treated CHO-K1^{wt} cells, thus making less probable that Sial-T1 activation was due to the presence of some glycolipid activator in CHO-K1$^{Sial\text{-}T2}$ cells. It was also considered that some soluble activator of Sial-T1 could have been formed in CHO-K1$^{Sial\text{-}T2}$ cells. This possibility was discarded because upon mixing equal parts of CHO-K1^{wt} and CHO-K1$^{Sial\text{-}T2}$ cell homogenates the activity determined was essentially the sum of the activities of the mixed parts (Table 1); in case of presence of such an activator (in excess) the activity in the mixture should have been higher than the sum.

3.2. Sial-T1 mRNA levels in CHO-K1$^{Sial\text{-}T2}$ cells are comparable to those in CHO-K1^{wt} cells

To analyze the possibility of a specific transcriptional activation of Sial-T1 genes by the expression of Sial-T2, the level of Sial-T1 transcripts was determined in CHO-K1^{wt} and CHO-K1$^{Sial\text{-}T2}$ cells by RT-PCR using specific primers. Fig. 1 shows that Sial-T1 transcript levels in CHO-K1$^{Sial\text{-}T2}$ cells were not higher but rather were slightly lower than in CHO-K1^{wt} cells, thus discarding the possibility that a transcriptional activation was the main cause for the observed increase of Sial-T1 activity.

3.3. Sial-T1 stabilization is not the cause for its increased activity

Transfected Sial-T2, by participating in the formation of a complex with Gal-T1 and Sial-T1 [10], may have increased the Sial-T1 half life and hence the enzyme pool size. To approach this possibility, CHO-K1^{wt} and CHO-K1$^{Sial\text{-}T2}$ cells were cultured in the absence and presence of 60 μg/ml of cycloheximide during 3 h before Sial-T1 activity determination. This time approximates the half life (3 h) of a β galactoside α sialyl transferase from a rat hepatoma cell line [13]. Although the incorporation into endogenous acceptors dropped to 53% and 49% in cycloheximide treated CHO-K1^{wt} and CHO-K1$^{Sial\text{-}T2}$ cells, respectively, the activity of Sial-T1 decreased only about 10% but maintaining the difference in activity in favor of CHO-K1$^{Sial\text{-}T2}$ cells already shown in Table 1 (results not shown). This result indicates that stabilization of Sial-T1 is not a main cause for its increased activity in cells expressing Sial-T2.

3.4. The N-terminal domain of Sial-T2 is unable to activate Sial-T1

Gal-T1, Sial-T1 and Sial-T2 form a multi-enzyme complex and their N-terminal domains (Ntds) are involved in the formation of the complex [10]. To analyze if complex formation *per se* promotes the increased activity of Sial-T1, a clone of CHO-K1 cells stably expressing the Ntd of Sial-T2 (CHO-K1^{ST2Ntd}) was generated and Sial-T1 activity was determined in CHO-K1^{wt}, CHO-K1$^{Sial\text{-}T2}$ and CHO-K1^{ST2Ntd} cells. Surprisingly, the Ntd of Sial-T2 did not activate Sial-T1 (Fig. 2), indicating that the Sial-T2 C-terminal, lumenal domain containing the catalytic domain is required for the

Table 1
In vitro incorporation of sialic acid into endogenous glycolipid acceptors and into LacCer (Sial-T1 activity) by homogenates from CHO-K1^{wt} and CHO-K1$^{Sial\text{-}T2}$ cells grown in the presence or absence of P4.

Cell clone and condition	Sialic acid incorporation (pmol mg protein h^{-1}) into		
	Endogenous acceptors[a]	Endogenous acceptors + LacCer[b]	LacCer[b–a]
CHO-K1^{wt}	110 ± 3	235 ± 23	125 ± 22
CHO-K1$^{Sial\text{-}T2}$	272 ± 4	576 ± 39	304 ± 38
CHO-K1^{wt} + P4*	50 ± 1	256 ± 28	205 ± 22
CHO-K1$^{Sial\text{-}T2}$ + P4*	41 ± 2	410 ± 45	365 ± 47
CHO-K1^{wt} CHO-K1$^{Sial\text{-}T2}$**	218 ± 11	454 ± 15	236 ± 15

After 90 min of incubation at 37 °C, the radioactivity of ^{3}H-sialic acid incorporated into the lipid fraction was determined as indicated under the Section 2 section. The incorporation into endogenous glycolipids in samples run in parallel in the absence of added LacCer (a), but in otherwise identical conditions was discounted from that in samples with added LacCer (b) to calculate the incorporation into exogenous LacCer (b–a).

* Homogenates from cells cultured 4 days in the presence of P4 in the culture medium.

** Incubates contained half volume of each CHO-K1^{wt} and CHO-K1$^{Sial\text{-}T2}$ cell homogenates.

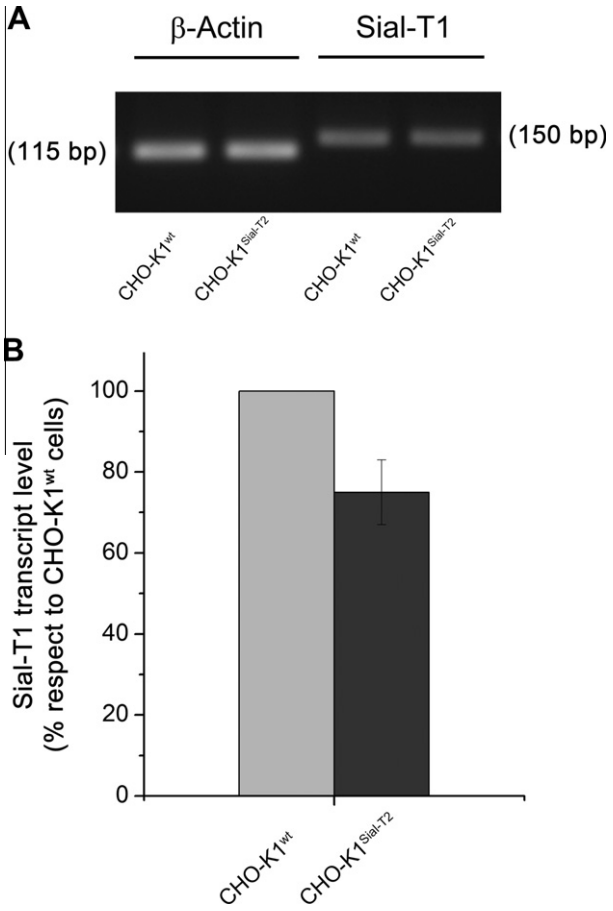

Fig. 1. Sial-T1 mRNA level in CHO-K1$^{Sial\text{-}T2}$ cells is comparable to that in CHO-K1^{wt} cells. (A) Fragments of the sequence of Sial-T1 were amplified by PCR using specific primers as indicated under Section 2. Housekeeping actin gene was used as a DNA loading control. (B) The amplified fragments in (A) were quantified by densitometry using ImageJ software. Results are mean ± SD, for three independent experiments.

activation. This result also minimizes the possibility that we are dealing with a particular cell clone having intrinsically elevated activity of Sial-T1.

3.5. Sial-T1 activity products in CHO-K1$^{Sial\text{-}T2}$ cells

Considering that Gal-T1, Sial-T1 and Sial-T2 participate of a multi protein complex, the possibility that we were measuring the activities of both, Sial-T1 and Sial-T2 in the same reaction assay was examined by analyzing the reaction products. The main product of sialylation of endogenous acceptors was exclusively GM3 in CHO-K1^{wt} cells but GM3 and GD3 in about the same proportion, and trace amounts of GT3, in CHO-K1$^{Sial\text{-}T2}$ cells (Fig. 3). In the presence of added LacCer, GM3 was the only radioactive product in CHO-K1^{wt} cells while in CHO-K1$^{Sial\text{-}T2}$ cells 56% of radioactivity was in GM3, 38% in GD3 and 6% in GT3 (Fig. 3). From the distribution of radioactivity in Fig. 3, the incorporation values of sialic acid given in Table 1 and taking into account 1, 2 and 3 mol of sialic acid per mol of LacCer in respectively GM3, GD3 and GT3, it was estimated that the amount of LacCer converted into gangliosides in CHO-K1$^{Sial\text{-}T2}$ cells almost doubled that converted in CHO-K1^{wt} cells (Table 2). These results indicate that Sial-T2 in CHO-K1$^{Sial\text{-}T2}$ cells can efficiently use the product of Sial-T1 (GM3) for further sialylation reactions, passing through GD3 and ending in GT3. However, the most noticeable observation emerging from this

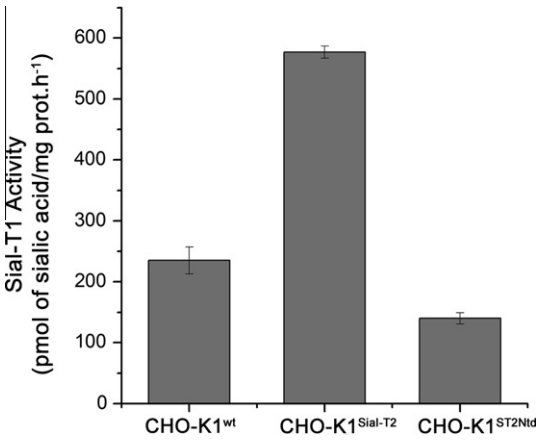

Fig. 2. The N-terminal domain of Sial-T2 fails to promote the increase of Sial-T1 activity. Sial-T1 activity was determined in CHO-K1^{wt}, CHO-K1$^{Sial\text{-}T2}$ and in cells expressing the N-terminal domain of Sial-T2 (CHO-K1^{ST2Ntd} cells) as described in Section 2. Results are shown as mean ± SD pmol of sialic acid/mg. prot h^{-1}, for three independent experiments.

result is that Sial-T1 was able to convert twice as much LacCer into GM3 when working with co-expressed Sial-T2.

3.6. GM3 substrate of Sial-T2 may not be released to the reaction medium

An interesting question emerging from the above results is how GM3 formed by Sial-T1 is used by Sial-T2. Among different possibilities, GM3 once synthesized may be released to the reaction medium and Sial-T2 may use it to synthesize GD3/GT3 as if it were provided exogenously to the reaction mixture. Alternatively, taking into consideration the multi-protein organization of Gal-T1/Sial-T1/Sial-T2 it may occur that de novo synthesized GM3 is kept bound to the complex and in this way be channeled to the synthesis of GD3 and GT3 by Sial-T2. To discern between these possibilities, Sial-T2 activity was measured in CHO-K1$^{Sial\text{-}T2}$ cell homogenates but in a reaction system containing the amount of GM3 at the concentration it reaches when formed from LacCer by the activity of Sial-T1 (0.34 μM). It is clear from the results in Table 3 that adding the amount of GM3 formed from LacCer (10.2 pmol) to the reaction mixture as GM3 source results in no detectable formation of GD3/GT3. The simplest interpretation of this experiment is that GM3 formed from LacCer in the assay has been kept bound to the multienzyme complex and in this location is taken by Sial-T2 (that is interacting with Sial-T1) to synthesize GD3/GT3.

4. Discussion

The hypothesis of "cooperative sequential specificity" in multiglycosyltransferase systems [14] was experimentally demonstrated for the first time in mammalian cells and in the glycolipid pathway for GalNAc-T and Gal-T2 [2], and for Gal-T1, Sial-T1 and Sial-T2 [10]. For the case of the GalNAc-T and GalT2N-terminal domains, Gal-T2 but not GalNAc-T form homo complexes, and in the GalNAc-T/Gal-T2 heterocomplex the molar ratio of the two enzymes is 1:2 [15].

Functional homo- and heteromeric complexes were described for Golgi glycosyltransferases of the N- and O-glycosylation pathway, with co-expression of GalT1 and ST6Gal-1 resulting in a 2.4-fold increase of GalT1 activity towards the acceptor ovalbumin [16].

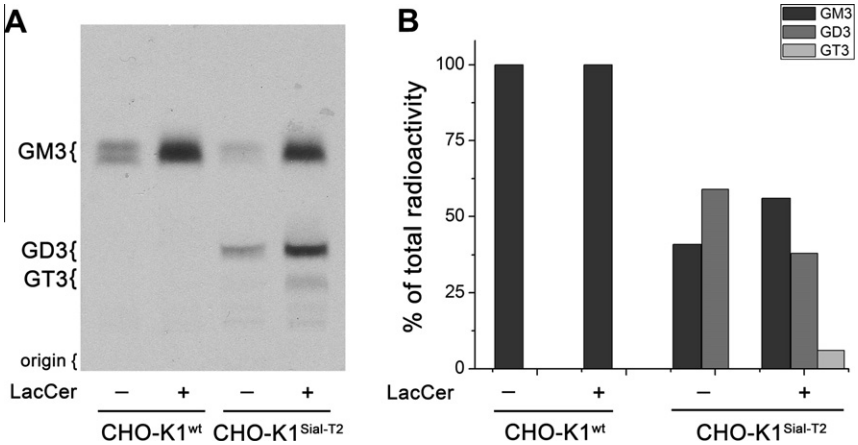

Fig. 3. Chromatographic analysis of Sial-T1 assay lipid products. (A) Sial-T1 assays as those described in Table 1 but scaled up 2- to 4-fold were processed for separation by HPTLC as indicated under Section 2. For each cell homogenate, radioactivity spotted in the chromatogram was 900 cpm and 2500 cpm for, respectively, samples minus (−) or plus (+) LacCer. The radioactivity in lipid products was detected by autoradiography. (B), Densitometric quantification of radioactive bands in (A) using ImageJ software.

Table 2
Radioactive ganglioside species synthesized from exogenous LacCer by CHO-K1^{wt} and CHO-K1$^{Sial\text{-}T2}$ cell clone homogenates.

Cell Clone Homogenate	Sial-T1 activity (pmol sialic acid incorporated . mg of protein . h^{-1}) into*			Total LacCer converted**
	GM3	GD3	GT3	
CHO-K1^{wt}	125	ND	ND	125
CHO-K1$^{Sial\text{-}T2}$	170	116	18	234

* Values were calculated from the incorporation values given in Table 1 and the percentage distribution of radioactive species in the HPTLC shown in Fig. 3B.
** The amount of exogenous lactosylceramide converted was calculated considering a sialic acid:LacCer molar ratio of 1, 2 and 3 for, respectively, GM3, GD3 and GT3. ND: not detected.

Table 3
Sial-T2 activity in CHO-K1$^{Sial\text{-}T2}$ cell clone homogenate at the concentration of GM3 formed from exogenous LacCer in the Sial-T1 assay.

Acceptor added	Product formed in the assay			
	GM3 (Sial-T1 activity)		GD3 (Sial-T2 activity)	
	pmol	μM	pmol	μM
LacCer[a] (400 μM)	10.2	0.34	2.9	0.1
GM3[b] (400 μM)	–	–	963.67	32.12
GM3[c] (0.34 μM)	–	–	ND	–

ND: not detected.
[a] Sial-T1 activity was determined in the conditions described in Section 2, and the amount of GM3 and GD3 formed in the assay was determined after separation of the lipid fraction by HPTLC as in Fig. 3.
[b] Sial-T2 activity was determined in the conditions described in Section 2 and product formation (not shown) analyzed by HPTLC as in Fig. 3.
[c] Sial-T2 activity was determined in the conditions described in Section 2, except that the acceptor GM3 was added at the concentration it reached in the Sial-T1 assay (a) (0.34 μM).

Sial-T2 works as a polysialyltransferase that use more efficiently GM3 than GD3 to synthesize GD3 and GT3, respectively [17]. CHO-K1$^{Sial\text{-}T2}$ cells stably expressing Sial-T2, show a glycolipid pattern containing only GD3 and GT3, (Fig. 3) because they lack the GalNAc-T necessary for elongation of the oligosaccharide [18].

Here we show that the activity of Sial-T1 in CHO-K1$^{Sial\text{-}T2}$ cells was about 2.5-fold higher than in CHO-K1^{wt} cells. In a search for probable causes of the activation it was found that the product of the Sial-T1 activity was mainly the expected GM3 with CHO-K1^{wt} homogenate but similar proportions of GM3 and of GD3, and some GT3, with the CHO-K1$^{Sial\text{-}T2}$ homogenate. This suggested that GM3 synthesized by Sial-T1 was used as substrate by Sial-T2 to form GD3 and some GT3. However, lowering the concentration of added GM3 in the Sial-T2 assay to a concentration equivalent to the one that it reached in the Sial-T1 assay resulted in undetectable formation of GD3 (Table 3). The simplest interpretation of this result is that the product of Sial-T1 (GM3) do not leave the multienzyme complex formed by Gal-T1/Sial-T1/Sial-T2, but remain bound to it and used as substrate by Sial-T2 for a second and third sialylation steps. Of course, this does not explain the consumption of twice LacCer molecules in the Sial-T1 assay with CHO-K1$^{Sial\text{-}T2}$ cell homogenate (Table 2). In this respect, it may be hypothesized that conversion of the GM3 formed by Sial-T1 into GD3/GT3 by Sial-T2 may facilitate the progression of acceptors and products along the complex, leaving unoccupied the acceptor site in Sial-T1 for occupancy with new acceptor LacCer molecules.

Several reports have described that complex formation between glycosyltranferases improve the enzymatic activity of one of the partners [2–5], but possible causes have not been analyzed. This knowledge is necessary to better understand postrancriptional levels of regulation of glycosyltransferase activities and hence of the pattern of glycolipids expressed by different cells.

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