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Trans-activity of Plasma Membrane-associated Ganglioside Sialyltransferase in Mammalian Cells*

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Gangliosides are acidic glycosphingolipids that contain sialic acid residues and are expressed in nearly all vertebrate cells. They are synthesized at the Golgi complex by a combination of glycosyltransferase activities followed by vesicular delivery to the plasma membrane, where they participate in a variety of physiological as well as pathological processes. Recently, a number of enzymes of ganglioside anabolism and catabolism have been shown to be associated with the plasma membrane. In particular, it was observed that CMP-NeuAc:GM3 sialyltransferase (Sial-T2) is able to sialylate GM3 at the plasma membrane (cis-catalytic activity). In this work, we demonstrated that plasma membrane-integrated ecto-Sial-T2 also displays a trans-catalytic activity at the cell surface of epithelial and melanoma cells. By using a highly sensitive enzyme-linked immunosorbent assay combined with confocal fluorescence microscopy, we observed that ecto-Sial-T2 was able to sialylate hydrophobically or covalently immobilized GM3 onto a solid surface. More interestingly, we observed that ecto-Sial-T2 was able to sialylate GM3 exposed on the membrane of neighboring cells by using both the exogenous and endogenous donor substrate (CMP-N-acetylneuraminic acid) available at the extracellular milieu. In addition, the trans-activity of ecto-Sial-T2 was considerably reduced when the expression of the acceptor substrate was inhibited by using a specific inhibitor of biosynthesis of glycolipids, indicating the lipidic nature of the acceptor. Our findings provide the first direct evidence that an ecto-sialyltransferase is able to trans-sialylate substrates exposed in the plasma membrane from mammalian cells, which represents a novel insight into the molecular events that regulate the local glycosphingolipid composition.

Glycosphingolipids are amphipathic molecules consisting of a ceramide lipid moiety linked to a glycan chain of variable length and structure. Among these are found gangliosides, which are sialosylated glycosphingolipids mainly located in the outer layer of the plasma membrane of vertebrate cells (1, 2) and have been implicated in many physiological processes, including growth, differentiation, migration and apoptosis through modulating both cell signaling processes and cell-to-cell and cell-to-matrix interactions (3–6). Furthermore, gangliosides have been associated with a wide range of pathological processes, being receptors for both viruses and antibodies (7, 8).

The biosynthesis of gangliosides is mainly carried out in the lumen of the Golgi cisternae by a complex system of membrane-bound glycolipid acceptors, nucleotide sugar donors, glycosyltransferases, and nucleotide sugar transporters (1, 9). These neosynthesized gangliosides move through the Golgi complex to the plasma membrane via the lumenal surface of transport vesicles (10–12). It is very well documented that glycosphingolipid expression, including gangliosides, is mainly regulated at the transcriptional and posttranscriptional levels of glycolipid glycosyltransferases and specific transport proteins in the lumen of the Golgi complex. However, regulation of glycosphingolipid expression has also been demonstrated to occur at the plasma membrane, with the existence of a plasma membrane-associated sialidase (Neu3), β-hexosaminidase, β-glucosidase, and β-galactosidase (1, 13) having been reported. In addition, we recently showed that both ectopically and endogenously expressed CMP-NeuAc:GM3 sialyltransferase (Sial-T2)[4] are able to sialylate GM3 at the plasma membrane (cis-catalytic activity) by using both the exogenous and endogenous donor (CMP-NeuAc) and acceptor (GM3) substrates (14). Thus, the current scenario shows the presence of both ganglioside glycosyltransferases and glycohydrolases at the plasma membrane, which locally modulate cellular glycolipid expression and, consequently, different signaling processes.

In this work, we focused our attention on investigating if plasma membrane-integrated ecto-Sial-T2 displays a trans-catalytic activity in living cells. By using a specific ELISA combined with confocal fluorescence microscopy, we demonstrated that ecto-Sial-T2 was able to sialylate when GM3 was hydrophobically or covalently immobilized onto a solid surface (cell surface-matrix interaction). Furthermore, it was also observed that ecto-Sial-T2 displayed enzymatic activity on the GM3 substrate belonging to the surface of neighboring cells, which did not express endogenous Sial-T2 (cell-cell interaction). Interest-

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[4] The abbreviations used are: Sial-T2, CMP-NeuAc:GM3 sialyltransferase; P4, d,l-threo-1-fenyl-2-hexadecanoilamino-3-pirrolidino-1-propanol-HCl; CHO, Chinese hamster ovary; CMP-NeuAc, cytidine monophospho-N-acetylneuraminic acid; GM3, NeuAcα2,3Galβ1,4Glc-ceramide; GD3, NeuAcα2,8NeuAcα2,3Galβ1,4Glc-ceramide.

ingly, ecto-Sial-T2 was able to synthesize GD3 at the cell surface of adjacent cells by using both the exogenous and endogenous donor substrate (CMP-NeuAc) available at the extracellular milieu. Finally, the trans-activity of ecto-Sial-T2 was considerably reduced when the expression of the substrate was inhibited by using a specific inhibitor of biosynthesis of glycolipids, demonstrating the lipidic nature of the acceptor. Taken together, our findings provide the first direct evidence that an ecto-sialyltransferase is able to trans-sialylate substrates immobilized on a solid surface or expressed in membranes from mammalian cells, which represents a novel insight into the molecular events that regulate the local glycosphingolipid composition.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Cultures—CHO-K1 cell clones expressing different ganglioside glycosyltransferases had been obtained previously in our laboratory. The following cells were used: wild-type CHO-K1 (CHO-K1^{wt}) cells (ATCC); clone 2 (CHO-K1$^{Sial\text{-}T2+}$), a stable chick Sial-T2 (tagged at the C terminus with the YPYDVPDYA nanopeptide epitope of the viral HA) transfectant expressing the gangliosides GD3 and GT3 (6, 15) and the SK-Mel 28 human melanoma cell line (ATCC), which endogenously expresses Sial-T2. Cells were grown and maintained at 37 °C in 5% CO_2 in DMEM supplemented with 10% FBS.

GD3 Detection by ELISA—GM3 was purified in our laboratory from dog erythrocytes. GD3 purified from chicken brain and glycolipids standards were kindly provided by G. Nores (Centro de Investigaciones en Química Biológica de Córdoba, Universidad Nacional de Córdoba, Argentina). The quality and purity of the gangliosides were assessed by chromatography on high performance thin-layer chromatography (HPTLC) plates (Merck) using chloroform:methanol:0.2% $CaCl_2$ (55:45:10 v/v) as a solvent and revealed by orcinol staining. Different amounts of GM3 (50 and 100 pmol) or GD3 (0.75, 1.5, 3.0, and 6.0 pmol) were coated on polystyrene microtitration plates in methanol and dried overnight at 37 °C. After ganglioside coating, wells were saturated with PBS/2% BSA for 2 h. The reactivity to GD3 was measured using the specific mouse monoclonal antibody anti-GD3 (IgG3) clone R24 (ATCC no. HB-8445). After washes, primary R24 antibody was detected by incubating it overnight at 4 °C with mouse IgG antibody conjugated with HRP and diluted 1:1000 in PBS/2% BSA. After more washes, HRP activity was revealed using 0.5 mg/ml *o*-phenylenediamine in 0.1 M citrate-citric acid buffer (pH 5.5) containing 0.03% H_2O_2. The reaction was stopped by addition of H_2SO_4 (0.5 N final concentration), and the optic density (*A*) was measured at 490 nm in a microplate reader (Bio-Rad, model 680). The GD3 synthesis was calculated by subtracting the nonspecific GD3 binding from each measurement.

Determination of Sial-T2 Activity Using GM3 Substrate Absorbed into a Solid Surface—The synthesis of GD3 by ecto-Sial-T2 activity was measured by ELISA using the specific mouse monoclonal antibody R24. First, GM3 (0.05 nmol) was coated onto polystyrene microtitration plates in methanol and dried overnight at 37 °C. Then, wells were saturated with PBS/2% BSA for 2 h. Also, cells from clone 2 (CHO-K1$^{Sial\text{-}T2+}$) and SK-Mel 28 cells were grown on coverslips and treated for 5 days with 2.4 or 1.8 μM, respectively, of d,l-threo-1-fenyl-2-hexadecanoilamino-3-pirrolidino-1-propanol-HCl (P4) (Matreya, Inc., PA) to reduce GM3, GD3, and the neutral glycolipid content (16). After treatment with P4, coverslips (6-mm diameter) containing 12,000 CHO-K1$^{Sial\text{-}T2+}$ or SK-Mel 28 cells were placed in contact with wells coated with GM3 and incubated at 37 °C in an incubation system containing 10 mM $MnCl_2$, 1 mM $MgCl_2$, 100 mM sodium cacodylate-HCl buffer (pH 6.5) and 30 μM CMP-NeuAc in a final volume of 70 μl DMEM. After 2 h of incubation, coverslips were removed and processed for confocal microscopy analysis. In addition, wells were washed thoroughly with PBS/1% BSA and incubated overnight with antibody to GD3 (R24) at 4 °C. After washes, primary R24 antibody was detected by ELISA as described above. The GD3 synthesis was calculated by subtracting the nonspecific GD3 binding from each measurement.

Ecto-Sial-T2 Activity on Chemically Immobilized GM3—50 pmol of GM3 in methanol was coated onto the photo reactive Universal-BIND™ surface microtitration plates (Corning, Lowell, MA) and dried overnight at 37 °C. Covalent immobilization of GM3 to the polystyrene surface was made via abstractable hydrogen using UV illumination, resulting in a carbon-carbon bond. Then, wells were washed and saturated with PBS/2% BSA for 2 h. Next, CHO-K1$^{Sial\text{-}T2+}$ cells previously grown on coverslips (6-mm diameter) and treated with P4 for 5 days were placed in contact with wells coated with GM3 and incubated at 37 °C for 90 min in an incubation system containing 10 mM $MnCl_2$, 1 mM $MgCl_2$, 100 mM sodium cacodylate-HCl buffer (pH 6.5), and 30 μM CMP-NeuAc in a volume of 70 μl DMEM. After incubation, the coverslips were removed, and the GD3 synthesis was detected as described above.

Assessment of GD3 Synthesis by Ecto-Sial-T2 Trans-activity on GM3 Expressed in Plasma Membrane from CHO-K1^{wt} Cells—CHO-K1^{WT} cells and CHO-K1$^{Sial\text{-}T2+}$ were grown on 96-well polystyrene flat-bottom plates and coverslips (6 mm diameter), respectively, with or without P4 for 5 days. Then, CHO-K1$^{Sial\text{-}T2+}$ cells treated with P4 were incubated "face-to-face" for 2 h at 37 °C with CHO-K1^{WT} cells, previously treated or untreated with P4, in an incubation system containing 10 mM $MnCl_2$, 1 mM $MgCl_2$, 100 mM sodium cacodylate-HCl buffer (pH 6.5), and 30 μM CMP-NeuAc in a volume of 80 μl of DMEM. Where indicated, CHO-K1^{WT} cells were incubated for 90 min at 37 °C with CM from CHO-K1$^{Sial\text{-}T2+}$ cells maintained in the incubation system indicated above. Next, the coverslips were removed, and the wells containing CHO-K1^{WT} cells were washed and incubated with antibody to GD3 (R24) at 4 °C for 60 min. Then, cells were washed, fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and incubated at 4 °C with secondary antibody conjugated to HRP in PBS/2% BSA. After washes, HRP activity was revealed using 0.5 mg/ml *o*-phenylenediamine in 0.1 M citrate-citric acid buffer (pH 5.5) containing 0.03% H_2O_2. The reaction was stopped by addition of H_2SO_4 (0.5 N final concentration) and *A* was measured at 490 nm. The GD3 synthesis was calculated by subtracting the nonspecific GD3 binding (*A* value from a well containing CHO-K1^{wt} cells treated with P4) from each measurement.

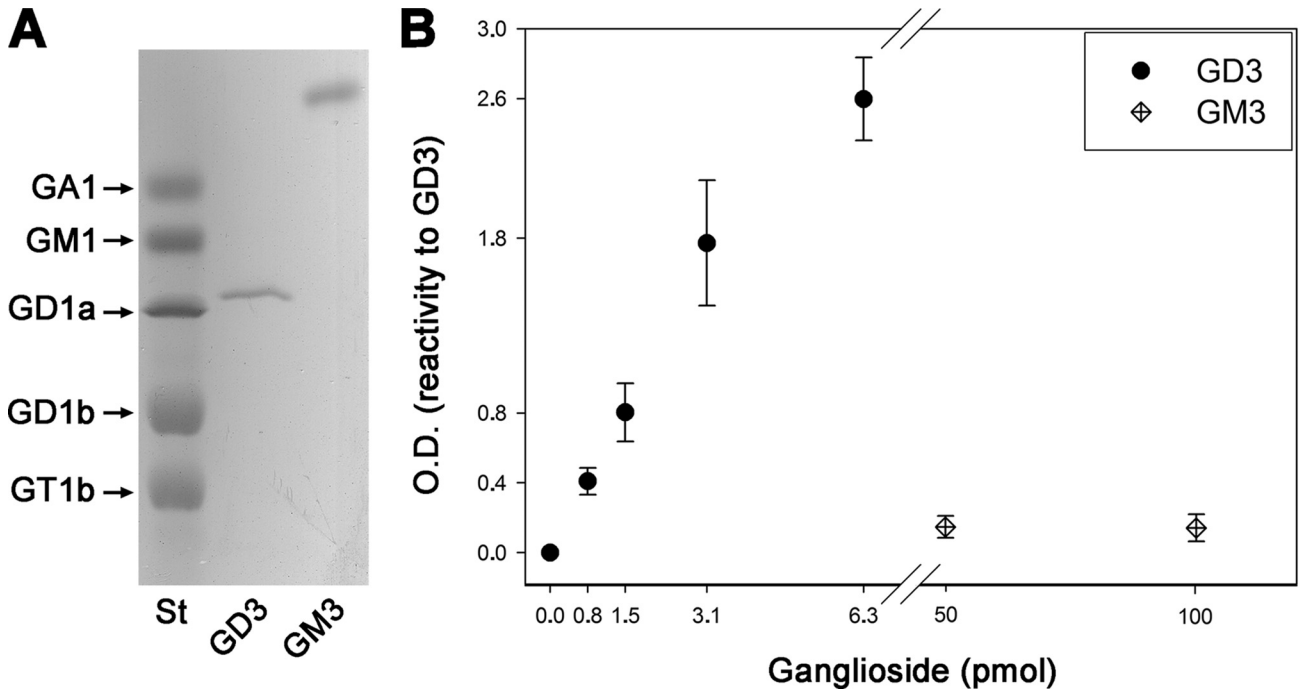


FIGURE 1. **GD3 detection by ELISA.** *A*, chromatographic analysis of GM3 and GD3 gangliosides used in experiments shown in *B*. Glycolipids were chromatographed on high-performance thin-layer chromatography and revealed by orcinol staining. The positions of the glycolipid standards (*St*) are indicated on the left. *B*, different amounts of GM3 or GD3 were loaded on polystyrene microtitration plates in methanol and dried overnight at 37 °C. After ganglioside coating, wells were saturated with PBS/2% BSA, washed thoroughly with PBS, and incubated with antibody to GD3 (R24) at 4 °C. GD3-antibody complex was detected with mouse IgG antibody conjugated with HRP in PBS/2% BSA. After washes, HRP activity was revealed and the *A* measured at 490 nm as indicated under "Experimental Procedures." Results are mean ± S.E. of duplicates of three independent experiments.

Determination of Ecto-Sial-T2 Activity at the Cell Surface—The ecto-Sial-T2 activity was measured as recently described by Crespo *et al.* (14) with minor modifications. Briefly, CHO-K1$^{Sial\text{-}T2+}$ cells were grown on coverslips and treated with 2.4 μM P4 for 4 days. Then, cells were incubated for 2 h with 25 μM GM3. Next, cells were washed repeatedly with 0.2% BSA in PBS to remove the GM3 and then incubated for 30 or 90 min in an incubation system containing 10 mM $MnCl_2$, 1 mM $MgCl_2$, 100 mM sodium cacodylate-HCl buffer (pH 6.5), and 30 μM CMP-NeuAc in a volume of 30 μl of DMEM. Finally, coverslips were processed for immunodetection of the synthesized GD3 and confocal microscopy analysis.

Confocal Immunofluorescence Microscopy—Cells grown on coverslips (12-mm diameter, 30,000 cells) were washed twice with PBS, incubated at 4 °C for 1 h with mouse monoclonal antibody anti-GD3 (R24) diluted 1:130, fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, and then incubated at 37 °C for 90 min with goat antibody to mouse IgG-Alexa^{488} diluted 1:1000 (Santa Cruz Biotechnology, Inc., CA). After washes with PBS, cells were mounted in FluorSave reagent (Calbiochem, EMD Biosciences, La Jolla, CA).

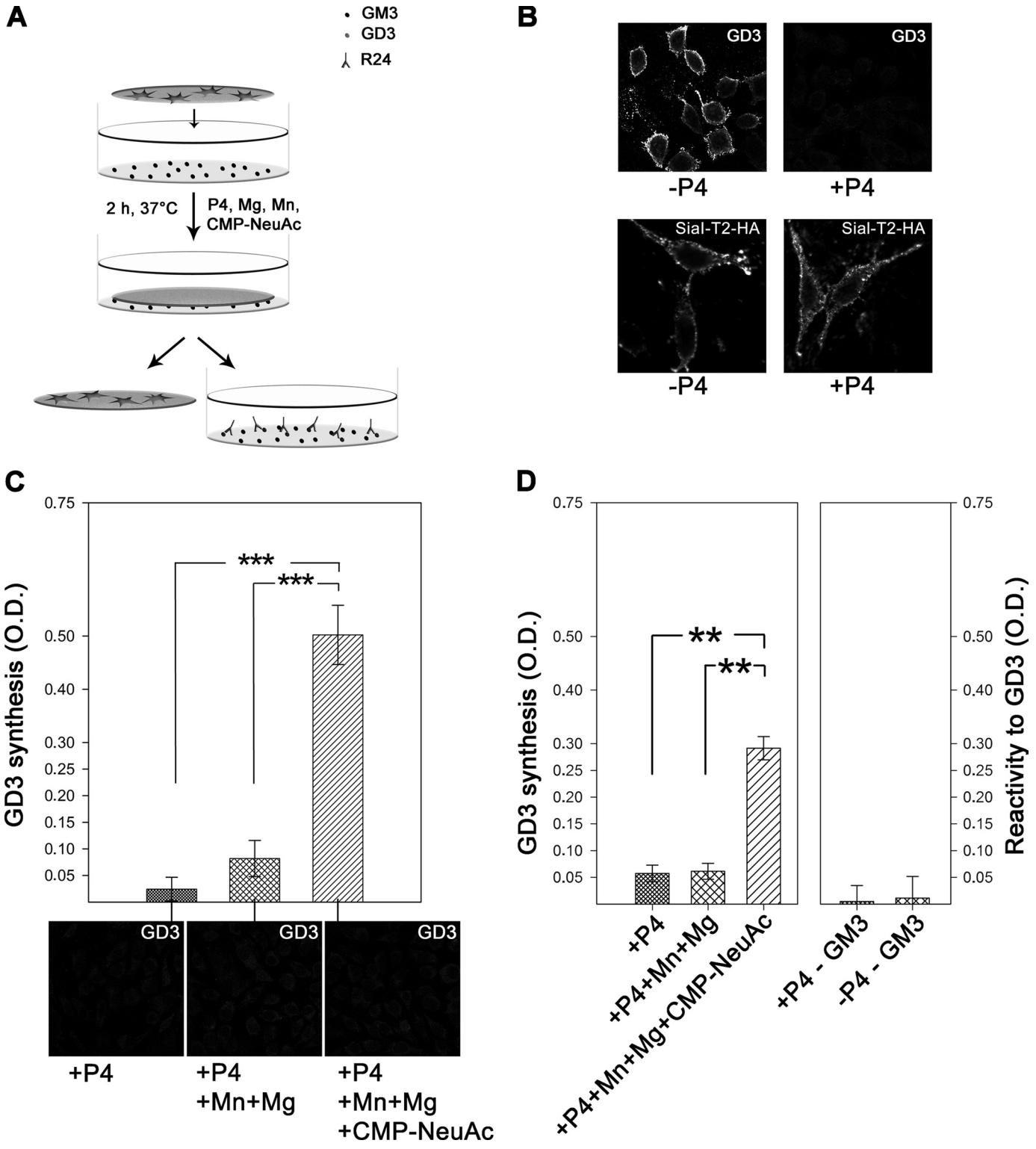
Confocal images were collected using a Carl Zeiss LSM5 Pascal laser-scanning confocal microscope (Carl Zeiss AG, Germany) or an Olympus FluoView FV300 confocal microscope (Olympus Latin America, Miami, FL) equipped with an argon/helium/neon laser and a ×63 (numerical aperture = 1.4) oil immersion objective (Zeiss Plan-Apochromat). Single confocal sections of 0.8 μm were taken parallel to the coverslip (*xy* sections). Final images were compiled with Adobe Photoshop 9.0. The confocal fluorescence micrographs shown in this manuscript are representative of at least three independent experiments.

RESULTS

Ecto-Sial-T2 from CHO-K1$^{Sial\text{-}T2+}$ Cells Sialylates GM3 Ganglioside Immobilized onto a Solid Surface—Many reports have described the significance of the regulation of the glycolipid metabolism at the plasma membrane (13, 14, 17, 18). However, the possibility that ecto-Sial-T2 may catalyze the conversion of GM3, belonging to the surface of neighboring cells, to the ganglioside GD3 has not yet been described. To explore this hypothesis, we first set up a very sensitive and specific ELISA to detect GD3 using the monoclonal antibody R24. This assay allowed us to detect values lower than 0.8 pmol of disialoganglioside GD3 (Fig. 1). As expected, the ELISA signals were drastically decreased when the microtitration plates were coated with different amounts of GM3, which clearly indicated the specificity of R24 antibody binding (Fig. 1*B*).

Next, we attempted to demonstrate trans-activity of ecto-Sial-T2 by using the ELISA procedure and following the experimental protocol described in Fig. 2*A*. Thus, CHO-K1 cells expressing Sial-T2 (CHO-K1$^{Sial\text{-}T2+}$) were grown on coverslips and treated with P4 (a potent inhibitor of ceramide glucosyltransferase) for 5 days to reduce GM3, GD3, and the neutral glycolipid content (Fig. 2*B*, *+P4*, *upper panel*). Under these experimental conditions, ecto-Sial-T2 expression was even observed at the cell surface (Fig. 2*B*, *lower panels*) (14). Next, P4-treated cells were washed and incubated at 37 °C for 150 min with GM3 (50 pmol) absorbed on polystyrene microtitra-

tion plates in a medium containing CMP-NeuAc, Mn^{+2}, Mg^{+2}, and P4 inhibitor. As shown in Fig. 2*C* (*third column*), we were able to detect a significant GD3 synthesis by ecto-Sial-T2 activity. On the other hand, GD3 synthesis was drastically reduced when P4-treated CHO-$K1^{Sial\text{-}T2+}$ cells were incubated in a medium containing the bivalent cations (10 mM Mn^{+2} and 1 mM Mg^{+2}) but without exogenous CMP-NeuAc (Fig. 2*C*, *second column*). A similar result was obtained when P4-treated CHO-$K1^{Sial\text{-}T2+}$ cells were incubated in DMEM (culture medium containing 0.814 mM Mg^{+2}) (Fig. 2*C*, *first column*). Additionally, the coverslips containing the cells that had been used in the different experimental conditions were fixed and processed for immunodetection of GD3 and later analysis by confocal fluorescence microscopy. As shown in Fig. 2*C* (*lower*



panels), the fluorescent signal was under the limit of detection in all the conditions, with these results being similar to those obtained with P4-treated CHO-K1$^{Sial\text{-}T2+}$ cells (Fig. 2*B*, *+P4*, *upper panel*). This suggests that GM3 was not taken and processed (addition of sialic acid moiety) in membranes from P4-treated CHO-K1$^{Sial\text{-}T2+}$ cells. Instead, the results support the idea that synthesis of GD3 by ecto-Sial-T2 might have been occurring in immobilized GM3 on the solid surface.

To study the trans-activity of ecto-Sial-T2 and to attempt to rule out the possibility of GM3 release from the microtitration plates with later uptake by the cells, we performed a chemical GM3 immobilization via the carbon-carbon bond using photo reactive Universal-BIND™ surface microtitration plates as described under "Experimental Procedures." As shown in Fig. 2*D* (*left columns*), synthesis of GD3 was detected when P4-treated CHO-K1$^{Sial\text{-}T2+}$ cells were incubated at 37 °C for 90 min with covalently immobilized GM3 in a medium containing bivalent cations (10 mM Mn^{+2} and 1 mM Mg^{+2}) and exogenous CMP-NeuAc. On the other hand, GD3 synthesis was drastically reduced when P4-treated CHO-K1$^{Sial\text{-}T2+}$ cells were incubated in a medium containing the bivalent cations (10 mM Mn^{+2} and 1 mM Mg^{+2}) or incubated in DMEM only. Interestingly, the ELISA signals obtained from microtitration plates (without GM3) incubated with CHO-K1$^{Sial\text{-}T2+}$ cells (-P4-GM3) or P4-treated CHO-K1$^{Sial\text{-}T2+}$ cells (+P4-GM3) in DMEM were weak, indicating both the specificity of the detection and that GD3 from CHO-K1$^{Sial\text{-}T2+}$ cells was not released and absorbed on the surface plates and therefore not significantly contributing to the positive signal in the studied experimental conditions (Fig. 2*D*, *right columns*). Similar results were obtained in ELISA assays using GM3 absorbed on the surface plate (results not shown). Taken together, these results further indicate that the addition of sialic acid residue on GM3 immobilized or absorbed on an artificial surface occurs via trans-sialylation, catalyzed by ecto-Sial-T2 expressed on the plasma membrane of CHO-K1$^{Sial\text{-}T2+}$ cells.

Sialylation of Immobilized GM3 Ganglioside by Ecto-Sial-T2 from SK-Mel-28 Human Melanoma Cells—Next, we examined trans-sialylation catalyzed by the ecto-Sial-T2 expressed on the cell surface of SK-Mel-28 human melanoma cells, which endogenously synthesize the sialyltransferase and express the ganglioside GD3 (Fig. 3) (14, 19). GD3 synthesis by ecto-Sial-T2 activity on the SK-Mel-28 cell surface was measured following essentially the same protocol as that described above for CHO-K1$^{Sial\text{-}T2+}$ cells. When P4-treated SK-Mel-28 cells were incubated at 37 °C with immobilized GM3 in a medium containing only DMEM or bivalent cations in the absence of exogenous CMP-NeuAc, no appreciable synthesis of GD3 was observed

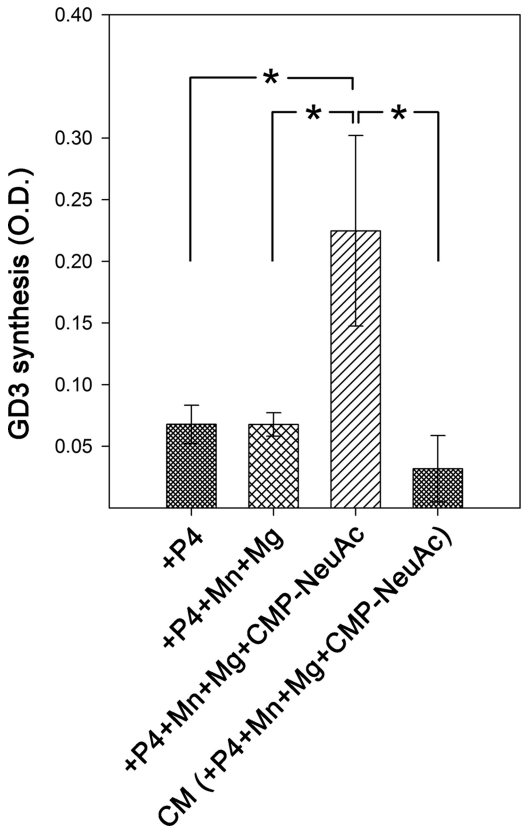


FIGURE 3. **Ecto-Sial-T2 from SK-Mel-28 human melanoma cells sialylates the GM3 ganglioside absorbed onto a solid surface.** GM3 (0.05 nmol) was coated on polystyrene microtitration plates in methanol and dried at 37 °C. Then, SK-Mel-28 cells previously grown on coverslips with P4 (*+P4*) for 5 days were incubated for 90 min at 37 °C on the immobilized GM3 in a medium containing only DMEM (*+P4*) or in a medium containing Mn^{2+} and Mg^{2+} (*+P4+Mn+Mg*) or CMP-NeuAc, Mn^{2+}, and Mg^{2+} (*+P4+Mn+Mg+CMP-NeuAc*). Also, absorbed GM3 was incubated for 90 min at 37 °C with CM from SK-Mel-28 cells previously incubated with CMP-NeuAc plus cations. After 90 min, the coverslips were removed, and the GD3 synthesis for all experimental conditions was detected as described in Fig. 2 and under "Experimental Procedures." Results are mean ± S.E. of two independent experiments. Note that GD3 synthesis was significantly higher in the medium containing exogenous CMP-NeuAc plus cations than in the medium containing only DMEM, cations, or conditioned medium. *, $p < 0.05$.

FIGURE 2. **Ecto-Sial-T2 from CHO-K1$^{Sial\text{-}T2+}$ cells sialylates GM3 ganglioside absorbed or chemically immobilized onto a solid surface.** *A*, schematic representation of the experimental protocol employed to study trans-activity of ecto-Sial-T2 described in *C* and *D*. *B*, *upper panels*, CHO-K1$^{Sial\text{-}T2+}$ cells treated with P4 (*+P4*) or vehicle (*-P4*) were immunostained with antibody to GD3 and fixed and incubated with secondary antibody conjugated to Alexa Fluor 488. Representative fluorescent confocal sections of 0.8 μm were taken parallel to the coverslip. *Lower panels*, CHO-K1$^{Sial\text{-}T2+}$ cells were grown on coverslips and treated with P4 (*+P4*) or without P4 (*-P4*) for 5 days. Then, Sial-T2-HA expressing cells were immunostained with antibody to HA at 4 °C for 60 min and fixed and incubated with a secondary antibody conjugated to Alexa Fluor 488. Representative fluorescent confocal sections of 0.8 μm were taken parallel to the coverslip. *C*, GM3 (0.05 nmol) was coated on polystyrene microtitration plates in methanol and dried at 37 °C. Then, wells were saturated with PBS/2% BSA for 2 h. Next, CHO-K1$^{Sial\text{-}T2+}$ cells previously grown on coverslips with P4 (*+P4*) for 5 days at 37 °C were incubated for 2 h with the immobilized GM3 in a medium containing only DMEM (*+P4*) or in a medium containing Mn^{2+} and Mg^{2+} (*+P4+Mn+Mg*) or CMP-NeuAc, Mn^{2+}, and Mg^{2+} (*+P4+Mn+Mg+CMP-NeuAc*). After incubation, coverslips were removed and processed for immunodetection of antibody to GD3, whereas wells were washed and processed for ELISA. ***, $p < 0.005$. *D*, GM3 (0.05 nmol) was coated on the photoreactive Universal-BIND™ surface microtitration plates in methanol and dried at 37 °C. Then, the covalent immobilization of GM3 was made using UV illumination. Next, wells were saturated with PBS/2% BSA for 2 h. CHO-K1$^{Sial\text{-}T2+}$ cells previously grown on coverslips with P4 (*+P4*) for 5 days were incubated for 90 min on the immobilized GM3 under the same conditions mentioned in *C*. After incubation, coverslips were removed and the GD3 synthesis was immunodetected by ELISA procedure (***left panel***). Note that GD3 synthesis was significantly higher in the medium containing exogenous CMP-NeuAc plus cations than in the medium containing only DMEM or DMEM plus cations. **, $p < 0.01$. Additionally, CHO-K1$^{Sial\text{-}T2+}$ cells previously grown on coverslips and treated with (*+P4*) or without P4 (*-P4*) were incubated on uncoated GM3 microtitration plates (*+P4-GM3* and *-P4-GM3*, respectively) (*right panel*). Then, coverslips were removed, and GD3 was immunodetected by ELISA procedure. Results (*O.D.*) are mean ± S.E. of duplicates of four independent experiments (*C*) or means ± S.E. of three samples (*D*).

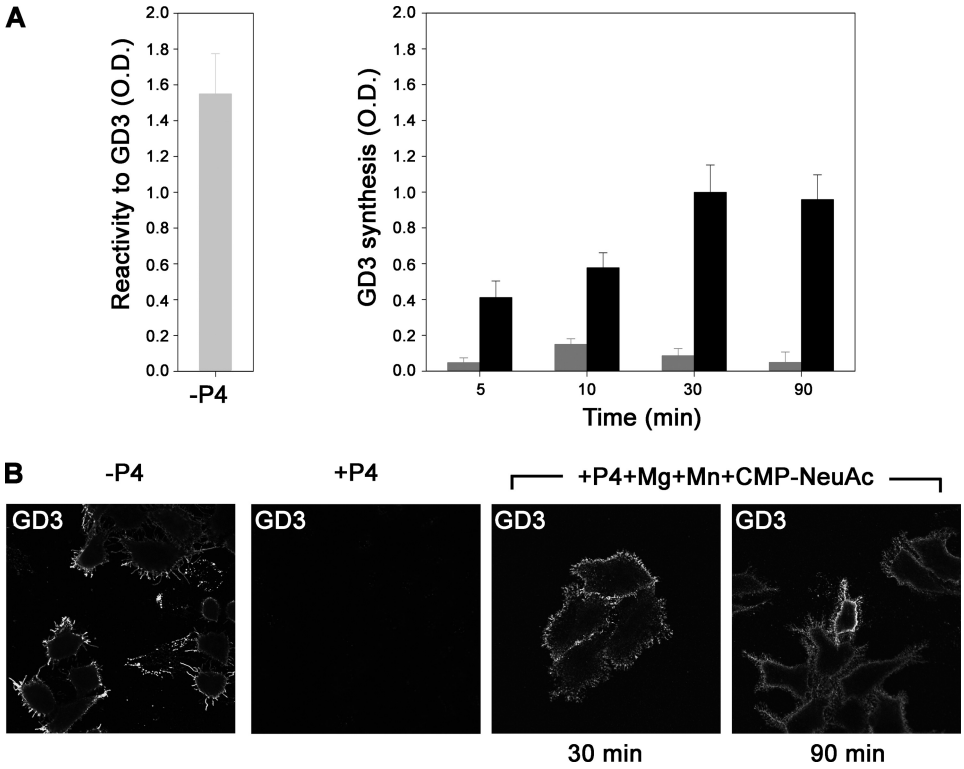


FIGURE 4. **Detection of GD3 synthesis on the cell surface of CHO-K1$^{Sial\text{-}T2+}$ cells by ELISA.** *A*, right panel, CHO-K1$^{Sial\text{-}T2+}$ cells were grown on 96-well polystyrene flat-bottom plates with P4 (12,000 cells) or without P4 (5000 cells) for 4 days. Then, cells were treated with 25 μM GM3, washed, and incubated at 37 °C for 5, 10, 30, or 90 min in a medium containing only DMEM (*gray bars*) or in a medium containing CMP-NeuAc, Mn^{+2}, and Mg^{+2} (*black bars*). The P4 inhibitor remained present throughout the experiments. Then, wells were washed and incubated with antibody to GD3 (R24) at 4 °C for 60 min and fixed and incubated with secondary antibody conjugated to HRP at 4 °C. After washes, HRP activity was revealed, and the *A* was measured at 490 nm as indicated under "Experimental Procedures." *Left panel*, as a positive control of the assay, CHO-K1$^{Sial\text{-}T2+}$ cells were grown on 96-well polystyrene flat-bottom plates without P4 (−*P4*) for 4 days, and GD3 expression was revealed by ELISA. Results are mean ± S.E. of three samples. *B*, CHO-K1$^{Sial\text{-}T2+}$ cells grown with P4 (+*P4*, *second* to *fourth panels*) or without P4 (−*P4*, *first panel*) for 4 days were treated with 25 μM GM3, washed, and incubated at 37 °C for 90 min in a medium containing only DMEM (+*P4*) or for 30 and 90 min in a medium containing CMP-NeuAc, Mn^{+2}, and Mg^{+2} (+*P4*+*Mn*+*Mg*+*CMP-NeuAc*). The P4 inhibitor remained present throughout the experiments. Then, cells were washed, immunostained with antibody to GD3 at 4 °C for 60 min, and fixed and incubated with secondary antibody conjugated to Alexa Fluor 488. Representative confocal sections of 0.8 μm taken parallel to the coverslip are shown.

(Fig. 3). As already seen in CHO-K1$^{Sial\text{-}T2+}$ cells, the addition of exogenous CMP-NeuAc further increased the synthesis of disialoganglioside GD3 (Fig. 3).

Soluble Sial-T2 Does Not Significantly Contribute to GD3 Synthesis on a Solid Surface—Several reports have described the secretion of active and proteolytically processed sialyltransferases in different cell types (15, 20–22). In fact, we previously described that the secreted Sial-T2 present in the culture medium from CHO-K1$^{Sial\text{-}T2+}$ cells grown for 48 h accounted for 8% of the total activity *in vitro* (15). Next, we attempted to investigate the contribution of soluble Sial-T2 in the catalytic conversion of GM3 (immobilized in the solid surface) to GD3. When GM3 coated polystyrene plates were incubated at 37 °C for 90 min with CM obtained from P4-treated SK-Mel-28 cells previously incubated with medium containing bivalent cations and exogenous CMP-NeuAc, no significant signal of GD3 synthesis was observed (Fig. 3, *right column*). This suggests that secreted and soluble Sial-T2 does not appreciably contribute to the addition of sialic acid residue in GM3 absorbed on the solid surface.

Overall, the results shown in Figs. 2 and 3 indicate that endogenously (SK-Mel-28 cells) and ectopically (CHO-K1$^{Sial\text{-}T2+}$ cells) expressed Sial-T2 are able to exert trans-sialylation toward the gangliosides immobilized in an artificial matrix, thus leaving open the possibility of trans-sialylation by means of the substrate exposed on the cell surface of adjacent cells.

GM3 Expressed at the Cell Surface of CHO-K1^{WT} Cells Is Catalytically Converted to GD3 by Ecto-Sial-T2 from Neighboring CHO-K1$^{Sial\text{-}T2+}$ Cells—We recently demonstrated by biochemical approaches, combined with confocal microscopy and flow cytometric analysis, the expression and cis-activity of ecto-Sial-T2 at the cell surface of epithelial and melanoma cells (14). In this study, these results were further confirmed and validated by using the very sensitive and specific ELISA procedure. CHO-K1$^{Sial\text{-}T2+}$ cells were grown on 96-well polystyrene flat-bottom plates with P4 (+*P4*) or without P4 (-*P4*) for 5 days. Then, cells were incubated for 2 h with 30 μM GM3 before being washed and incubated in a medium containing CMP-NeuAc, Mn^{+2}, and Mg^{+2} at 37 °C for 5, 10, 30, and 90 min in the presence of P4 inhibitor. As shown in Fig. 4*A* (*right panel*, *black bars*), GD3 synthesis was detected at the cell surface of P4-treated CHO-K1$^{Sial\text{-}T2+}$ cells at 5 min and continued to rise, reaching a plateau at 30 min. Thus, these results indicate that Sial-T2 was able to use the exogenously incorporated acceptor (GM3) to catalytically convert it to disialoganglioside (GD3) in a fast process of sialylation at the cell surface. In contrast, a reduced amount of GD3 synthesis was seen when P4-treated CHO-K1$^{Sial\text{-}T2+}$ cells were fed with GM3 and incubated in DMEM only (culture

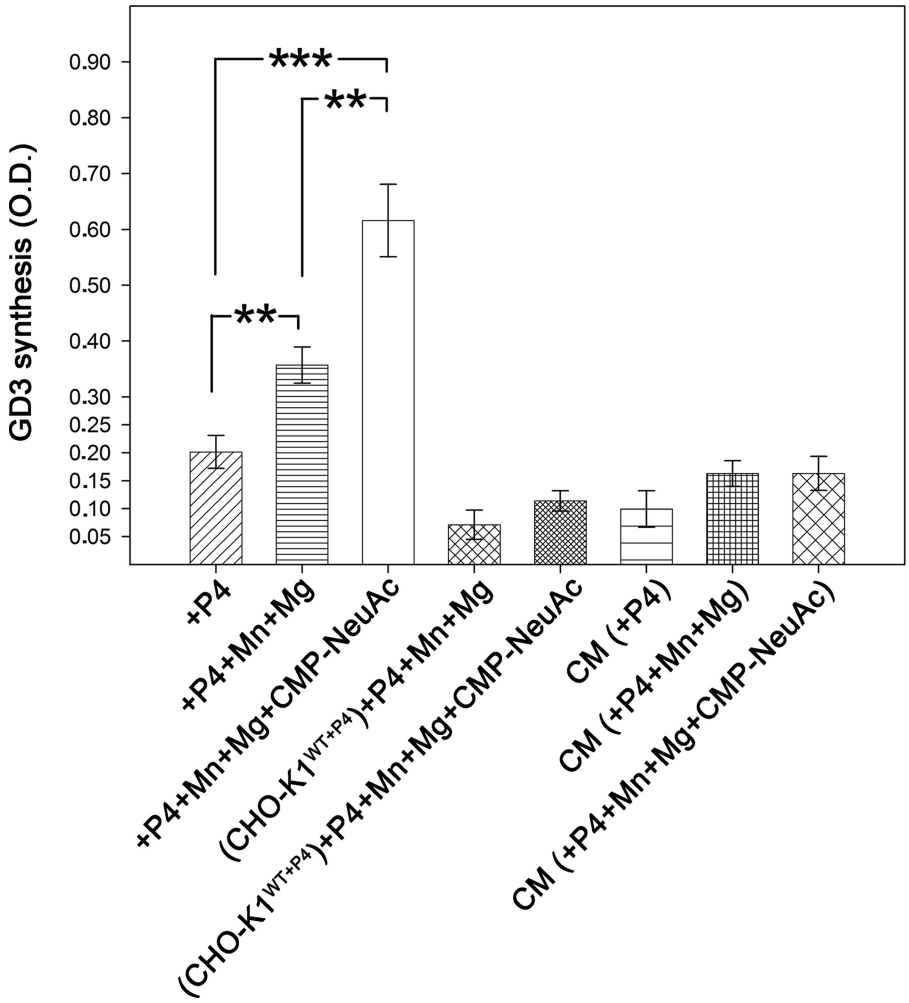


FIGURE 5. **Ecto-Sial-T2 from CHO-K1$^{Sial\text{-}T2+}$cells sialylates GM3 expressed at the cell surface of CHO-K1^{WT}cells.** CHO-K1^{WT} and CHO-K1$^{Sial\text{-}T2+}$ cells were grown on 96-well polystyrene flat-bottom plates or coverslips, respectively, with P4 (*+P4*) or without P4 (*−P4*) for 5 days. Then, P4-treated CHO-K1$^{Sial\text{-}T2+}$ cells were incubated face to face for 90 min at 37 °C with P4-untreated CHO-K1^{WT} cells (*first*, *second*, and *third columns*) or with P4-treated CHO-K1^{WT} (*CHO-K1^{WT+P4}*, *fourth* and *fifth columns*) in a medium containing only DMEM (*+P4*); Mn^{2+} and Mg^{2+} (*+P4+Mn+Mg*); or CMP-NeuAc, Mn^{2+}, and Mg^{2+} (*+P4+Mn+Mg+CMP-NeuAc*). Additionally, CHO-K1^{WT} cells were incubated for 90 min at 37 °C with conditioned medium obtained from CHO-K1$^{Sial\text{-}T2+}$ cells previously incubated at the conditions indicated in the *first*, *second*, and *third columns*. Next, the coverslips (*first* to *fifth columns*) were removed and wells (*first* to *eighth columns*) were washed and incubated with antibody to GD3 (R24) at 4 °C for 60 min and then fixed and incubated with secondary antibody conjugated to HRP at 4 °C. After washes, HRP activity was revealed, and the *A* was measured at 490 nm as indicated under "Experimental Procedures." Results are mean ± S.E. of three to five independent experiments. Note that the GD3 synthesis on CHO-K1^{WT} cells was significantly higher in the medium containing exogenous CMP-NeuAc plus cations than in the medium containing DMEM only or DMEM plus cations (***, $p < 0.005$; **, $p < 0.01$, respectively). GD3 synthesis on CHO-K1^{WT} cells was also significantly higher in the medium containing DMEM plus cations than DMEM only (**, $p < 0.01$).

medium containing 0.814 mM Mg^{+2}) (Fig. 4*A*, *right panel*, *gray bars*). As expected, P4-untreated cells showed high levels of GD3-immnoreactivity (Fig. 4*A*, *left panel*), and, interestingly, similar results were obtained by confocal fluorescence microscopy analysis. Cells grown on coverslips with P4 (*+P4*) were fed for 2 h with 30 μM GM3 before being washed and then incubated at the same conditions mentioned above, with GD3 synthesis being immunodetected using antibody R24 (Fig. 4*B*).

Next, we attempted to evaluate whether ecto-Sial-T2 from CHO-K1$^{Sial\text{-}T2+}$ cells was able to catalyze the transfer of sialic acid moiety to the GM3 ganglioside expressed on the plasma membrane from wild-type CHO-K1 cells (CHO-K1 ^{WT}, Sial-T2, and GD3-negative). Interestingly, the results showed a considerable increase in GD3 synthesis in CHO-K1^{WT} cells after incubation face-to-face with P4-treated CHO-K1$^{Sial\text{-}T2+}$ cells (grown on coverslips) for 2 h in a medium containing bivalent cations (10 mM Mn^{+2} and 1 mM Mg^{+2}) and exogenous CMP-NeuAc (Fig. 5, *third column*, *+P4+Mn+Mg+ CMP-NeuAc*). In contrast, although a reduced amount of GD3 synthesis was detected in CHO-K1^{WT} cells after incubation face-to-face with P4-treated CHO-K1$^{Sial\text{-}T2+}$ cells in a medium containing only DMEM (Fig. 5, *first column*), a significant increase of GD3 synthesis was also observed in CHO-K1^{WT} cells after incubation face-to-face with P4-treated CHO-K1$^{Sial\text{-}T2+}$ in a medium containing bivalent cations in the absence of exogenous CMP-NeuAc (Fig. 5, *second column*). Furthermore, when the biosynthesis of glycolipids (including the substrate GM3) was inhibited in CHO-K1^{WT} cells by P4 treatment, GD3 synthesis was drastically reduced in these cells after incubation with P4-treated CHO-K1$^{Sial\text{-}T2+}$ cells in a medium containing bivalent cations or in an incubation system containing cations plus exogenous CMP-NeuAc (Fig. 5, *fourth* and *fifth columns*, respectively). Taken together, these results strongly suggest the existence of specific trans-sialylation catalyzed by ecto-Sial-T2,

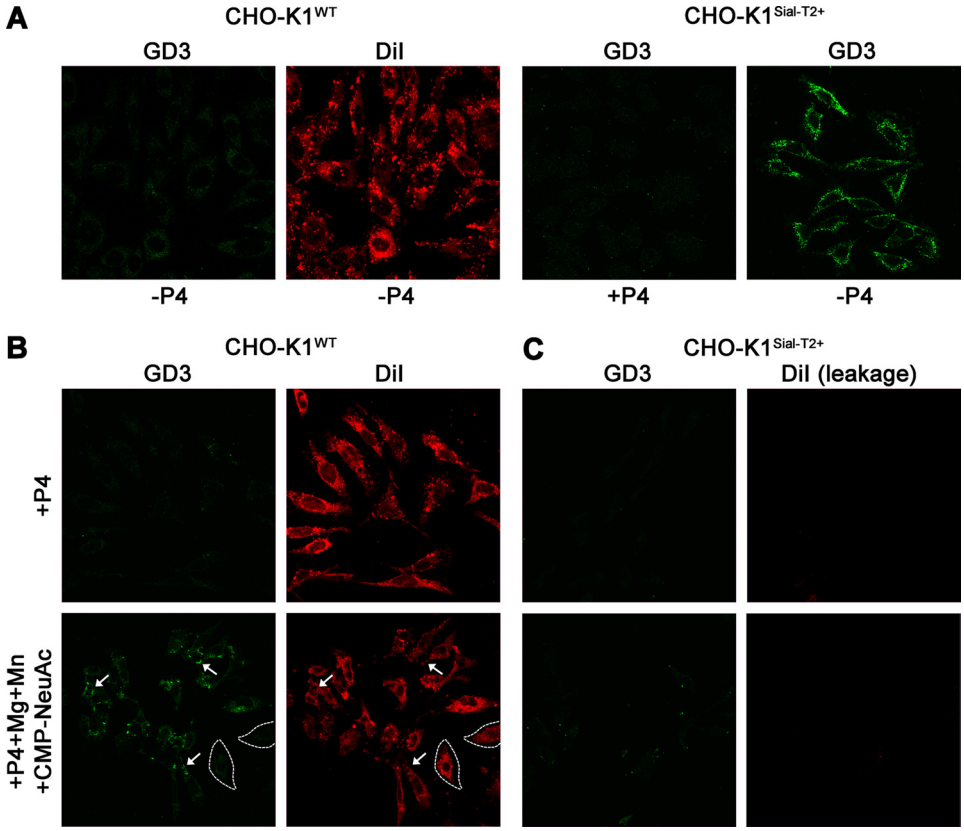


FIGURE 6. **Trans-sialylation of ecto-Sial-T2 demonstrated by immunofluorescence.** *A*, CHO-K1^{WT} cells and CHO-K1$^{Sial\text{-}T2+}$ cells were grown on coverslips for 5 days in DMEM (−*P4*; *first*, *second*, and *fourth panels*). To reduce the content of glycolipids, CHO-K1$^{Sial\text{-}T2+}$ cells were grown on coverslips for 5 days in DMEM containing P4 (+*P4*; *third panel*). Then, cells were fixed and immunostained for GD3 using the R24 antibody as indicated under "Experimental Procedures." For CHO-K1^{WT} cells, they were labeled additionally with 0.2 μM DiI at 37 °C for 30 min before fixation (*DiI*, *second column*). *B*, CHO-K1$^{Sial\text{-}T2+}$ cells treated with P4 were incubated face-to-face at 37 °C for 90 min with CHO-K1^{WT} cells previously labeled with 0.2 μM DiI in a medium containing only DMEM (+*P4*, *first row*) or CMP-NeuAc, Mn^{2+}, and Mg^{2+} (+*P4*+*Mn*+*Mg*+*CMP-NeuAc*, *second row*). After 90 min, coverslips were removed (used for experiments shown in *C*), and cells were immunostained with antibody to GD3 (R24) at 4 °C for 60 min and then fixed and incubated with secondary antibody conjugated to Alexa Fluor 488. Single confocal sections were taken every 0.8 μm parallel to the coverslip. Cell boundaries (*white lines*) are indicated. The GD3 synthesis is also shown (*arrows*) in CHO-K1^{WT} cells. The fluorescence micrographs are representative of three independent experiments. *C*, coverslips from the experiment mentioned in *B* and containing P4-treated CHO-K1$^{Sial\text{-}T2+}$ cells were processed for GD3 immunostaining and analyzed by confocal microscopy in fluorescence channels for Alexa Fluor 488 and DiI detection.

which was able to exert its enzymatic activity on the GM3 belonging to the surface of neighboring cells by using both the exogenous and endogenous donor substrate (CMP-NeuAc) available at the extracellular milieu.

As already described above for SK-Mel-28 cells (Fig. 3), the incubation of CHO-K1^{WT} cells, with conditioned medium from P4-treated CHO-K1$^{Sial\text{-}T2+}$ cells previously incubated with medium containing bivalent cations and exogenous CMP-NeuAc, did not significantly enhance GD3 synthesis above the levels observed in control conditions (medium only or medium containing bivalent cations) (Fig. 5). Thus, this suggests that the secreted and soluble form of Sial-T2 from CHO-K1 cells did not make a significant contribution to the addition of sialic acid residue in GM3 from the surface of adjacent cells.

Trans-sialylation catalyzed by ecto-Sial-T2 in CHO-K1 cells was further investigated by immunofluorescence and confocal microscopy analysis. The lack of expression of GD3 in wild-type CHO-K1 cells and the drastic reduction of GD3 synthesis in P4-treated CHO-K1$^{Sial\text{-}T2+}$ cells is shown in Fig. 6*A*. GD3 synthesis at the cell surface of CHO-K1^{WT} (previously labeled with the lipophilic fluorescent carbocyanine DiI dye) was low when incubated face-to-face with P4-treated CHO-K1$^{Sial\text{-}T2+}$ cells for 90 min in DMEM (Fig. 6*B*, *top row*, +*P4*). However, a noticeable increase in GD3 synthesis at the cell surface of CHO-K1^{WT} was observed when the cells were incubated in a complete system containing bivalent cations (10 mM Mn^{+2} and 1 mM Mg^{+2}) and exogenous CMP-NeuAc (Fig. 6*B*, *bottom row*, +*P4*+*Mg*+*Mn*+ *CMP-NeuAc*), which is in complete agreement with results obtained by the ELISA procedure (Fig. 5). Additionally, these experiments also indicated that lipid transfer or leakage from CHO-K1^{WT} to CHO-K1$^{Sial\text{-}T2+}$ cells was very restricted because no significant DiI fluorescence was detected in CHO-K1$^{Sial\text{-}T2+}$ cells (Fig. 6*C*). Therefore, the poor expression of GD3 at the surface of P4-treated CHO-K1$^{Sial\text{-}T2+}$ cells (Fig. 6*C*, *bottom row*, +*P4*+*Mg*+*Mn*+ *CMP-NeuAc*) probably represents a reduced synthesis of GD3 that used the remaining amount of GM3 after P4 treatment.

DISCUSSION

Ganglioside sialylation is a process that involves numerous Golgi-associated sialyltransferases which also contribute to diversity in the linkage and the chemical structure of sialic acid residues on the cell surface. Although ganglioside sialylation

was first reported more than 30 years ago, the development of new molecular and biochemical approaches in recent years has produced a notable growth in information about the presence and significance of the sialyltransferases and glycohydrolases associated with the outer layer of the plasma membrane. In this sense, we recently demonstrated in epithelial and melanoma cells that Sial-T2 is able to sialylate GM3 at the plasma membrane by using both the exogenous and endogenous donor (CMP-NeuAc) and acceptor (GM3) substrates (14). In this work, we report for the first time that ecto-Sial-T2 expressed at the cell surface of CHO-K1 cells is able to sialylate GM3 ganglioside absorbed on an artificial surface using the exogenous donor CMP-NeuAc. Furthermore, ecto-Sial-T2 trans-activity was also detected when GM3 was covalently immobilized on a solid surface, thus discarding the possibility that GM3 could have been removed from the solid surface during the period of incubation and later modified by "cis" interaction with the enzyme expressed at the cell surface. All these observations were also confirmed using SK-Mel-28 human melanoma cells, which endogenously express Sial-T2 both at the Golgi complex and at the cell surface (14). More interestingly, we obtained strong experimental evidence indicating a specific trans-sialylation catalyzed by ecto-Sial-T2 that was able to exert its enzymatic activity on the GM3 belonging to the surface of neighboring cells (see simplified representation in Fig. 7).

The highly sensitive (able to detect less than 0.8 pmol of disialoganglioside GD3) and specific ELISA employed in this study allowed us to observe GM3 cis-sialylation (Fig. 4) and trans-sialylation (Fig. 5) at 5–10 min and 90 min of incubation, respectively, which indicates a fast enzymatic process. Rapid cell surface sialylation was also reported in other cell types such as polymorphonuclear leukocytes, which express the sialidase and sialyltransferase activities that permit modulation of their surface sialylation and hence adhere to and migrate across the endothelium (22), or in human brain-derived TE671 cells, where there is a rapid GD3 synthesis and trafficking during amyloid β-induced apoptosis (23). Thus, the fast transformation of GM3 to GD3 is in agreement with the hypothesis that enzymes such as sialyltransferases can act quickly and locally by modifying the pattern of glycolipids on the cell surface or the sialylating substrates expressed in membranes from neighboring cells in response to a range of dissimilar environmental stimuli.

Several reports have described the secretion of active and proteolytically processed sialyltransferases into extracellular media of different cell types (15, 20–22). However, we report here that the secreted and soluble form of Sial-T2 does not make a significant contribution to the addition of sialic acid residue on GM3 immobilized on artificial surfaces or expressed at the surface of neighboring cells, which strongly suggests that sialylation is mainly mediated by ecto-Sial-T2 trans-activity. Moreover, the absence of GD3 synthesis by secreted sialyltransferase could be indicating differences in enzymatic parameters (*i.e.* in the enzyme acceptor or donor substrate specificity or affinity) between secreted Sial-T2 and ecto-Sial-T2 enzymes or that the amount of Sial-T2 released to the culture medium in the period of time used in our experimental conditions was insufficient to sialylate GM3. Nevertheless, we cannot discard

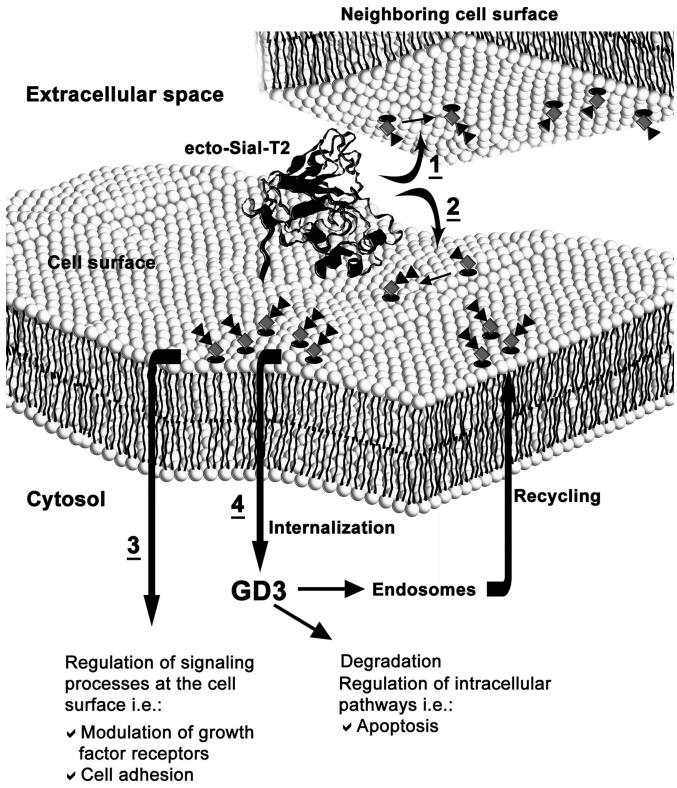


FIGURE 7. **Schematic representation of cis- and trans-catalytic activity exerted by ecto-Sial-T2 and putative intracellular routes and the biological implications of plasma membrane-synthesized GD3.** The schematic shows plasma membranes from two opposing cells, one of which expressed ecto-Sial-T2 (*lower membrane*). GM3 substrate, expressed in both cell membranes or incorporated from the extracellular medium, was able to be used for synthesis of GD3. Sialic acid transfer to GM3 is exposed in membranes from neighboring cells by ecto-Sial-T2 (trans-catalytic activity) (*1*). Sialic acid transfer to GM3 is exposed at the same cell surface as ecto-Sial-T2 (cis-catalytic activity) (*2*). GD3 regulates cell signaling processes at the cell surface (*3*). GD3 is eventually internalized to modulate intracellular pathways, degraded, or recycled back to the plasma membrane via endosomes (*4*). The structural representation of ecto-Sial-T2 corresponds to another mammalian sialyltransferase (porcine ST3Gal-I) described by Rao *et al.* (39), with minor modifications. GM3 oligosaccharide is represented as a *black oval* (glucose), *gray rhombus* (galactose), and ▲ (sialic acid). GD3 is represented with an additional ▲ (sialic acid).

that the local concentration of soluble Sial-T2 in tissues and intercellular spaces may be high enough to contribute to GD3 synthesis at the cell surface. Supporting the first assumption, we observed that the *N*-glycan processing status of cell surface-located Sial-T2 was different to that of the secreted form of Sial-T2 (14), which probably affects the enzymatic properties. On this regard, *N*-linked oligosaccharides in UDP-GalNAc:lactosylceramide/GM3/GD3 N-acetylgalactosaminyltransferase (GalNAc-T) (24), UDP-Gal:GA2/GM2/GD2 galactosyl transferase (Gal-T2) (25), and Sial-T2 (15, 26) were found to be critical for the activity of these ganglioside glycosyltransferases.

Ganglioside synthesis is highly regulated during cell differentiation, development, apoptosis, and oncogenic transformation, suggesting that gangliosides play a fundamental role in these processes. In particular, Sial-T2 and its enzymatic product GD3 are prevalently expressed at the early developmental stages of the central nervous system (for references, see Ref. 27. Sial-T2 gene knockout mice were shown to exhibit impairment in the regeneration of the lesioned hypoglossal nerves and ther-

mal hyperalgesia (28, 29), and the double knockout mice of the GalNAc-T and Sial-T2 genes showed reduced motor and sensory function and emotional response with progressive neurological deterioration (30, 31) and susceptibility to the induction of lethal seizures by sound stimulus (32, 33). In recent years, GD3 has emerged as a cell death effector because of its role in apoptosis signaling in different cellular types by inducing mitochondrial damage (34, 35). However, GD3 has also been reported to be overexpressed in a variety of tumors, especially in melanomas, where GD3 promotes tyrosine phosphorylation of paxillin, thereby resulting in an increased cell growth and an increased invasion by adhesion signals via integrin (36, 37). Recently, it was also suggested that the signal transduction in ganglioside GD3-enriched lipid microdomains is involved in the growth cone morphology of cerebellar granule cells (38).

The biological effects of gangliosides depend on their site of production and on the subsequent subcellular distribution. In this context, both the cis- and trans-activity of ecto-Sial-T2 emerge as potential levels of regulation of ganglioside composition (see the schematic representation in Fig. 7) which, unlike the biosynthetic machinery associated with the Golgi complex, might spatially and rapidly modulate the plasma membrane physicochemical properties by expression of GD3 and, consequently, the different cell signaling processes.

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