

The Plasma Membrane-associated Sialidase MmNEU3 Modifies the Ganglioside Pattern of Adjacent Cells Supporting Its Involvement in Cell-to-Cell Interactions*

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We describe herein the enzyme behavior of MmNEU3, the plasma membrane-associated sialidase from mouse (*Mus musculus*). MmNEU3 is localized at the plasma membrane as demonstrated directly by confocal microscopy analysis. In addition, administration of the radio-labeled ganglioside GD1a to MmNEU3-transfected cells, under conditions that prevent lysosomal activity, led to its hydrolysis into ganglioside GM1, further indicating the plasma membrane topology of MmNEU3. Metabolic labeling with [1-³H]sphingosine allowed the characterization of the ganglioside patterns of COS-7 cells. MmNEU3 expression in COS-7 cells led to an extensive modification of the cell ganglioside pattern, *i.e.* GM3 and GD1a content was decreased to about one-third compared with mock-transfected cells. At the same time, a 35% increase in ganglioside GM1 content was observed. Mixed culture of MmNEU3-transfected cells with [1-³H]sphingosine-labeled cells demonstrates that the enzyme present at the cell surface is able to recognize gangliosides exposed on the membrane of nearby cells. Under these experimental conditions, the extent of ganglioside pattern changes was a function of MmNEU3 transient expression. Overall, the variations in GM3, GD1a, and GM1 content were very similar to those observed in the case of [1-³H]sphingosine-labeled MmNEU3-transfected cells, indicating that the enzyme mainly exerted its activity toward ganglioside substrates present at the surface of neighboring cells. These results indicate that the plasma membrane-associated sialidase MmNEU3 is able to hydrolyze ganglioside substrates in intact living cells at a neutral pH, mainly through cell-to-cell interactions.

Glycosphingolipids (GSLs)¹ expressed at the cell surface are well known as modulators of several aspects of signal trans-

duction processes involved in the control of cell proliferation, survival, and differentiation (1). GSLs in the plasma membrane are able to interact laterally with other membrane molecules modulating their properties (*cis*-interactions). Lipid rafts or membrane microdomains result from dynamic clustering of sphingolipids and cholesterol to form the so-called sphingolipid-enriched domain (SED) or lipid rafts (2). These structures move within the fluid bilayer and function as platforms for the attachment of proteins when membranes are moved around the cell and during signal transduction (3, 4). In addition, the expression pattern of GSLs in several cells and tissues undergoes deep changes during development and neoplastic transformation (5). These events are usually characterized by dramatic changes in cell recognition, suggesting that GSLs as cell surface antigens could play relevant roles as receptor sites in cell-cell recognition (*trans*-interaction). The receptor role of GSLs has been hypothesized in the case of microbial infections based on their ability to interact with bacterial toxins and microbial lectins (6, 7). Conformational analysis confirms that the orientation of the oligosaccharide chains of glycolipids at the cell surface complies with the possibility to interact with extracellular molecules (8, 9). Indeed, synthetic analogues of GSL oligosaccharides are able to block cell-cell recognition (10). In the case of mammalian cells, *trans*-GSL-GSL interactions are important in determining the motility and metastatic potential of tumor cells (11). For example, in B16 melanoma cells, characterized by high levels of GM3,² the adhesion to endothelial cells is a GM3-dependent phenomenon. GM3 is closely associated with c-Src, Rho, and Ras within SED, and interactions with globoside Gg3 or the use of anti-GM3 antibody stimulate focal adhesion kinase phosphorylation and c-Src activity. In these conditions, not only adhesion but also spreading and enhancement of cell motility occur (12). Similar results have been obtained in the case of globoside-dependent adhesion in human embryonal carcinoma 2102 cells (13).

On the other hand, several different classes of cell surface proteins potentially can interact with specific carbohydrate sequences of GSLs, including animal lectins and receptors with lectin sequence homologies such as selectin family receptors and human lymphocyte IgE receptor (1). It has been shown that in the nervous system gangliosides represent functional

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¹ The abbreviations used are: GSL, glycosphingolipid; 4-MU-NeuAc, 4-methylumbelliferyl α -N-acetyl-D-neuraminic acid; HA, hemagglutinin; PBS, phosphate-buffered saline; HPLC, high performance liquid

chromatography; HPTLC, high performance silica gel-precoated thin-layer chromatography; SED, sphingolipid-enriched domain; DMEM, Dulbecco's modified Eagle's medium; ESI, electrospray ionization.

² The ganglioside nomenclature proposed by Svennerholm was followed (see Ref. 61).

ligands for the myelin-associated glycoprotein, a sialic acid binding lectin involved in myelin-axon interactions (14). Other cell surface proteins that are natural candidates for the interaction with GSL oligosaccharides are glycosyltransferases and glycohydrolases. In the past, little attention was paid to these proteins concerning this aspect, because their main localization was believed to be restricted inside the cell. Today, increasing evidence of the presence of such proteins at the level of the plasma membrane suggests their possible role as a cell recognition site for GSLs. Sialidases or neuraminidases (EC 3.2.1.18) are hydrolytic enzymes that remove sialic acid residues from gangliosides and other natural substrates. Among these proteins, plasma membrane-associated sialidase has been described in different tissues (15–17) and cell types (18–20), and a form linked to the membrane via glycosylphosphatidylinositol anchor has also been identified (21, 22). Evidence of a possible involvement of this enzyme in the regulation of the sialic acid levels of the cell surface has been produced. For example, in neuroblastoma cells the enzyme triggers selective ganglioside desialylation, and such surface glycolipid modulations are involved in cell growth control and differentiation (23–25). The plasma membrane sialidase NEU3³ is characterized by high substrate specificity for ganglioside substrates in the acidic range of pH (26). In addition, MmNEU3 has been demonstrated to be associated with SED in the neuroblastoma cell line SK-N-MC (27) as well as in HeLa and COS-1 cells, where it is closely associated with caveolin (28).

Herein we report the first direct evidence that: (i) MmNEU3 has enzyme activity toward gangliosides in intact living cells; and (ii) the enzyme activity of MmNEU3 is able to modulate the expression of gangliosides at the surface of adjacent cells.

EXPERIMENTAL PROCEDURES

Materials—Commercial chemicals were the purest available, common solvents were distilled before use, and deionized water obtained by a MilliQ system (Millipore) was distilled in a glass apparatus. High performance silica gel-precoated thin-layer plates (HPTLC Kiesegel 60, 20 × 10 cm) were purchased from Merck GmbH.

Sphingosine was prepared from cerebroside (29). [³H]Sphingosine (radiochemical purity >98%, specific radioactivity 2.08 Ci/mmol) was prepared by specific chemical oxidation of the primary hydroxyl group of sphingosine followed by reduction with sodium borohydride (30).

Ganglioside GD1a was purified from the total ganglioside mixture extracted from bovine brains (31). Radioactive GD1a containing erythro-C18-sphingosine, isotopically tritium-labeled at position 3, [³H(Sph18)]GD1a, was prepared by the dichloro-dicyano-benzoquinone/sodium borohydride method followed by reversed phase HPLC purification (homogeneity >99%, specific radioactivity of 1.2 Ci/mmol) (32, 33).

Radioactive sphingolipids were extracted from cells fed with [³H]sphingosine, purified, characterized as described previously (34), and used as chromatographic standards. Standard molecular biology techniques were carried out as described by Sambrook and Russell (35).

Tissue Culture and Expression of MmNEU3 in COS-7—COS-7 cells were grown in Petri dishes (100 mm diameter) using Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (Sigma). A sialidase expression plasmid coding for MmNEU3 with a hemagglutinin (HA) tag at the C terminus was constructed as follows. The entire open reading frame of the enzyme was amplified with the HA epitope at the C terminus by PCR using MmNEU3 cDNA in pBluescript (Stratagene) as template and subcloned into pcDNA1 mammalian expression vector (Invitrogen), giving rise to the construct pcDNA1-MmNEU3 (36). The sequence of the construct was confirmed by automated DNA sequencing. Transfections were performed overnight with

pcDNA1-MmNEU3 construct or with pcDNA1 vector and LipofectAMINE reagent in accordance with the manufacturer's guidelines (Invitrogen) using 4–5 × 10⁵ cells/dish. Cells were harvested at 48 h after transfection by scraping, washed in PBS, and resuspended in the same buffer containing 1 mM EDTA, 1 μg/ml pepstatin A, 10 μg/ml apoprotinin, and 10 μg/ml leupeptin. Total cell extracts were prepared by sonication. Lipid extraction and analysis of sphingolipid component were performed on total cell extracts. The supernatant obtained after centrifugation at 800 × g for 10 min corresponded to the crude cell extract and was subsequently centrifuged at 200,000 × g for 15 min on an Optima TL 100 Ultracentrifuge (Beckman). Aliquots of the crude cell extract, 200,000 × g supernatant and pellet, were used for assays for protein (Coomassie Protein Assay Reagent; Pierce) and sialidase activity, as well as for Western blot analysis. Cell vitality was determined by the trypan blue procedure.

Treatment of Cell Cultures with [³H]Sphingosine—[³H]Sphingosine dissolved in methanol was transferred into a sterile glass tube and dried under a nitrogen stream. The residue was then dissolved in an appropriate volume of pre-warmed (37 °C) 10% fetal bovine serum DMEM medium to obtain a final concentration of 3 × 10⁻⁸ M (corresponding to 0.4 μCi/100-mm dish). Transfected COS-7 cells were incubated for a 2-h pulse followed by a 40-h chase. In the case of co-culture experiments, 2 × 10⁵ cells were plated in 100-mm dishes and transfected with pcDNA1-MmNEU3 or pcDNA1 vector alone, and 3 × 10⁵ COS-7 were separately subjected to a 2-h pulse with [³H]sphingosine. After labeling, cells were detached from dishes using 0.25% trypsin-EDTA, resuspended in 10% fetal bovine serum DMEM, and finally added to plates containing just transfected COS-7 cells. Cells were then co-cultured for 48 h, resulting in 48 h of post-transfection time and 60 h of chase. A time course analysis of metabolically labeled [³H]sphingolipids was performed after adding freshly [³H]sphingosine-labeled COS-7 cells to MmNEU3-HA and mock-transfected cells after 48 h of post-transfection time. Cells were co-cultured for up to 30 h, corresponding to 78 h post-transfection time and 72 h of chase. Cells were harvested by scraping, washed in PBS, snap-frozen, lyophilized, and subjected to lipid extraction and sphingolipid analyses, together with the dialyzed medium collected from the cell cultures.

Immunofluorescence Staining and Confocal Analysis of MmNEU3—COS-7 cells were plated onto glass coverslips and grown for 24 h before transfection. Cells were transfected as previously described. After 16 h of incubation in DMEM in the presence of 10% fetal bovine serum, cells were washed in PBS, fixed for 40 min in 3% paraformaldehyde in PBS, and quenched for 10 min with 50 mM NH₄Cl in PBS. For permeabilization, cells were incubated for 15 min in the presence of 0.5% saponin (w/v) in PBS. Cells were then incubated with anti-HA mouse monoclonal antibody 12CA5 (15 μg/ml) (Roche Applied Science) in 0.5% saponin in PBS for 1 h and, after extensive washes with the same buffer, incubated with donkey anti-mouse Cy2 (1:400) in the same buffer. Fluorescence microscopy was carried out using an MRC-1024 confocal imaging system (Bio-Rad Laboratories). Image processing was performed with Adobe Photoshop software.

Treatment of Cell Cultures with [³H(Sph18)]GD1a—[³H(Sph18)]GD1a dissolved in propanol/water, 4:1 (v/v), was transferred into a sterile glass tube and dried under a nitrogen stream. The residue was then dissolved in an appropriate volume of pre-warmed (37 °C) DMEM to obtain [³H(Sph18)]GD1a at a final molar concentration of 3 × 10⁻⁷ M (corresponding to 2 nmol of GD1a/100-mm dish). Transfected COS-7 cells were incubated for up to 24 h in the presence of 10 mM NH₄Cl. Cells were harvested by scraping and washed in PBS, and total lipids were extracted from the cells and media (data not shown) and analyzed.

Lipid Extraction and Analyses—Total lipids from lyophilized cells and lyophilized cell media were extracted twice with chloroform/methanol 2:1 (v/v) and with chloroform/methanol/water 20:10:1 (v/v), respectively (37). The resulting lipid extracts were dried under a nitrogen stream and dissolved in chloroform/methanol 2:1 (v/v). In some experiments the lipid extracts were treated with 0.5 M NaOH in methanol to remove glycerophospholipids by selective hydrolysis (38). Lipid extracts were analyzed by HPTLC carried out with the solvent system chloroform-methanol-0.2% aqueous CaCl₂ 50:42:11 (v/v). The total lipid extracts were subjected to a two-phase partitioning in chloroform-methanol-water, 2:1 (v/v), and 20% water (38); the aqueous and organic phases obtained were counted for radioactivity and analyzed by HPTLC. [³H]Sphingolipids of aqueous and organic phases, respectively, were separated by HPTLC using the solvent systems chloroform-methanol-0.2% aqueous CaCl₂, 60:40:9 (v/v), and chloroform-methanol-water, 55:20:3 (v/v), respectively. [³H]Sphingolipids were identified by referring to radiolabeled standards and quantified by radiochromatography (Beta-Imager 2000, Biospace, Paris, France).

³ NEU is the official human gene symbol approved by HUGO Gene Nomenclature Committee. To avoid misinterpretation, the NEU symbol indicates the gene product (that is, sialidase), NEU3 is the symbol for plasma membrane-associated sialidase, and the two-letter code preceding NEU3 indicates the species of origin (e.g. Mm indicates *Mus musculus*). The NEU3 mRNA sequence is available in the GenBank™ data base under accession number NM_016720.

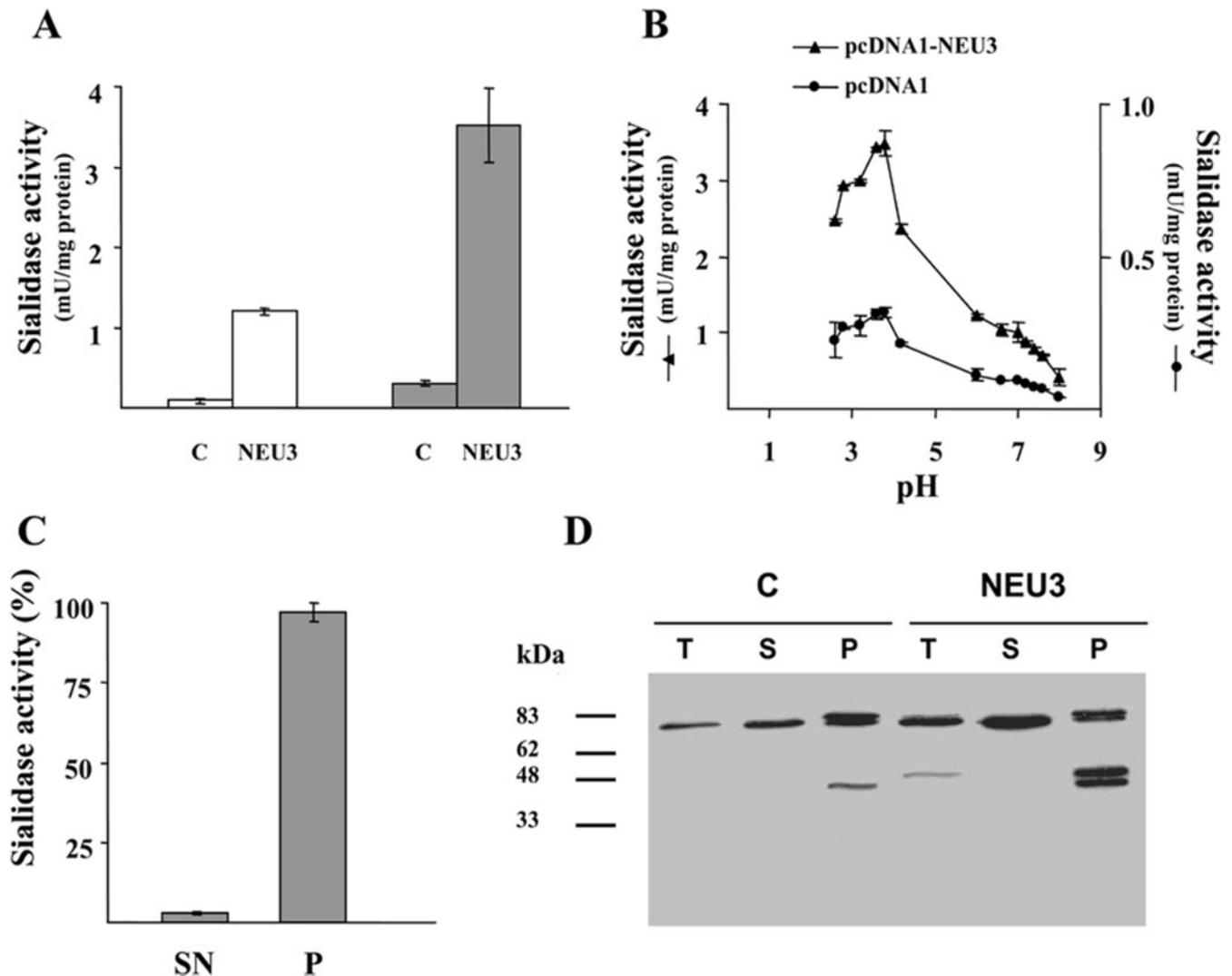


FIG. 1. **MmNEU3 expression in COS-7 cells.** *A*, COS-7 cells transfected overnight with pcDNA1 vector alone (*C*) or pcDNA1-MmNEU3 construct (*NEU3*) were grown for 48 h, and sialidase-specific activities toward the substrate 4-MU-NeuAc (white columns) and ganglioside GD1a (gray columns) were determined in the total cell lysate. Variations in the observed activity are indicated by the error bar ($n = 3$). *B*, rate of hydrolysis of ganglioside [$3\text{-}^3\text{H}(\text{Sph18})$]GD1a over the pH range of 2.8 to 8.0 in 12.5 mM sodium citrate/phosphate buffer using aliquots of the crude cell homogenate of pcDNA1-MmNEU3-transfected cells (▲) and pcDNA1 mock-transfected cells (●) as the enzymatic source. *C*, sialidase activity of the supernatant (SN) and pellet (P) obtained by ultracentrifugation at $200,000 \times g$ of the total lysate of pcDNA1-MmNEU3-transfected cells. The rates of hydrolysis of 4-MU-NeuAc were expressed as a percentage of the value detectable in the total cell lysate ($n = 3$). *D*, Western blot analysis of protein samples (10 μg) obtained by cells transfected with pcDNA1-MmNEU3 (*NEU3*) and mock-transfected cells (*C*). Total homogenate (*T*), supernatant (*S*), and pellet (*P*) obtained by ultracentrifugation were analyzed. The blot was carried out using anti-HA monoclonal antibody. Detection of antibodies bound to HA epitope was carried out using peroxidase-conjugated isotype-specific antibodies followed by ECL developing reagents. Protein standard positions (in kDa) are shown on the left.

The structural characterization of the ganglioside mixture from COS-7 cells was determined by reversed phase HPLC-ESI mass spectrometry. ESI mass spectrometry of the ganglioside species was carried out in negative mode on a ThermoQuest Finnigan LCQ_{deca} mass spectrometer equipped with an electrospray ion source (39).

Sialidase Assay—The enzymatic activity of MmNEU3 in total cell lysates and in cellular subfractions was determined as described previously using ganglioside [$3\text{-}^3\text{H}(\text{Sph18})$]GD1a and 4-MU-NeuAc as substrates (40). Assays were performed in triplicate with 25 μg of total protein in a final volume of 100 μl and in the presence of 12.5 mM sodium-citrate/phosphate buffer, pH 3.8. One unit of sialidase activity is defined as liberation of 1 μmol of Neu5Ac/min at 37 $^{\circ}\text{C}$.

Statistical Analyses—Values are presented as means \pm S.D. Statistical analyses were made using unpaired Student's *t* test. Significance was attributed at the 95% level of confidence ($p < 0.05$).

RESULTS

MmNEU3 Activity and Structure of Gangliosides in COS-7 Cells—Sialidase activity assay performed at pH 3.8 on GD1a ganglioside, without detergent in the reaction mixture, conclusively allows determination of the plasma membrane-associ-

ated sialidase activity with very minor or no contamination by lysosomal enzyme(s) activity (41). Under these experimental conditions, MmNEU3 activity on GD1a ranged from 0.1 to 0.3 milliunits/mg cell protein. The ganglioside mixture from COS-7 cells was analyzed by HPLC-ESI mass spectrometry, and the percentage of each species was determined by densitometry and radioimaging (see below) after HPTLC separation. GD1a, GM1, GM2, and GM3 were the main gangliosides and represented 41.8, 31.5, 15.8, and 10.9% of the total main ganglioside mixture, respectively. Each ganglioside species contained C18 sphingosine and by HPTLC analysis was split in two spots. The molecular species containing palmitic acid showed a lower mobility, whereas molecular species containing C24:0 and C24:1 fatty acids showed a slightly higher mobility in our solvent system.

Transient Overexpression of Membrane-bound MmNEU3 in COS-7 Cells—In this study a tagged form of MmNEU3 was used (36, 42). The recombinant vector pcDNA1-MmNEU3 al-

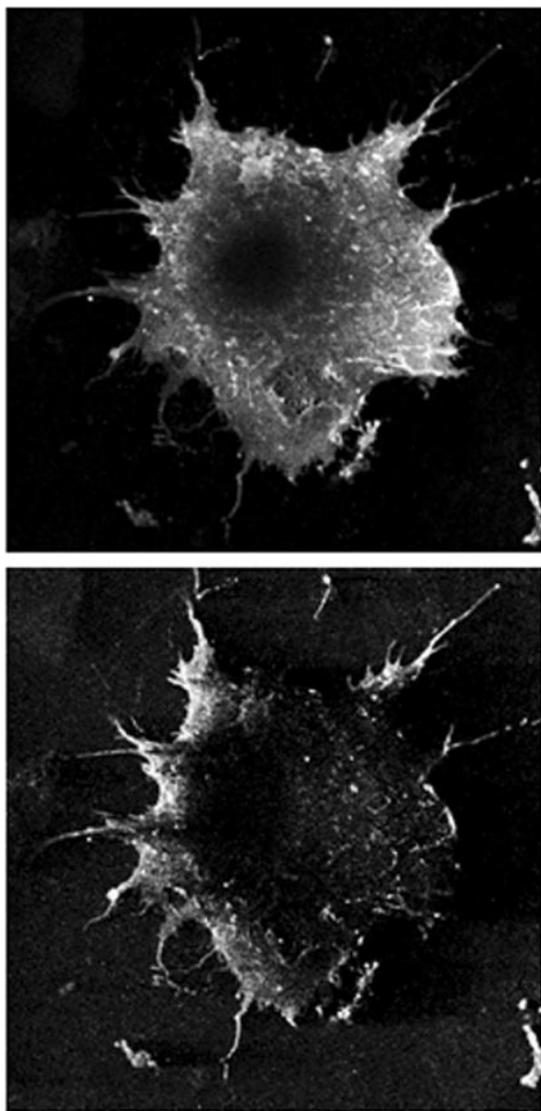


FIG. 2. Immunofluorescence detection of MmNEU3 in COS-7 cells. COS-7 cells grown on glass coverslips were transfected with pcDNA1-MmNEU3 and after 16 h were processed for indirect immunofluorescence using mouse anti-HA as primary antibodies. Specimens were analyzed using a MRC-1024 confocal laser system (Bio-Rad), and images were processed with Adobe Photoshop software. Magnification, 250 \times .

lowed the expression in mammalian cells of MmNEU3 as a chimera protein characterized by the HA epitope linked at the C terminus of the original open reading frame. This protein can be detected easily using antibodies against the HA epitope, and in addition, MmNEU3 retains its original enzyme activity.

Upon transfection MmNEU3 activity increased in a time-dependent manner up to 48 h and remained quite stable until 60 h after transfection. Starting from this time point, the enzyme specific activity gradually decreased and disappeared almost completely at about 72 h post-transfection time (data not shown).

Sixty hours after transfection, COS-7 cells showed an 11- and 35-fold increase in sialidase activity *ex vivo* toward 4-MU-Neu5Ac and ganglioside GD1a, respectively, as compared with mock-transfected cells (Fig. 1A). The pH curve showed an optimal value in the acidic range corresponding to 3.8, the same value observed in the case of the endogenous plasma membrane-associate sialidase (Fig. 1B). More than 96% of the expressed sialidase activity in the crude homogenates was recovered in the rough particulate fraction obtained by ultracentrifugation (Fig. 1C). The membrane association of MmNEU3 was confirmed by Western blot analysis using an anti-HA monoclonal antibody. Fig. 1D shows the staining of a protein band with the expected molecular mass of 48 kDa in the transfected cells, the protein being associated to the 200,000 \times g cell pellet. Additional protein bands of 80 and 44 kDa were detected by the monoclonal antibody. The presence of the same bands in the cell fractions obtained from mock-transfected cells indicated that they were unrelated to MmNEU3.

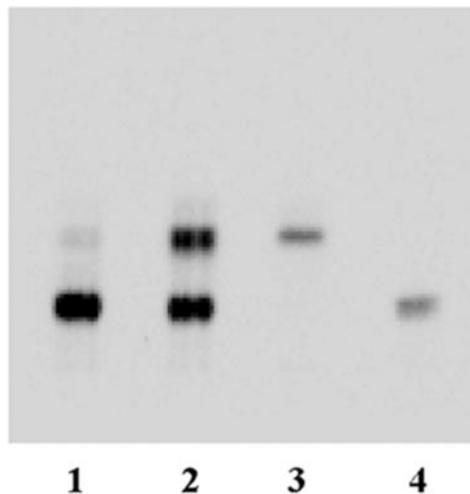


FIG. 3. GD1a hydrolysis in mock- and pcDNA1-MmNEU3-transfected cells. [^3H (Sph18)]GD1a was administered directly to COS-7 cells (2 nmol of GD1a/100-mm dish). Cells were incubated for up to 24 h in presence of 10 mM NH_4Cl , harvested by scraping, and washed with PBS, and total lipids were extracted from cells and analyzed. Solvent system chloroform-methanol-0.2% aqueous CaCl_2 , 60:40:9 (v/v). Lane 1, mock-transfected cells; lane 2, MmNEU3-transfected cells; lanes 3 and 4, standard radiolabeled GM1 and GD1a, respectively.

To confirm the association with the plasma membrane, COS-7 cells transiently transfected with expression plasmid pcDNA1-MmNEU3 were analyzed by confocal microscopy. Fig. 2 shows a cell surface staining in distinct confocal planes of the same cell expressing the enzyme, with negligible labeling in the intracellular membranous structures. Based on the ratios between MmNEU3-expressing cells and their total number, determined by fluorescence microscopy, the average yield of transfection corresponded to about 20–30%.

Direct administration into the culture media of [^3H (Sph18)]GD1a to mock- and pcDNA1-MmNEU3-transfected COS-7 cells led to its incorporation into the cell membranes. Under these conditions, because of the slow ganglioside turnover, only a minor portion of the radioactive GD1a reaches the lysosomes and is catabolized (43). COS-7 cells expressing MmNEU3 showed the conversion of GD1a to the corresponding GM1 in the presence of NH_4Cl , a condition in which the activity of the lysosomal compartment is strongly reduced (Fig. 3) (44). Overall, these results confirm the plasma membrane topology of MmNEU3.

MmNEU3 Activity in Living Cells—Administration of [^3H]sphingosine to cultured cells at 3×10^{-8} M final concentration led to an extensive labeling of the sphingolipids, namely gangliosides, neutral glycosphingolipids, sphingomyelin, and ceramide (43). In addition, a portion of the [^3H]sphingosine was catabolized by the cells to radioactive ethanolamine and then recycled for the biosynthesis of radioactive phosphatidylethanolamine. After a 2-h pulse with [^3H]sphingosine followed by a 24-h chase, free sphingosine was scarcely detectable within the radioactive lipid mixtures, and stable radioactive lipid patterns overlapping the endogenous one were observed in mock-transfected cells (Fig. 4, A and B, lane 1). Instead, the

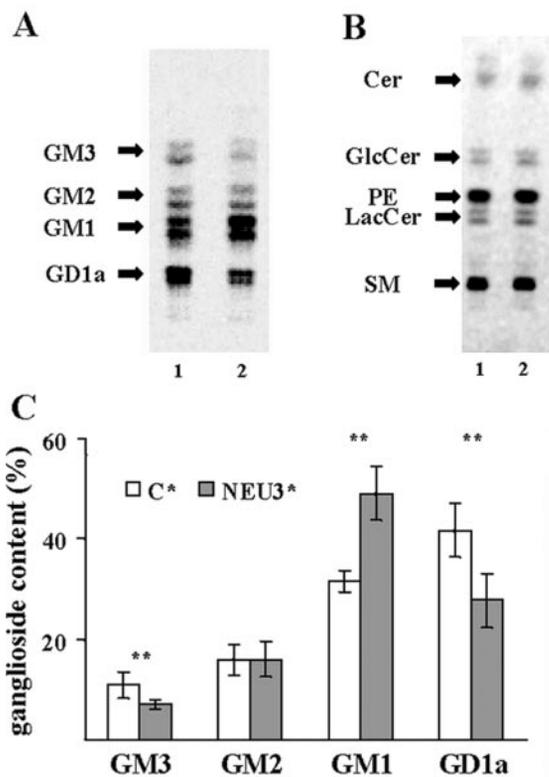


FIG. 4. Ganglioside pattern in COS-7 cells and effect of MmNEU3 overexpression on ganglioside contents. Cells (3×10^5) were pulsed for 2 h with [$1\text{-}^3\text{H}$]sphingosine, 3×10^{-8} M final concentration. After a 40-h chase, cells were harvested and treated for lipid analysis (see "Experimental Procedures"). **A**, HPTLC separation of the aqueous phase. Solvent system chloroform-methanol-0.2% aqueous CaCl_2 , 60:40:9 (v/v). **Lane 1**, mock-transfected cells; **lane 2**, pcDNA1-MmNEU3-transfected cells. **B**, HPTLC separation of the organic phase obtained by lipid extraction and fractionation. Solvent system chloroform-methanol- H_2O 55:20:3 (v/v). **Lane 1**, mock-transfected cells; **lane 2**, pcDNA1-NEU3-transfected cells. **C**, ganglioside relative contents observed after a 40-h chase in mock (C-) and pcDNA1-MmNEU3 (NEU3)-transfected cells. Data are the means \pm S.D. of four different experiments carried out in triplicate. Significance according to Student's *t* test: **, $p < 0.001$.

sphingolipid pattern from MmNEU3-expressing cells showed a marked variation (Fig. 4A, lane 2). In fact, 60 h after transfection, a 34 and 35% decrease of ganglioside GD1a and GM3 relative contents, respectively, and a 36% increase of ganglioside GM1 content were detectable (Fig. 4C). No statistically significant differences were observed between the radioactive lipid patterns of organic phases from normal and transfected cells (Fig. 4B, lanes 1 and 2).

To investigate the possible activity of MmNeu3 toward ganglioside substrates exposed on the cell surface of adjacent cells, a co-culture experiment was engineered by co-culturing MmNEU3-expressing COS-7 cells with previously [$1\text{-}^3\text{H}$]sphingosine-labeled untransfected cells. As we envisioned, the modification of the ganglioside relative abundance followed the variations of MmNEU3 specific activity induced by transient expression of the enzyme. In fact, the ganglioside content modification reached a maximum at 60 h after transfection and than slowly returned to the endogenous value (Fig. 5A). Fig. 5B shows the radioactive ganglioside distribution from co-culture experiments at 60 h after transfection. The ganglioside pattern is very similar to that observed in [$1\text{-}^3\text{H}$]sphingosine-labeled MmNEU3-transfected cells. Overall, these results indicate that the plasma membrane sialidase activity is exerted mainly toward gangliosides inserted in the membrane of neighboring cells. Moreover, we found only small amounts of radioactivity

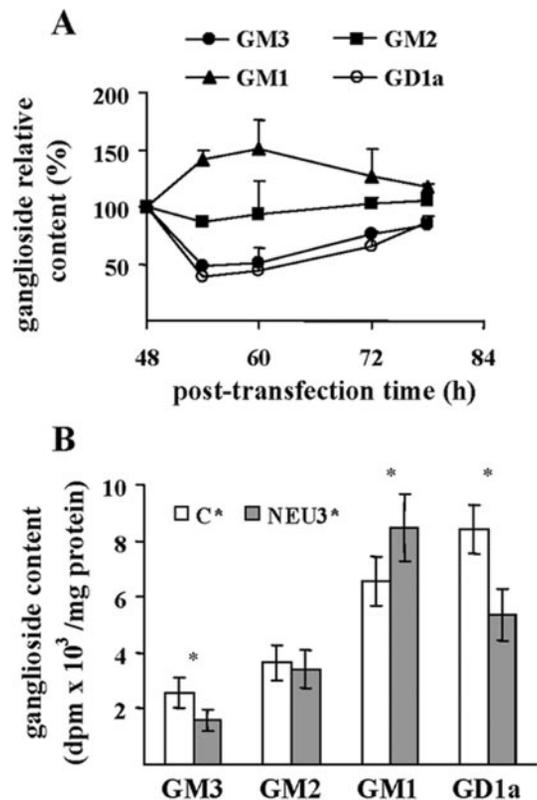


FIG. 5. Co-cultured experiments with pcDNA1-NEU3-transfected cells in the presence of [$1\text{-}^3\text{H}$]sphingosine-labeled untransfected COS-7 cells. Cells (2×10^5) transfected with pcDNA1-MmNEU3 or pcDNA1 vector alone were plated in 100-mm dishes. Meanwhile, (3×10^5) cells were separately subjected to a 2-h pulse with [$1\text{-}^3\text{H}$]sphingosine. After labeling, cells were detached from dishes and added to plates containing the previously transfected COS-7 cells. At different time, cells were harvested and treated for lipid analysis as described previously. **A**, time course analysis of metabolically of ganglioside relative content up to 78 h post-transfection. **B**, ganglioside content of mock (C-) and pcDNA1-MmNEU3 (NEU3)-transfected cells. Data are the means \pm S.D. of three different experiments carried out in triplicate. Significance according to Student's *t* test: *, $p < 0.05$.

in the cell media. In fact, the metabolically labeled lipids found in the media amounted to about 2% of the total radiolabeled ones detectable in the cell lipid extracts. By HPTLC analysis we found that about 90% of this negligible radioactivity was due to sphingomyelin.

DISCUSSION

As already reported in the case of its human counterpart (45), mouse MmNEU3 was demonstrated to be a membrane-associated enzyme with an optimum pH in the acid range (pH 3.8) when measured using classical *ex vivo* assays. Confocal microscopy of COS-7 cells expressing MmNEU3 showed an extensive labeling of the cell surface with a negligible intracellular content of the enzyme. To further confirm the plasma membrane association of MmNEU3 and its possible activity at the cell surface, we embedded the plasma membrane with GD1a and followed its hydrolysis to GM1. Conversion of GD1a to GM1 occurred without the involvement of the lysosomal compartment as demonstrated by the fact that the hydrolysis process was not affected by NH_4Cl treatment, a condition that strongly reduces lysosomal activity.

Moreover, MmNEU3 is an enzyme that is active toward gangliosides inserted in the plasma membrane of living cells. In fact, cells expressing MmNEU3 and metabolically labeled with [$1\text{-}^3\text{H}$]sphingosine showed a marked decrease of GD1a and GM3 and an increase of GM1 in comparison with mock-transfected cells. Similar variations of ganglioside relative contents

were detectable when sialidase substrates were represented by metabolic radiolabeled gangliosides at the cell surface of untransfected cells (see co-culture experiments). In addition, the modification of the ganglioside pattern was reversible and strictly dependent on MmNEU3 transient expression. Furthermore, the finding of a negligible amount of radiolabeled lipids in the culture media excludes significant ganglioside shedding (46) under our experimental conditions. Therefore, the discovery that MmNEU3 is able to modify the ganglioside content of neighboring cells supports MmNEU3 involvement in cell-to-cell interaction, implications that are still unclear but are worthy of further studies. The ability of plasma membrane-associated sialidase to recognize a substrate that is not part of the same cell plasma membrane in which it resides was suggested some time ago by studies done on cell adhesion (47, 48) and, more recently, by studies on a GD1a ganglioside derivative covalently linked to bovine serum albumin (49).

The capability of MmNEU3 to work through cell-to-cell interactions could be predicted by looking at the three-dimensional structures of the sialidase enzyme available thus far. Actually, mammalian sialidases show a high degree of amino acid sequence homology and share highly conserved amino acid motifs throughout the evolutionary scale from viral neuraminidase to bacterial enzymes (26, 50). In addition, all of the microbial sialidase structures available thus far show a typical β -propeller (or barrel) structure, with the active site located in a shallow crevice on the top of the barrel containing roughly a dozen of highly conserved amino acids (51). Most of these residues are present in topologically equivalent positions in all of the mammalian sialidases cloned thus far, strongly suggesting that the enzymes exist in a similar three-dimensional structure. In addition, MmNEU3 does not show either multiple stretches of hydrophobic amino acids or potential glycosylphosphatidylinositol modification or palmitoylation or myristoylation sites along the primary structure, thus rendering rather puzzling the issue of its anchorage to the plasma membrane. From this perspective, the presence of short hydrophobic amino acid stretches at the C terminus of the protein could suggest their involvement in the membrane association of MmNEU3 as a tail-anchored protein (52, 53). The structures of HsNEU1 (54) and HsNEU2 (55) carried out using computer modeling approaches, positioned the C-terminal portion of the molecule on the opposite site of the catalytic crevice. Thus, based on the assumption of a common three-dimensional structure of sialidases, the MmNEU3 barrel could stand on the lipid bilayer with the active site toward the extracellular space, far away from the oligosaccharide chains of the sphingolipids inserted in the outer leaflet of the membrane. For example, the terminal Gal-Neu5Ac linkage of GD1a, which is hydrolyzed by MmNEU3, should be about 20 Å away from the membrane surface (56–58), whereas the enzyme active site on the top of the barrel is located about 50 Å from the surface of the cell (55). Evidently, our results do not exclude the possibility that an enzyme activity can occur toward gangliosides of the same membrane, assuming that the barrel is able to lie down on the membrane surface, and/or in area of the cell surface where the plasma membrane is sharply bent, allowing the exposition of the substrate oligosaccharide chains directly to MmNEU3 active site. In fact, NEU3 activity toward self-membrane substrate has been suggested to occur in diluted cultures of neuroblastoma cell lines (36, 59) as well as in hippocampal neurons (42).

The finding that MmNEU3 is able to recognize and hydrolyze gangliosides belonging to neighboring cells is very attractive. In fact, both MmNEU3 (28) and gangliosides are segregated, together with cholesterol and the other sphingolipids, in

SED or lipid rafts (2). From this perspective, MmNEU3 activity might modify the ganglioside relative content within SED and thus participate in important functional membrane events such as signal transmission, cell adhesion, and lipid/protein sorting (60).

Overall, MmNEU3 activity seems to be an important factor in the modulation of the cell surface ganglioside pattern directly *in situ* without the intervention of the lysosomal compartment. Such a surface plasticity could be very important for cell adaptation to altered environmental conditions. Experiments aimed at better characterizing the effects exerted by MmNEU3 on the ganglioside composition lipid raft could provide new insight into sialidase biology, leading toward a comprehensive picture of the ganglioside roles within the lipid rafts and, more generally, on the cell surface.

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