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NEU3 Sialidase Protein Interactors in the Plasma Membrane and in the Endosomes*

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NEU3 sialidase has been shown to be a key player in many physio- and pathological processes, including cell differentiation, cellular response to hypoxic stress, and carcinogenesis. The enzyme, peculiarly localized on the outer leaflet of the plasma membrane, has been shown to be able to remove sialic acid residues from the gangliosides present on adjacent cells, thus creating cell to cell interactions. Nonetheless, herein we report that the enzyme localization is dynamically regulated between the plasma membrane and the endosomes, where a substantial amount of NEU3 is stored with low enzymatic activity. However, under opportune stimuli, NEU3 is shifted from the endosomes to the plasma membrane, where it greatly increases the sialidase activity. Finally, we found that NEU3 possesses also the ability to interact with specific proteins, many of which are different in each cell compartment. They were identified by mass spectrometry, and some selected ones were also confirmed by cross-immunoprecipitation with the enzyme, supporting NEU3 involvement in the cell stress response, protein folding, and intracellular trafficking.

NEU3 sialidase (1, 2) is a glycohydrolytic enzyme often referred to as the "ganglioside sialidase" (3), because it preferentially removes sialic acid residues from gangliosides. It is topologically associated with the outer leaflet of the plasma membrane, where its natural gangliosidic substrates are present (2). The localization of NEU3 on the plasma membrane has been recently defined to be dynamic, because the enzyme is distributed between detergent-resistant membranes and non-detergent-resistant membranes, in a cholesterol-dependent manner, suggesting its presence in supramolecular organized structures (2, 4). Moreover, NEU3 could be found also in vesicular structures corresponding to the endosomes (2). Thus, the presence of NEU3 in these two different cell districts supports a possible dynamic equilibrium between different pools of the enzyme, suggesting that NEU3 may have different roles in the cell and, consequently, compartmental-specific interactors. Along this line, NEU3 has been shown to participate in various cellular processes, including the differentiation of skeletal myoblasts, where its activity is needed for myotube formation and apoptosis protection (5, 6). Moreover, NEU3 was shown to be up-regulated under hypoxic conditions, and the overexpression of the enzyme greatly increases cell resistance to oxygen deprivation opposing cell death (7). Also, NEU3 plays an important role during axonal growth and neuronal differentiation, presumably by causing a local change of the plasma membrane sphingolipid composition (8–10). Furthermore, NEU3 participates in the control of insulin signaling via the modulation of ganglioside content and by interacting with Grb2 (11). In fact, the overexpression of the human ortholog NEU3 in a mouse model induces the development of insulin-resistant diabetes mellitus (11). Also, an excessive up-regulation of NEU3 has been detected in various neoplasms, including colon (12), renal (13, 14), ovarian (15), melanoma (16), and prostate cancers (17), although a down-regulation of the enzyme has been observed in acute lymphoblastic leukemia (18).

Although the effects of NEU3 on gangliosides have been extensively studied, less is known about the direct interaction of NEU3 with other proteins (11, 19–22). Thus, to investigate NEU3 interactors both in the plasma membrane and in the endosomes, a lentiviral system was constructed to overexpress the human NEU3 sialidase bearing an histidine tag at the N terminus in HEK293a and HeLa cells. This allowed the identification of several NEU3-specific protein interactors via a global proteomic approach with LC-MS/MS. Selected proteins were also confirmed by immunoprecipitation, and in the case of GRP78 (glucose-regulated protein 78), it was shown that its interplay with NEU3 has a functional role.

Experimental Procedures

Cell Culture and Stable Overexpression of NEU3—HEK293a and HeLa cell lines were purchased from Thermo Fisher Scientific and ATCC, respectively. All cells were maintained in DMEM (Sigma-Aldrich) with high glucose (4.5 g/liter) supplemented with 10% (v/v) FBS (Sigma-Aldrich), 2 mM glutamine (Sigma-Aldrich), penicillin/streptomycin 1× (Euroclone) at 37 °C in 5% CO_2 and 95% air-humidified atmosphere. For the metabolic shift experiments, the cells were cultured under normal conditions until they reached 90% confluence. Thereafter, the cells were incubated for additional 24 h with or without 50 μM (−)-epigallocatechin gallate (EGCG)[2] under various glucose concentration: (*a*) high glucose (DMEM with glucose 25

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[2] The abbreviations used are: EGCG, (−)-epigallocatechin gallate; WB, Western blotting; HG, high glucose; NG, normo-glucose; LG, low glucose; 4-MU-NeuAc, 4-methylumbelliferyl-*N*-acetylneuraminic acid; UPR, unfolded protein response.

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mM; Sigma-Aldrich), (*b*) normo-glucose (DMEM with glucose 5 mM; Sigma-Aldrich), and (*c*) low glucose (DMEM with glucose 2.5 mM; Sigma-Aldrich). The fresh medium was replaced every 12 h to avoid acidosis arising from high cell density. cDNA encoding human NEU3, with a histidine tag (His-N3), was subcloned into pLenti6.3/V5-TOPO TA Cloning (Thermo Fisher Scientific), which was successively transfected into 8×10^5 HEK293a and HeLa cells using Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer's instructions. HEK293a and HeLa, stably overexpressing NEU3 were isolated after selection with 5 and 2 μg/ml blasticidin (Thermo Fisher Scientific), respectively.

RNA Extraction and Real Time PCR—Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific), and 1 μg of the extracted RNA was reverse transcribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer's instructions. Real time PCR was performed with 10 ng of cDNA template, 0.2 μM primers, and 1× Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) in 20 μl of final volume using a StepOnePlus® real time PCR system (Applied Biosystem). The following primers were used: NEU3, forward 5′-TGGTCATCCCTGCGTATACC-3′ and reverse 5′-TCACCTCTGCCACTTCACAT-3′; and hypoxanthine-guanine phosphoribosil-transferase, forward 5′-TGACACTGGCAAAACAATGCA-3′ and reverse 5′-GGTCCTTTTCACCAGCAAGCT-3′, which was used as the housekeeping gene. The amplification program consisted in a initial denaturation at 95 °C for 3 min, followed by 40 cycles of 5 s each at 95 °C and 30 s at 57 °C. Relative quantification of target genes was performed in triplicate and calculated by the equation $2^{-\Delta\Delta Ct}$ (23).

Sialidase Activity Assay—WT and NEU3-overexpressing cells (His-N3) were washed in PBS, harvested by scraping, centrifuged, and resuspended in PBS containing protease inhibitor and phosphatase inhibitor cocktails (Sigma-Aldrich). The cells were lysed by sonication and then centrifuged at 800 × *g* for 10 min at 4 °C, to eliminate intact cells and nuclear components. The crude extract was centrifuged at 200,000 × *g* for 20 min at 4 °C with a TLC100 Ultracentrifuge (Beckman Coulter) to obtain cytosolic and particulate fractions. Total protein content was determined with the BCA protein assay kit (Pierce) following the manufacturer's instructions. The pH-dependent activity curve of NEU3 was assayed in the particulate and the endosomal fractions toward 4-MU-NeuAc in the presence of 12.5 mM sodium citrate phosphate (pH 2.7–7.7) or in the presence of 25 mM acetate (pH 3.8–5.7). NEU3 enzymatic activity was also determined after sucrose gradient centrifugation in membrane and endosome fractions using [3-^{3}H] GD1a as the substrate (24). Assays were performed in triplicate with 40 μg of total protein in a final volume of 100 μl and in presence of 12.5 mM sodium citrate phosphate buffer, at pH 3.8 for the plasma membrane and at pH 3.8–6.2 for the endosomes, according to the measured pH levels in the early and late endosomes (25). One unit of sialidase activity was defined as the quantity needed for the liberation of 1 μmol of Neu5Ac/min at 37 °C (24).

Subcellular Fractionation—The cells were washed three times with ice-cold PBS, scraped, and pelleted at 200 × *g* for 5 min. The cell pellet was then resuspended in a homogenization buffer (HB) (26) consisting of 250 mM sucrose, 3 mM imidazole, 1 mM EDTA, pH 7.4, in the presence of a protease inhibitor and a phosphate inhibitor cocktail (Sigma-Aldrich). Then it was pelleted again at 1,300 × *g* for 10 min at 4 °C. The cells were resuspended in HB with 0.03 mM cycloheximide (Sigma-Aldrich) and homogenized by seven passes through a 22-gauge needle. Postnuclear supernatant was obtained by centrifugation at 2,000 × *g* for 10 min at 4 °C. The sucrose concentration of the postnuclear supernatant was adjusted to 40.6% by adding 62% sucrose and loaded on the bottom of an SW41 ultracentrifuge tube (Beckman Coulter) and overlaid with sucrose in HB (7 ml). HB was added to fill the tube (3 ml), and the sample was centrifuged at 100,000 × *g* for 3 h at 4 °C. Proper separation of plasma membrane and endosomes was assessed by Western blotting (WB) using antibodies for proteins specific for each cell compartment, as described in the next paragraph.

Western Blot Analyses—Proteins were denaturated by boiling for 10 min in sample buffer (0.6 g/100 ml Tris, 2 g/100 ml SDS, 10% glycerol, 1% β-mercaptoethanol, pH 6.8) and loaded into 10% SDS-PAGE gel, and then transferred to a nitrocellulose membrane (Trans-blot; Bio-Rad) by electroblotting. Nitrocellulose membranes were incubated with a blocking solution containing 5% (w/v) nonfat dry milk or 5% (w/v) BSA (Sigma-Aldrich) in Tris-buffered saline with 0.1% Tween® 20 (TBS-T) for 1 h. Blots were incubated with primary antibodies for 2 h at room temperature. The following primary antibodies were used: anti-His-HRP conjugated (1:5000 dilution; Thermo Fisher Scientific), anti-NEU3 (1:700 dilution; Abcam), anti-EE1a (1:1000 dilution; Cell Signaling), anti-Na^+/K^+ ATPase (1:1000 dilution; Cell Signaling), anti-G3BP1 (1:1000 dilution; Abcam), anti-GRP78 (1:1000 dilution; Abcam), anti-SNX9 (1:1000 dilution; Abcam), anti-Flot1 (1:1000 dilution; Abcam), and GAPDH (1:5000 dilution; Millipore). Membranes were washed three times for 10 min with TBS-T and then incubated with the appropriate anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (1:2000 dilution; Dako) for 1 h at room temperature. After three washes for 10 min with TBS-T, the immunoreactive bands were visualized using the enhanced chemiluminescence detection kit reagents (ECL Advance; GE Healthcare) following the manufacturer's instructions.

Immunoprecipitation and Western Blot—For immunoprecipitation, 500 μg of protein from both endosome and membrane fractions were used. After a preclearing step with 25 μl of PureProteome™ protein A/G mix magnetic beads (Merck-Millipore) for 30 min at room temperature on a mixing rotator, proteins were incubated at 4 °C overnight, rotating with 1.5 μg of the selected primary antibody: anti-His (Thermo Fisher Scientific), anti-G3BP1 (Abcam), anti-GRP78 (Abcam), anti-Flot1 (Abcam), or anti-SNX9 (Abcam). The antibody-antigen complex was precipitated with 25 μl of PureProteome™ protein A/G mix magnetic beads (Merck-Millipore) (15 min, room temperature, rotating). After washing the samples two times with 200 μl of washing buffer (TBS 1× + 1 M NaCl) and two times with washing buffer with 0.5% Tween® 20, the beads were resuspended in 30 μl of sample buffer and boiled for 10 min. Magnetic beads were pulled down using a magnet, and proteins were resolved by SDS-PAGE, as described above.

NEU3 Interacting Protein Identification—After protein separation, the gel was stained by Coomassie Brilliant Blue R (Sig-

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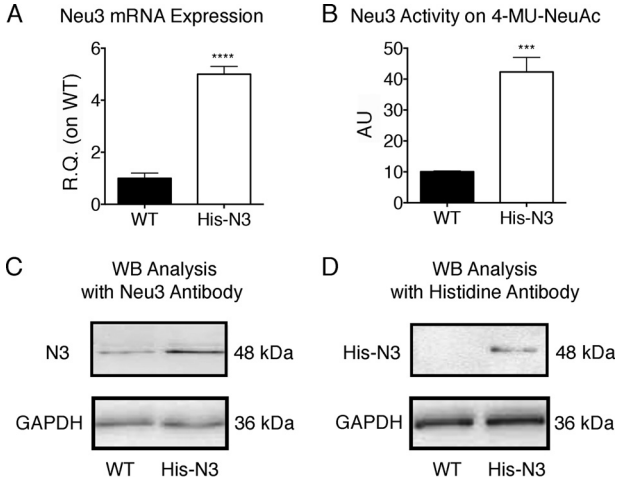

FIGURE 1. **NEU3 Overexpression in HEK293a.** NEU3 overexpression was analyzed using real time PCR (*A*), sialidase activity toward 4-MU-NeuAc at pH 3.8 (*B*), and Western blot using two different antibodies with specificity for endogenous NEU3 (*C*) and for the histidine tag (*D*). Four replicates were performed for each experiment, and the data were expressed as fold change in comparison to WT cells. ***, $p < 0.001$; ****, $p < 0.0001$. *R. Q.*, relative quantity.

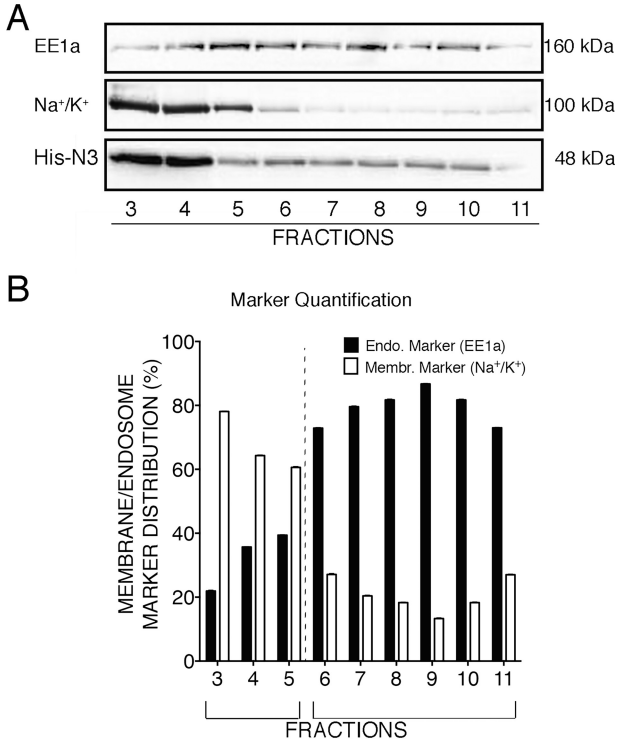

FIGURE 2. **Separation of cell endosomes from membranes by sucrose gradient.** His-N3 cell endosomes were separated from cell membranes by collecting 11 sucrose gradient fractions, using EE1a (endosomes) and Na^+/K^+ (plasma membranes) antibodies as markers. Western blot analysis (*A*) and quantification (*B*) revealed the prevalence of cell membrane markers in fractions 3–5 and of endosome markers in fractions 6–11. *Endo.*, endosome; *Membr.*, membrane.

ma-Aldrich), and bands were manually excised and subjected to *in situ* hydrolysis (27). Extracted peptides were lyophilized and resuspended in 1% formic acid before LC-MS analysis. Peptide mixtures were injected onto a 0.075 × 150-mm nanoC18 column (Zorbax 300SB, Agilent Technologies) loaded onto an Easy-nLC system (Proxeon) coupled to an AmaZon Speed ETD ion trap (Bruker Daltonics). Each sample was eluted with acetonitrile/0.1% formic acid gradient (5–55% in 120 min). Mass spectrometer parameters were: capillary voltage set to −1400 V, and data-dependent MS/MS acquisitions performed on precursors with charge states of +2, +3, or +4 over a survey mass range of 300–1500. Proteins were identified by correlation of uninterpreted MS/MS to *Homo sapiens* entries in SwissProt 51.6 database (257964 sequences; 93947433 residues), using MASCOT software. Peptide false discovery rates were determined by searching against the reversed sequence decoy database version of the human proteome using the application in the MASCOT software. No mass and pI constraints were applied. One missed cleavage per peptide was allowed, the fragment ion tolerance window was set to 0.3 Da for precursor masses and 0.5 for fragmented masses, and trypsin was indicated as enzyme. Carbamidomethylation of cysteine was set as fixed modification, whereas methionine oxidation as variable modification. Only proteins with at least two identified unique peptides were considered.

Statistical Analysis—The Student's *t* test was used to determine significance using GraphPad Prism 6 software. *p* values of less than 0.05 were considered to be significant. All *p* values were calculated from data obtained from at least three independent experiments. All error bars represent the standard deviation of the mean.

Results

Stable Overexpression of NEU3 in HEK293a Cell Line—HEK293a cell model was used to study NEU3 interactors by overexpressing the enzyme with a histidine tag using a pLenti 6.3/V5 TOPO lentiviral system, as described under "Experimental Procedures." Initially, the mRNA expression and the catalytic activity of NEU3-overexpressing HEK293a cells (His-N3) was determined and compared with that of WT control cells (Fig. 1, *A* and *B*). The results showed a 5-fold increase in NEU3 mRNA levels by real time PCR as compared with WT (Fig. 1*A*). His-N3 revealed a 4.2-fold catalytic-activity increase toward the artificial substrate 4-MU-NeuAc as compared with WT cells (Fig. 1*B*). NEU3 protein content increase was then determined in His-N3 by WB using anti-NEU3 (Fig. 1*C*) and anti-His (Fig. 1*D*) specific antibodies, confirming that NEU3 overexpression caused a marked increase of the enzyme also at a protein level.

Separation and Characterization of Plasma Membrane and Endosome Fractions—To assess NEU3 interactors on the cell plasma membrane and in the endosomes, a sucrose gradient separation was performed, and 11 fractions were collected, as described under "Experimenta Procedures." The localization of the Na^+/K^+ pump and of EE1a was used to check the correct separation of the plasma membrane from the endosomes, respectively. In particular, for the plasma membrane, collected gradient fractions from 3 to 5 were combined, because they contained a major enrichment in the Na^+/K^+ pump; for the endosomes, fractions from 6 to 11 were combined, according to the main localization of EE1a (Fig. 2). Successive analyses of the combined fractions revealed the presence of an endosomal contamination in the membrane fraction (~30%), whereas the endosomal fraction appeared essentially pure (Fig. 3, *A* and *B*). Moreover, it was observed that His-N3 was mostly concen-

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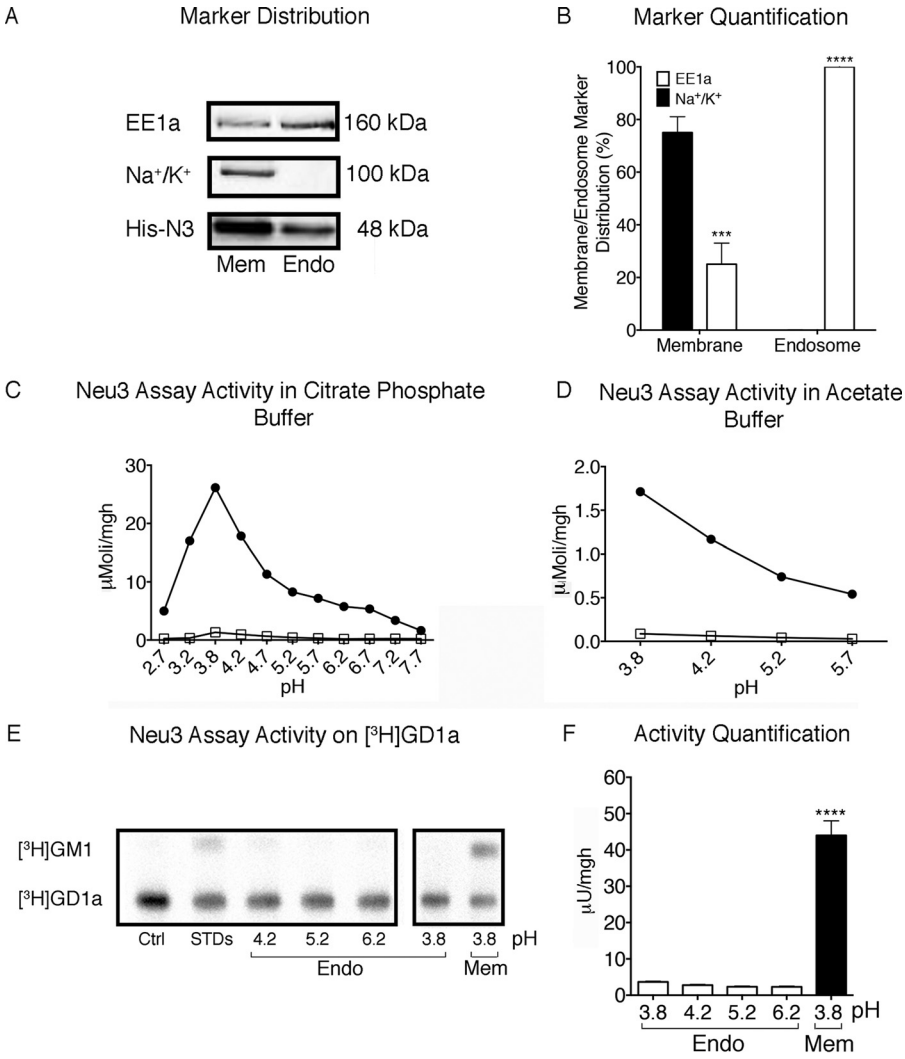

FIGURE 3. **Characterization of membranes and endosomes.** *A* and *B*, Western blot analysis (*A*) and quantification (*B*) of the subcellular localization of His-N3 after sucrose gradient, using EE1a as the endosome marker and Na^+/K^+ for the plasma membranes. *C* and *D*, sialidase activity toward 4-MU-NeuAc in presence of sodium-citrate 12.5 mM in a pH range of 2.7–7.7 (*C*) or in the presence of acetate 25 mM in a pH range of 3.8–5.7 (*D*). *E*, NEU3 activity assay on gangliosides [^{3}H]GD1a: HPTLC separation of radiolabeled gangliosides. *F*, quantification of sialidase activity on radiolabeled [^{3}H]GD1a in the membrane (at pH 3.8) and in the endosome fractions (at pH ranging from 3.8 to 6.2). ***, $p < 0.001$; ****, $p < 0.0001$. *Ctrl*, control; *Endo*, endosome; *Mem*, membrane; *STDs,* standards.

trated in the plasma membrane compartment (~80%), whereas the remaining 20% was localized in the endosomal compartment (Fig. 3*A*). Then an enzymatic sialidase activity assay was performed on the particulate and endosomal fractions using the artificial substrate 4-MU-NeuAc in presence of 12.5 mM sodium citrate (pH range, 2.7–7.7) or 25 mM acetate (pH range, 3.8–5.7). The sialidase activity on 4-MU-NeuAc was detected mainly in the plasma membrane fraction at pH 3.8 in both buffers (Fig. 3, *C* and *D*). Instead, very low detectable sialidase activity could be measured in the endosomes fraction under both buffer conditions. Moreover, the sialidase activity was also assayed on [^{3}H]GD1a, confirming that the catalytic activity (measured as microunits/mg of proteins) could be detected mainly in the plasma membrane fraction (Fig. 3, *E* and *F*).

Identification of NEU3 Interacting Proteins by LC-MS/MS—The plasma membrane and the endosomes fractions were incubated with an anti-His antibody to immunoprecipitate putative NEU3 interactors, as described under "Experimental Procedures." Then the immunoprecipitates were fractionated by SDS-PAGE, and the extracted peptides underwent LC-MS/MS analysis. These analyses identified 282 (189 with at least 2 identified unique peptides) and 66 (34 with at least 2 identified unique peptides) unique proteins for the endosome and membrane fractionations, respectively (Fig. 4), at a false discovery rate of 2%. Among them, 34 and 12 proteins were specific to endosomes and membranes, respectively (Table 1). A set of proteins was identified both in the plasma membrane and the endosomes but, in previous studies, it was demonstrated that these proteins were localized in both compartments (28–30). The co- immunoprecipitation methodology is the reference method to investigate protein-protein interaction or protein complexes. In fact, although it does bear some limitations, like nonspecific binding, direct and transient interactions, and their cellular compartmentalization.

Validation of NEU3 Interacting Proteins—A subset of NEU3-interacting proteins that were identified by mass spectrometry was then validated by cross-immunoprecipitation. Flotillin1 was chosen for the plasma membrane compartment, whereas

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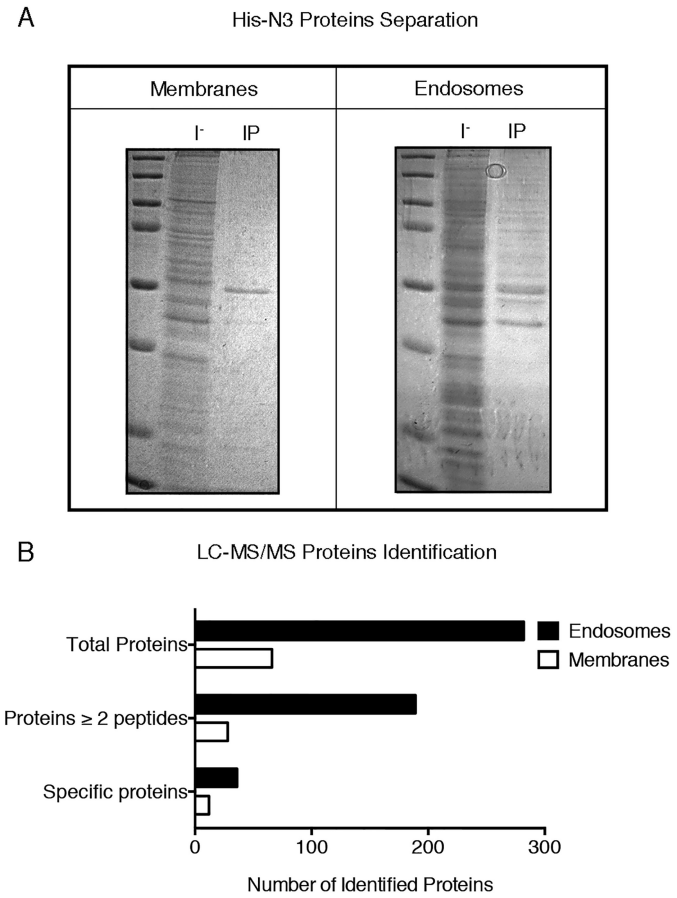

FIGURE 4. **Protein separation and LC-MS/MS analysis.** *A*, Coomassie Blue SDS-PAGE of immunodepleted (I^-) and His-N3 immunoprecipitation (*IP*) in membranes and endosomes compartments. *B*, total number of protein identified by LC-MS/MS analysis after immunoprecipitation in the membranes and endosome fractions.

G3BP1, SXN9, and GRP78 were chosen for the endosomes. The interaction of these proteins with NEU3 was validated by overexpressing NEU3 with a His tag in HEK293a (His-N3) to increase NEU3 cell content and have a reliable tag. In fact, the currently available antibody for NEU3 does not allow immunoprecipitation. Initially, it was checked whether the overexpression of NEU3 would alter the cell content of Flot1, G3BP1, SNX9, and GRP78 (Fig. 5, *A* and *B*) by WB analysis of wild-type and His-N3 cells with the appropriate antibodies (Fig. 5, *A* for membrane and *B* for endosome fractions). The results confirmed that NEU3 overexpression did not cause any significant change. Then His-N3 cell membranes were used for the validation of Flot1 interaction with NEU3. To this purpose, an immunoprecipitation was performed with the anti-His antibody followed by WB analysis with the Flot1 antibody, revealing the presence of the interacting protein in the immunoprecipitate (Fig. 5*C*, *left panel*). Then to confirm the interaction, the complementary experiment was performed on the same sample by immunoprecipitating with the anti-Flot1 antibody and then analyzing for NEU3 presence in the immunoprecipitate with the anti-His antibody by WB (Fig. 5*C*, *right panel*). Analogous experiments were performed for the endosomal fraction, confirming NEU3 interaction with G3BP1, SNX9, and GRP78 (Fig. 5*D*). The same cross-immunoprecipitation experiments were performed on NEU3-overexpressing HeLa cells, confirming the interaction of NEU3 with the same proteins (Fig. 6). Finally, the specificity of His antibody was confirmed by comparing the immunoprecipitates of His-N3 with wild-type cells that do not carry the His tag and should not interact with the His antibody. To this purpose, wild-type and His-N3 cell plasma membranes and endosomes were immunoprecipitated with the His antibody and then subjected to WB analysis with Flottillin1 (membranes; Fig. 5*E*) G3BP1, SXN9, and GRP78 (endosomes; Fig. 5*F*) antibodies. The results revealed the presence of Flot1, G3BP1, SNX9, and GRP78 in His-N3 immunoprecipitates (Fig. 5, *E* and *F*, immunoprecipitation/His-N3, *right lanes*), although barely detectable bands were observed in the wild-type samples, confirming only minor aspecific binding (Fig. 5, *E* and *F*, immunoprecipitation/WT, *left lanes*). The quantification was made using the "input" (2% of total protein) as the housekeeper.

Effects of GRP78 on NEU3 Folding and Translocation—To gain a better understanding of the possible role of GRP78 interaction with NEU3 in controlling sialidase folding and translocation from the endosomes to the cell membrane, His-N3 cells were grown in the presence of different glucose concentrations: (*a*) high glucose (25 mM glucose, HG, which is the standard concentration normally used for cell cultures *in vitro*), (*b*) normo-glucose (5 mM glucose, NG, which is the human physiological concentration), and (*c*) low glucose (2.5 mM glucose, LG, which is found, for instance, in poorly irrigated tumor areas). GRP78 and His-N3 protein levels were analyzed by WB in the endosomes and in the plasma membranes. The results revealed that GRP78 had a modest, although statistically significant, increase in the endosomes of ~1.2-fold in both NG and in LG, as compared with HG, which was used as control condition for normalization in all experiments (Fig. 7*A*). Moreover, to characterize the endosomal interaction between His-N3 and GRP78, the cells were collected, immunoprecipitated for NEU3 with His antibody and analyzed by WB with anti-GRP78 antibody. The results showed a significant increase in His-N3/GRP78 binding, which was inversely proportional to the glucose concentration in the medium. In fact, WB analyses revealed an increase of GRP78 in the immunoprecipitate, which was 1.2- and 1.5-fold in NG and in LG, as compared with HG, respectively (Fig. 7*B*). These results were confirmed by performing the reciprocal experiments, *i.e.* immunoprecipitating with the GRP78 antibody and then checking for NEU3 presence by WB using the anti-His antibody (Fig. 7*C*). The assay confirmed that GRP78/His-N3 binding is inversely proportional to the glucose concentration in the medium. In fact, WB analyses revealed an increase of His-N3 in the immunoprecipitate, which was 1.2- and 1.4-fold in NG and in LG, as compared with HG, respectively (Fig. 7*C*).

Then to test whether the increase in GRP78/His-N3 binding upon glucose reduction in the culture medium affected NEU3 distribution and activity, the sialidase protein levels were assessed by WB in the endosomes and in the cell membranes. The results revealed that in the endosomes, no statistically significant alterations of His-N3 protein levels could be detected by WB in NG or LG, as compared with HG (Fig. 8*A*). On the other hand, a significant increase in NEU3 protein levels could

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TABLE 1

Protein identification by LC-MS/MS

Unique proteins with at least two peptides after LC-MS/MS analysis of endosome and membrane eluates were identified. For each protein, UniProt ID, name, theoretical molecular mass and pI, and MASCOT scores are reported.

UniProt ID	Protein Name	Molecular mass	pI	MASCOT score
		kDa		
Endosome eluates				
ABCD3	ATP-binding cassette subfamily D member 3	75.4	9.9	127.7
APMAP	Adipocyte plasma membrane-associated protein	46.5	5.8	109.9
AT1A1	Sodium/potassium-transporting ATPase α-1 chain precursor	112.8	5.2	201.6
CKAP4	Cytoskeleton-associated protein4 (63-kDa membrane protein)	66.0	5.6	630.8
CLH1	Clathrin heavy chain 1	191.5	5.4	135.9
COPB	Coatomer subunit β	107.1	5.7	149.9
DNJA1	DnaJ homolog subfamily A member1 (heat shock 40-kDa protein 4)	44.8	6.7	189.1
EHD4	EH domain-containing protein 4	61.1	6.3	86.8
ENOA	α-Enolase	47.1	7.7	220.5
ENPL	Endoplasmin precursor	92.4	4.6	609.3
FLOT1	Flotillin-1	47.3	7.8	120.5
FLOT2	Flotillin-2	41.7	5.1	93.4
FUBP3	Far upstream element-binding protein 3	61.6	9.4	408.4
G3BP1	Ras GTPase-activating protein-binding protein 1	52.1	5.3	98.0
GANAB	Neutral α-glucosidase AB precursor	106.8	5.7	372.8
GRP78	78-kDa glucose-regulated protein precursor	72.3	4.9	1067.2
K0310	Uncharacterized protein KIAA0310	233.4	5.3	292.1
MYH10	Myosin-10	228.8	5.3	513.8
NEUR3	Sialidase-3	48.2	6.9	185.8
OST48	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48-kDa subunit precursor	48.8	5.3	277.2
PDIA1	Protein disulfide isomerase precursor	57.1	4.6	95.9
PDIA3	Protein disulfide isomerase A3 precursor	56.7	5.9	143.2
PDIA6	Protein disulfide isomerase A6 precursor	48.1	4.8	159.4
PGRC1	Membrane-associated progesterone receptor component 1	21.7	4.4	116.7
RCN1	Reticulocalbin-1 precursor	38.9	4.7	133.9
RIB1	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 67-kDa subunit precursor	68.5	5.9	534.7
RIB2	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 63-kDa subunit precursor	69.2	5.4	358.0
RL4	60S ribosomal protein L4	47.7	11.8	233.0
SC23A	Protein transport protein Sec23A	86.1	6.7	186.9
SC23B	Protein transport protein Sec23B	86.4	6.5	98.0
SERPH	Serpin H1 precursor	46.4	9.3	480.8
SNX9	Sorting nexin-9	66.5	5.3	193.8
SRP72	Signal recognition particle 72-kDa protein	74.6	9.9	136.5
VDP	General vesicular transport factor p115	107.8	4.7	121.1
Membrane eluates				
APMAP	Adipocyte plasma membrane-associated protein	46.5	5.8	98.5
AT1A1	Sodium/potassium-transporting ATPase α-1 chain precursor	112.8	5.2	182.6
DNJA1	DnaJ homolog subfamily A member1 (heat shock 40-kDa protein 4)	44.8	6.7	166.3
FLOT1	Flotillin-1	47.3	7.8	116.7
FLOT2	Flotillin-2	41.7	5.1	66
IQGA1	Ras GTPase-activating-like protein IQGAP1 (p195)	189.1	6.1	301.9
NEUR3	Sialidase-3	48.2	6.9	90.3
OST48	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48-kDa subunit precursor	48.8	5.3	230.0
PGRC1	Membrane-associated progesterone receptor component 1	21.7	4.4	116.7
RCN1	Reticulocalbin-1 precursor	38.9	4.7	105.4
SERPH	Serpin H1 precursor	46.4	9.3	395.0
TRIPB	Thyroid receptor-interacting protein 11	227.5	5.1	85.2

be detected in the membranes, of 1.5- and 2.8-fold in NG and LG, respectively, as compared with HG (Fig. 8*B*).

Finally, to further investigate the role of GRP78 in regulating NEU3 folding and activity, sialidase expression and activity was measured on the artificial substrate 4-MU-NeuAc under HG, NG, and LG conditions (Fig. 8, *C* and *D*). Moreover, NEU3 activity was also determined in the presence of 50 μM EGCG, an inhibitor of GRP78 (Fig. 8*D*). The results revealed 1.8- and 2.8-fold increases in NEU3 expression and activity, respectively, in LG, as compared with HG, whereas no significant changes in NEU3 expression and activity were observed in NG (Fig. 8, *C* and *D*). Moreover, treatment with 50 μM EGCG (+) induced a decrease of NEU3 activity of approximately one-third the initial value at all glucose concentrations (Fig. 8*D*).

Discussion

Although NEU3 sialidase has been often referred to as the "plasma-membrane associated" member of the sialidase family (31), the effects of NEU3 activity on key cellular processes, including cancerogenesis and cell differentiation, are not limited to its ability of modulating sialic acids content, but they can be also the result of a direct interaction of the enzyme with signaling molecules, such as caveolin-1, Rac-1, integrin β4, Grb-2, and EGFR (11, 19–22). Moreover, although NEU3 is mainly localized on the plasma membrane, it was reported that the enzyme is also present in the endosomal compartment (2). However, in the current study, separating endosomes from plasma membranes in HEK293a cells revealed that NEU3 possesses enzymatic activity mainly in the membrane compartment, supporting its known preferential specificity for gangliosides, that are inserted in the cell membrane. However, because a substantial amount (~30%) of the enzyme could be found in the endosomes, we investigated whether NEU3 could play a role not for a direct local enzymatic activity but for the specific interaction with the some proteins present in the same cellular district. Analyses by mass spectrometry of the protein partners

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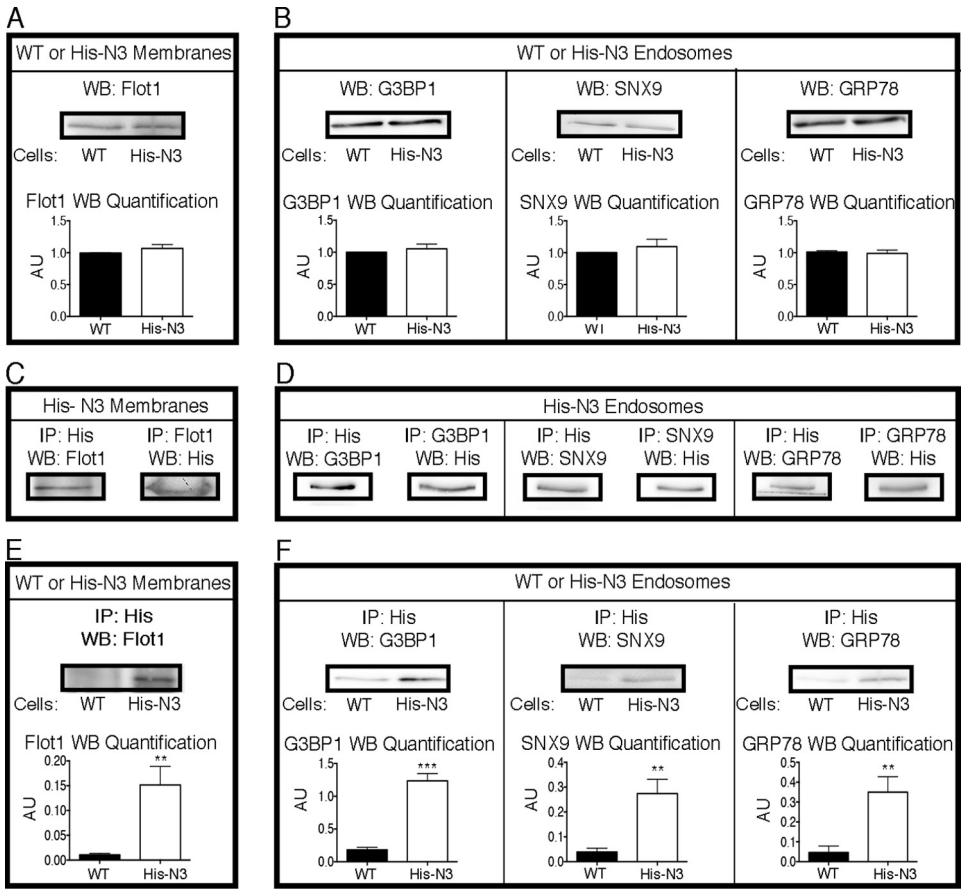

FIGURE 5. **Validation of NEU3 interactors.** *A* and *B*, comparison of Flot1, G3BP1, SNX9, and GRP78 protein expression in membrane (*A*) and endosomes (*B*) of WT and His-N3-overexpressing HEK293a cells by Western blot. *C* and *D*, validation of NEU3 interactors in HEK293a cells by immunoprecipitation with His antibody, followed by Western blot analysis with Flot1, G3BP1, SNX9, and GRP78 antibodies in the membrane (*C*, *left Western blots*) and in the endosome (*D*, *left Western blots*) fractions and vice versa, immunoprecipitation with Flot1, G3BP1, SNX9, and GRP78 followed by Western blot with His antibody (*C* and *D*, *right Western blots*); validation of His antibody specificity by comparison of the immunoprecipitates of His-N3 HEK293a with wild-type cells. *E* and *F*, wild-type and His-N3 cell membranes and endosomes were immunoprecipitated with the His antibody and then subjected to WB analysis with Flottillin1 (*E*) G3BP1, SXN9, and GRP78 (*F*) antibodies. **, $p < 0.01$; ***, $p < 0.001$. *IP*, immunoprecipitation.

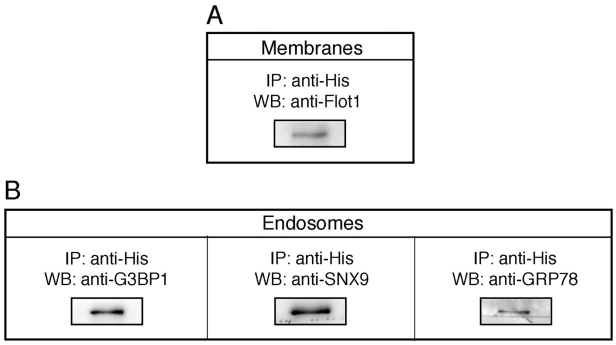

FIGURE 6. **NEU3 interactors in HeLa cells.** *A* and *B*, immunoprecipitation with His antibody followed by Western blot analysis with Flot1, G3BP1, SXN9, and GRP78 in membrane (*A*) and endosomal (*B*) cell fractions of His-N3-overexpressing HeLa cells. *IP*, immunoprecipitation.

that co-immunoprecipitated with NEU3 allowed the identification of 34 and 12 interactors of the enzyme in the endosomes and in the plasma membranes, respectively. The endosomal contamination in the plasma membrane fraction, which unfortunately could not be avoided during the separation process, did not seem to affect the successive analyses, because the few overlapping proteins between membranes and endosomes, like Flotillin 1, are known to be present in both cell compartments (32). This also proved that NEU3 interactors are predominantly cell compartment-specific, supporting the hypothesis that the enzyme could have different roles in the cell membrane and in the endosomes. The cross-immunoprecipitation assays validated the specificity of the interactions found by mass spectrometry, and the comparison of His-N3 with wild-type cells demonstrated that the overexpression of NEU3 does not change the levels of the interacting proteins, nor their subcellular distribution. Moreover, because the mechanisms regulating the enzyme folding, its transport to the cell membrane, and its recycling have yet to be elucidated, some of the newly discovered interactors (Flotillin1, which is present in both compartments, and SNX9, G3BP1, and GRP78 among those in the endosomes) were further investigated, because they appeared to play a role in those processes. Flotillin1 is a member of the "flotillins" family, which are considered to be scaffolding proteins of lipid rafts and are generally used as marker proteins of lipid microdomains (32). However, it has been demonstrated that they are also present in the endosomal compartment, in the phagosomes, in the Golgi, and in the exosomes (33–36). Previously, we demonstrated that NEU3 participates in a complex cell trafficking between the endosomes and the membrane, and, as it reaches the plasma membrane, it is initially

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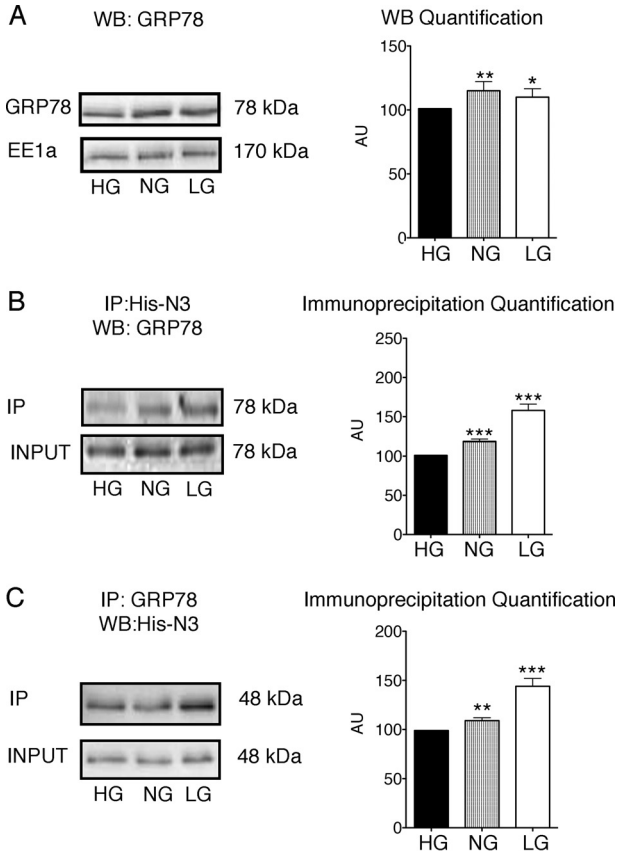

FIGURE 7. **Glucose shift: GRP78 protein level and binding with His-N3.** *A–C*, Western blot analysis and quantification of GRP78 in the endosomal fractions of His-N3 cells (*A*); immunoprecipitation with His-N3 antibody followed by Western blot analysis with GRP78 antibody (*B*); and cross-immunoprecipitation with GRP78 antibody followed by detection with His-N3 antibody (*C*). All the experiments were done using the endosomal fraction of His-N3 cells, cultured in the presence of three different glucose concentrations HG (25 mM), NG (5 mM), and LG (2.5 mM) for 24 h. Three replicates were performed for each experiment. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. *IP*, immunoprecipitation.

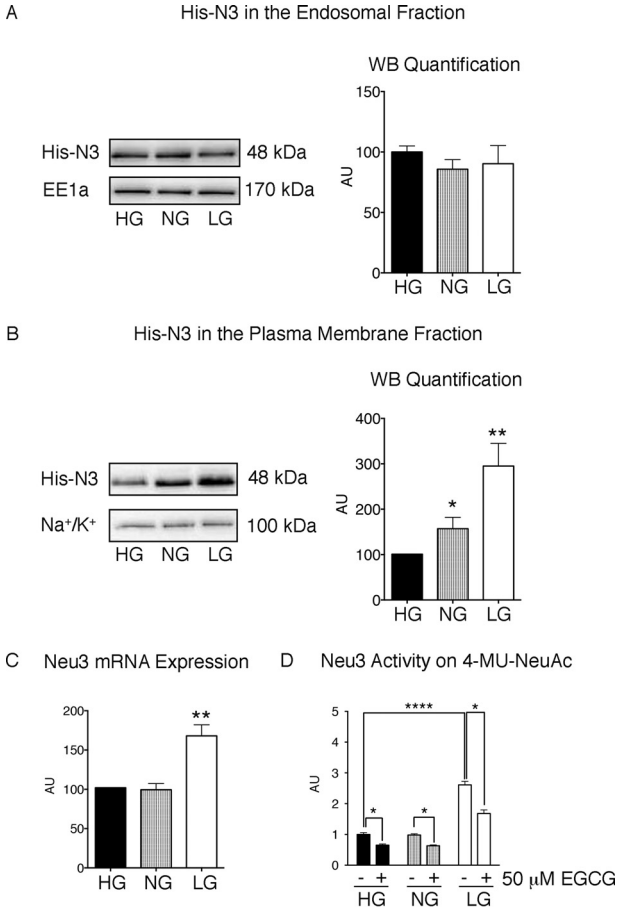

FIGURE 8. **His-N3 mobilization.** *A–C*, analysis of NEU3 protein content in the endosomal (*A*), in the plasma membrane (*B*) fractions and measurement of NEU3 mRNA expression level (*C*) in His-N3 cells, cultured in different glucose concentrations HG (25 mM), NG (5 mM), and LG (2.5 mM) at 24 h. *D*, sialidase activity toward the artificial substrate 4-MU-NeuAc of His-N3 cells, treated with 50 μM EGCG, was measured at 24 h at three different glucose concentrations HG, NG, and LG at 24 h. Three replicates were performed for each experiment. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

associated with non-detergent-resistant membrane areas, but eventually it partially translocates to detergent-resistant membranes, reaching an even distribution (4). Therefore, the interaction with Flotillin1 could be instrumental for the mobilization of NEU3 in the cell membrane. Moreover, because Flotillin 1 is known to promote endocytosis in a clathrin-independent way (37), its interaction with NEU3 could be important for the internalization of the enzyme. Another protein involved in endocytosis, but in a clathrin-dependent way, is SNX9 (sorting nexin 9), a multifunctional protein that remodels the membrane during the process, mediating the communication between dynamin-dependent membrane scission and actin dynamics (38). SNX9 might participate in the formation of the narrow neck of endocytic vesicles before scission occurs, and it could be also involved in NEU3 import in the endosomes. The interaction of NEU3 with both Flotillin 1 and SNX9 suggests the presence of more than one pathway for NEU3 endocytosis, and this feature could regulate NEU3 recycling in response to different stimuli. G3BP1 is a Ras-GTPase activating protein SH3 domain binding protein, the role of which is still quite unknown. It is implicated in Ras signaling (39), in protein degradation by modulating USP10 (40), in RNA metabolism (41, 42), and especially in stress response by regulating the assemble of stress granules, multimolecular aggregates of ribonucleoproteins, and mRNA encoding housekeeping genes that are generated in cells exposed to adverse environmental stresses (43). Actually, along this line, we recently reported that NEU3 plays a role in cellular response to hypoxic stress (7). Therefore, further investigations on the interaction between G3BP1 and NEU3 are currently underway in our laboratories.

Finally, GRP78, also known as Bip (immunoglobulin heavy chain binding protein), belongs to the HSP70 (heat shock protein 70) family (44, 45). GRP78 is an endoplasmic reticulum chaperone that is essential for the proper glycosylation, folding, and assembly of membrane-bound secreted proteins. It has been used extensively as a marker for the unfolded protein response (UPR) (45, 46). The transcription of GRP78 gene is induced by stress events, which can produce incorrectly folded or assembled proteins in the endoplasmic reticulum (44). These stresses include acidosis, endoplasmic reticulum calcium depletion, hypoxia, oxidative stress, and glucose starvation (47,

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48). In particular, glucose starvation causes the accumulation of specific unfolded proteins, which induce oligomerization and activation by autophosphorylation of the endoplasmic reticulum transmembrane endoribonuclease and kinase called IRE1 (49). IRE1 excise an intron from mRNA of XBP-1, and the spiced XBP-1 translocates into the nucleus, where it binds its target sequences, including GRP78 (50). Only the spliced form of XBP-1 protein is an active transcription factor (50). Moreover, it was demonstrated that GRP78 is implicated in the cellular trafficking, because it is able to translocate to the cell surface (51). Thus, to better understand the effects of glucose deprivation on NEU3, cells were cultured under high glucose concentration (which is the standard condition normally used to culture cells) and normo- and low glucose concentration. As expected, an up-regulation of the GRP78 protein was observed under normo- and low glucose conditions, confirming the activation of the UPR response. Actually, it is known that the UPR system is fundamental for the stabilization of unfolded proteins when a cell is under stress conditions (46). Interestingly, cross-immunoprecipitation with His-N3 or GRP78 antibodies showed a marked increase of their interaction in normo- and low-glucose concentration, supporting that GRP78 could be essential for the stabilization of unfolded NEU3. A validation of this hypothesis, *i.e.* UPR involvement in NEU3 stabilization, was given by mass spectrometry analysis, which identified DnaJA1, another important member of UPR system (44), among the interactors of NEU3. The effects of UPR on the stabilization and folding of His-N3 did not alter the content of NEU3 in the endosomal fraction at 24 h. The endosomal interaction of His-N3 with the UPR system, through GRP78, was fundamental for the translocation of His-N3 from the endosomes to the plasma membrane, where the enzyme is active. In fact, an important accumulation of His-N3 was observed in the membrane fraction in normo- and low glucose conditions at 24. Moreover, the proof that GRP78 is essential for the folding and, consequently, for the activity of His-N3 was given by the sialidase activity assay, where no alterations were detected in normo-glucose condition, whereas a significant increase of sialidase activity was observed in the low glucose concentration, showing that the recruitment of His-N3 in the plasma membrane is directly proportional to the cell stress. Moreover, NEU3 activity assays in the presence of EGCG, an inhibitor of GRP78, showed a decrease in NEU3 activity, thus further confirming the key role of GRP78 in NEU3 stabilization and folding.

In summary, herein we reported that sialidase NEU3 possesses enzymatic activity toward gangliosides mainly when it is exposed on the cell membrane. However, a substantial amount of the enzyme is present in the endosomal compartment, where it interacts with key proteins involved in stress response, protein folding, and intracellular trafficking. Overall, the current results support the hypothesis that the endosomes may constitute a cell reservoir of NEU3, which is ready, although inactive, to be translocated to the outer cell membrane when its activity is required, for instance in response to stress conditions. Further studies along this line are currently underway in our laboratories.

Author Contributions—F. C., A. G., and L. A. conceived the study and wrote the paper. F. C., A. G., C. F., E. T., and M. P. designed, performed, and analyzed the experiments. G. T., C. G., and L. A. designed and analyzed all the experiments. All authors reviewed the results and approved the final version of the manuscript.

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NEU3 Sialidase Protein Interactors in the Plasma Membrane and in the Endosomes

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