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*Membrane association and subcellular localization of sialidase NEU3***Sialidase NEU3 is a peripheral membrane protein localized on the cell surface and in endosomal structures**

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ADDRESSES

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ABBREVIATIONS:

*Mm*NEU3: *Mus musculus* membrane-associated sialidase NEU3

*Hs*NEU3: *Homo sapiens* membrane-associated sialidase NEU3

4MU-NeuAc: 4-Methylumbelliferyl-N-acetyl- α -D-neuraminic acid

[³H]GD1a: isotopically tritium-labeled ganglioside GD1a at position 3 of the sphingosine moiety

GPI: glycosyl-phosphatidyl-inositol

*Membrane association and subcellular localization of sialidase NEU3***ABSTRACT**

Sialidase NEU3 is also known as the plasma membrane-associated form of mammalian sialidases, exhibiting a high substrate specificity toward gangliosides. In this respect, sialidase NEU3 modulates cell surface biological events and plays a pivotal role in different cellular processes, including cell adhesion, recognition and differentiation. At the moment, no detailed studies concerning the subcellular localization of NEU3 are available and the mechanism of its association to cellular membranes is still unknown. Here we demonstrate that sialidase NEU3, besides its localization at the plasma membrane, is present in intracellular structures at least partially represented by a subset of the endosomal compartment. Moreover, we show that NEU3 present at the plasma membrane is internalized and locates then to the recycling endosomal compartment. The enzyme is associated to the outer leaflet of the plasma membrane, as shown by selective cell surface protein biotinylation. This evidence is in agreement with the ability of NEU3 in degrading gangliosides inserted in the plasma membrane of adjacent cells. Moreover, the association mechanism of the protein to the lipid bilayer was elucidated by carbonate extraction. Under these experimental conditions we succeeded in solubilizing NEU3, thus demonstrating that the enzyme is a peripheral membrane protein. In addition, Triton X-114 phase separation further demonstrates the hydrophilic nature of the protein. Overall, these results provide important information about the biology of NEU3, the most studied member of the mammalian sialidase family.

*Membrane association and subcellular localization of sialidase NEU3***INTRODUCTION**

Sialidases or neuraminidases (EC 3.2.1.18) are glycohydrolytic enzymes that remove sialic acid residues from sialoglycoconjugates, such as gangliosides, sialoglycoproteins and sialooligosaccharides. They are widely distributed in nature, from viruses and bacteria to vertebrates [1]. In mammals, four different forms of sialidases have been cloned so far and their classification is mainly based on their subcellular distribution: the lysosomal NEU1, the cytosolic NEU2, the plasma membrane-associated NEU3 and the lysosomal/mitochondrial NEU4 [2]. Among them, the plasma membrane-associated sialidase NEU3 has been shown to be preferentially active toward gangliosidic substrates [3-8]. Moreover, NEU3 is present within lipid rafts [9, 10] that represent specialized functional areas of the membranes, highly enriched in cholesterol and sphingolipids [11, 12]. Sphingolipids, and among them gangliosides, are known to affect different biological events including tumorigenic transformation [13-18], cell differentiation [19, 20] and motility [21-25]. The evidence that the over-expression of sialidase NEU3 induces dramatic changes in the cellular ganglioside pattern [6] is of particular interest because of the relevance of these amphiphilic molecules in mediating important cellular behaviours [26]. Indeed, sialidase NEU3 has been reported to play a pivotal role in different normal cellular processes including axonal growth and regeneration [27], and tumorigenic transformation [28]. Importantly, the activity of sialidase NEU3 is exerted also on gangliosides exposed on the extracellular leaflet of the plasma membrane of adjacent cells by cell-to-cell interaction [6]. This evidence has suggested the hypothesis that the enzyme is located at the external leaflet of the plasma membrane, facing the extracellular environment, thus allowing its interaction with the gangliosidic substrates exposed on the plasma membrane of neighbouring cells.

Detailed studies concerning the membrane anchoring of sialidase NEU3 have not been reported so far. Analysis of NEU3 aminoacid sequence reveals a striking similarity with the soluble counterpart NEU2. Indeed, based on the crystal structure of human NEU2 [29] and on the aminoacid sequence homology, a modeling of human NEU3 was performed, suggesting a common β -propeller folding for both enzymes [30]. The possible presence of canonical aminoacid motif(s) necessary for post-translational modification(s) potentially involved in NEU3 membrane anchoring can be explored by bioinformatic analysis [31-33]. In order to gain more information about the subcellular distribution of sialidase NEU3 and its mechanism of association to the lipid bilayer, we expressed the mouse protein (*MmNEU3*) in HeLa and COS-7 cells. We found that expressed sialidase NEU3 is present both at the plasma membrane and in intracellular tubulo-vesicular structures that represent a subset of the endosomal compartment. In addition, experiments of cell surface protein biotinylation and

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indirect immunofluorescence gave the first direct evidence that sialidase NEU3 is associated to the external leaflet of the plasma membrane. Finally, using different extraction and solubilization methods, we provide evidences that sialidase NEU3 has hydrophilic characteristics and behaves as a peripheral membrane protein.

Stage 2(a) POST-PRINT

*Membrane association and subcellular localization of sialidase NEU3***EXPERIMENTAL****Cell culture and transfection**

COS-7 and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) fetal bovine serum and maintained at 37°C and 5% CO₂ in a humidified incubator. Cells were transiently transfected with a C-terminal HA-tagged form of mouse NEU3 (*MmNEU3-HA*), subcloned into the pcDNA1/Amp (Invitrogen) vector. Transfection was performed in serum-free medium (OptiMEM; Gibco-BRL) employing FuGENE 6 (Roche) and incubating the cells at 37°C for 6 h. After transfection, cells were grown in DMEM for 36 h and then processed.

Antibodies

For indirect immunofluorescence and immunoblotting experiments the following primary antibodies were used: rabbit anti-HA (Sigma), mouse anti-EEA1 (Transduction Laboratories), mouse anti-TfR (Zymed), mouse anti-PDI (Stressgene), mouse anti-LBPA (from Dr. J. Gruenberg, Department of Biochemistry, University of Geneva, Switzerland) and rabbit anti-caveolin-1 (Santa Cruz). Detection of the human NEU3 in immunoblotting experiments was achieved using a rabbit anti-human NEU3, (from Dr. N. Stamatos, Institute of Human Virology, University of Maryland, Baltimore, U.S.A.). For immunofluorescence experiments, donkey anti-rabbit Alexa 488 and donkey anti-mouse Alexa 555 (Molecular Probes) secondary antibodies were used. For immunoblotting experiments, donkey anti-rabbit and sheep anti-mouse HRP-conjugated secondary antibodies (GE Healthcare) were used.

Indirect immunofluorescence and confocal microscopy analysis

Indirect immunofluorescence experiments were performed as already described with minor modifications [6]. COS-7 or HeLa cells were seeded onto glass coverslips and transfected with *MmNEU3-HA*. At 36 h post-transfection cells were fixed with 3% (w/v) paraformaldehyde in PBS for 15 min at room temperature. Paraformaldehyde was then quenched incubating the samples with 50 mM NH₄Cl in PBS for 15 min. After three washes with PBS, cells were permeabilized with 0.5% saponin in PBS (PBS/Sap) for 30 min and incubated with primary antibodies diluted in PBS/Sap for 1 h. Subsequently, cells were washed three times with PBS/Sap and incubated with secondary antibodies diluted in PBS/Sap for the same period. Finally, after three washes with PBS/Sap followed by washes with PBS, specimens were mounted using DakoCytomation Fluorescent Mounting Medium (DakoCytomation) and analyzed with confocal system LSM-510

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META (Carl Zeiss). For indirect immunofluorescence of non permeabilized cells, the same procedure was used substituting 0.5% saponin with 1% BSA. Images were processed with LSM Image Browser (Carl Zeiss) and Adobe Photoshop software.

Antibody uptake

COS-7 cells seeded onto glass coverslips were transfected with *MmNEU3*-HA as above. After 36 h of transfection, cells were incubated for 3 h at 37°C in growth medium supplemented with rabbit anti-HA antibody at 2.5 µg/ml final concentration. Cells were then chilled on ice, washed three times with ice-cold PBS and fixed with methanol. After saturation with 1% BSA in PBS (BSA/PBS), specimens were incubated with mouse anti-TfR antibody diluted in BSA/PBS for 1 h. After extensive washes with BSA/PBS, donkey anti-rabbit Alexa 488 and donkey anti-mouse Alexa 555 secondary antibodies were used. Specimens were then mounted and analyzed as above.

Immunoblotting

Proteins were separated by 12% SDS-PAGE and transferred to a PVDF membrane (Hybond-P; Amersham Biosciences). Membranes were then blocked with 5% (w/v) not-fat dry milk in PBS, washed three times with PBS containing 0.1% (v/v) Tween 20 (PBST) and incubated with primary antibody diluted in PBST containing 1% (w/v) milk for 2 h at room temperature. After three washes with PBST, membranes were incubated with HRP-conjugated secondary antibody diluted in PBST for 1 h at room temperature. Detection of the immunocomplexes was performed by enhanced chemiluminescent-based system (SuperSignal West Pico Chemiluminescent Substrate; Pierce), followed by densitometric analysis using GelPro 3.1 software (Media Cybernetics).

Cell surface protein biotinylation and purification

For biotinylation experiments all steps were carried out at 4°C. Surface proteins of subconfluent cultured COS-7 and HeLa cells expressing *MmNEU3*-HA for 36 h were labeled with 0.5 mg/ml of membrane-impermeable sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (Sulfo-NHS-SS-biotin; Pierce) in PBS for 30 min with gentle agitation, as indicated by the manufacturer's instructions. Cells were then washed three times with PBS and free biotin was quenched by one wash with 25 mM Tris-HCl pH 8.0, followed by three washes with PBS. Cells were then scraped in PBS and then lysed in RIPA buffer (25 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) by sonication. After lysis, total cell extracts were clarified by centrifugation at 800 x g for 10 min and biotinylated proteins were separated from non-biotinylated

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proteins using Immobilized Monomeric Avidin resin (Pierce). Fractions were adjusted to the same final volume and analyzed by immunoblotting.

Bioinformatic analysis

Analysis of the protein motifs involved in post-translational modifications was performed using the aminoacid sequence of *MmNEU3* (accession number Q9JMH7) [34] and *HsNEU3* (accession number Q9UQ49) [5] with public domain software. Prediction of GPI-anchor motifs was performed using the following softwares: big-PI Predictor - GPI Modification Site Prediction: http://mendel.imp.ac.at/sat/gpi/gpi_server.html, DGPI - Prediction of GPI-anchor and cleavage sites: http://129.194.185.165/dgpi/index_en.html, GPI-SOM - Identification of GPI-anchor signals by a Kohonen Self Organizing Map: <http://gpi.unibe.ch/>. Prediction of fatty acyl- or prenyl-anchor motifs was performed with the following softwares: Myristoylator Prediction of N-terminal myristoylation by neural networks: <http://www.expasy.org/tools/myristoylator/>, NMT - Prediction of N-terminal N-myristoylation: <http://mendel.imp.ac.at/myristate/SUPLpredictor.htm>, PrePS - Prenylation Prediction Suite: <http://mendel.imp.ac.at/sat/PrePS/index.html>. For the prediction of transmembrane sequences the following softwares were used: HMMTOP - Prediction of transmembrane helices and topology of proteins: <http://www.enzim.hu/hmmtop/>, SOSUI - Prediction of transmembrane regions: <http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html>, TMHMM - Prediction of transmembrane helices in proteins: <http://www.cbs.dtu.dk/services/TMHMM-2.0/>, TMPred - Prediction of transmembrane regions and protein orientation: http://www.ch.embnet.org/software/TMPRED_form.html. Finally, we analyzed the aminoacid sequences of *MmNEU3* with AmphipaSeeK (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_amphipaseek.html), a monotopic membrane protein prediction tool.

Membrane protein extraction

HeLa or COS-7 cells 36 h after transfection with *MmNEU3*-HA were broken by bundle sonication in ice-cold lysis buffer (10 mM Tris-HCl pH 7.5 containing 0.032 µg/ml aprotinin, 0.032 µg/ml leupeptin, 0.008 µg/ml pepstatin A, 0.06 µg/ml chymostatin and 0.2 mM PMSF as protease inhibitors). After cell lysis, samples were clarified by centrifugation at 800 x g for 10 min at 4°C and the resulting supernatant (total cell extract) was centrifuged at 100,000 x g for 1 h at 4°C in order to obtain a total cell membranes fraction. The resulting pellet was washed once with ice-cold distilled water, resuspended in the lysis buffer and split into identical aliquots. Extraction of peripheral proteins was performed by the exposure of total cell membranes either to pH 11.5 or 1 M

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KCl or 0.025 M EGTA and incubated on ice for 30 min. The above extraction conditions were achieved by the addition to the samples of an equal volume of 0.2 M Na₂CO₃ pH 12 or 2 M KCl or 0.05 M EGTA, respectively. As control sample, membranes were incubated in presence of lysis buffer alone and processed as above. Finally, solubilized and non-extractable proteins were separated by centrifugation at 100,000 x g and carbonate-containing samples were immediately neutralized to pH 7.5 by the addition of acetic acid. Soluble and membrane fractions were then adjusted to the same final volume before proteins repartition analysis by sialidase activity and/or immunoblotting assay.

Triton X-114 phase separation

Triton X-114 phase separation was performed as described [35]. Briefly, at 36 h post-transfection with *Mm*NEU3-HA, HeLa cells were chilled at 4°C. After three washes with cold PBS, cells were scraped and pelleted at 800 x g for 10 min. Cells were then resuspended in 10 mM Tris-HCl pH 7.4, lysed by sonication and the resulting total cell extracts were diluted to a protein concentration corresponding to 1.0 mg/ml in 0.1 ml in the same buffer. Proteins were extracted by adding 0.1 ml of 2% (v/v) precondensed Triton X-114 (Sigma) and incubating the sample for 1 h on ice. Detergent-extracted samples (200 µl) were then layered onto 6% (w/v) sucrose cushion (300 µl), incubated at 30°C for 3 min and finally centrifuged at 300 x g for 3 min. After centrifugation, the upper aqueous phase was removed, re-extracted with 1% Triton X-114 and subjected to a second separation through the same sucrose cushion. The detergent and aqueous phases were adjusted to the same final volume and *Mm*NEU3-HA repartition, together with the endogenous protein markers, was analyzed by immunoblotting.

Sialidase activity assay

The enzymatic activity of *Mm*NEU3-HA was determined as previously described [6] using radioactive GD1a ganglioside ([³H]GD1a), prepared according to Ghidoni et al. [36] (specific radioactivity, 1.2 Ci/mmol; homogeneity > 99%), and 4MU-NeuAc (Sigma) as substrates, in 100 mM sodium-citrate/phosphate buffer and performed at pH 3.8. Reaction mixtures were adjusted to the appropriate final concentrations of buffered-carbonate or Triton X-114 before the addition of the enzymatic source. Samples were incubated at 37°C for 30 min. For NEU3 enzymatic stability at alkaline pH, total membranes were incubated at pH 11.5 for different time periods (up to 30 min), neutralized to pH 7.5 and assayed as above without ultracentrifugation. NEU3 activity related to Triton X-114 fractions was measured both in the single aqueous and detergent phases, as well as in the pooled phases.

*Membrane association and subcellular localization of sialidase NEU3***RESULTS****Subcellular localization of sialidase NEU3.**

In order to study the subcellular distribution and localization of sialidase NEU3, the chimeric form of the mouse protein (*MmNEU3*) bearing the haemagglutinin epitope (HA) at the C-terminus (*MmNEU3-HA*) was transiently expressed in both HeLa and COS-7 cells. The expression of this construct gives rise to a fusion protein that is catalytically active and has already been extensively used in biochemical and functional studies [6-8, 10]. After 36 h expression, cells were fixed, permeabilized with saponin and *MmNEU3-HA* subsequently detected using anti-HA antibodies. Laser confocal microscopy analysis clearly shows that, irrespective to the cell type considered (Figures 1A and 1D), *MmNEU3-HA* localizes both at the plasma membrane (Figures 1B and 1E) and in intracellular vesicular structures that concentrate in the juxtannuclear region of the cells (Figures 1C and 1F). While the presence of NEU3 at the plasma membrane was already demonstrated [3, 5-7], an intracellular localization of the enzyme was only once reported without giving any information about the precise nature of this intracellular labelling [8].

In order to better characterize the intracellular distribution of NEU3, co-localization experiments were carried out using markers of different cellular compartments. When *MmNEU3-HA* distribution was related either to the endoplasmic reticulum marker protein disulfide isomerase (PDI), as well as to the Golgi complex marker GM-130, no co-localization was observed (data not shown). Instead, co-localization between *MmNEU3-HA* and markers of the endosomal compartment was observed. As shown in Figure 2, COS-7 cells showed intracellular vesicular structures labelled for both *MmNEU3-HA* and the early endosome antigen 1 (EEA1), a marker of the early-endosomes (Figure 2, upper row). Co-localization of *MmNEU3-HA* is even more striking with the juxtannuclear transferrin receptor (Tf-R) positive structures, representing the recycling-endosomal compartment (Figure 2, middle row). Interestingly, *MmNEU3-HA* does not colocalize with the late-endosomes markers lyso-bisphosphatidic acid (LBPA) (Figure 2, bottom row), as well as with the lysosomal associated membrane protein-1 (LAMP-1) (data not shown). Taken together these results indicate a restrict localization of sialidase NEU3 to a subset of the endosomal compartment. Moreover, the protein does not follow the degradative lysosomal pathway as demonstrated by the lack of co-localization with both LBPA and LAMP-1. A similar intracellular distribution and co-localization pattern was observed also in HeLa cells (data not shown). Overall, we can conclude that *MmNEU3-HA*, besides its localization at the plasma membrane, is present also in intracellular membranous structures that are mainly represented by the early- and recycling-endosomes.

*Membrane association and subcellular localization of sialidase NEU3***Sialidase NEU3 is associated to the external leaflet of the plasma membrane.**

In order to confirm the presence of sialidase NEU3 both at the plasma membrane and in intracellular compartments, COS-7 cells expressing *MmNEU3*-HA were subjected to cell surface protein biotinylation at 4°C, and labelled proteins were isolated by affinity chromatography using an avidin resin. Distribution of *MmNEU3*-HA between avidin-unbound and avidin-bound fractions was then analyzed by Western-blot. As shown in Figure 3(A), *MmNEU3*-HA was found both in the non-biotinylated and in the biotinylated fractions, confirming that the protein is present both in intracellular compartments and at the cell surface. Densitometric analysis of the immunoblotting indicates that $23.8\% \pm 5.4$ ($n=3$) of *MmNEU3*-HA localizes to the cell surface while the remaining portion of the protein is localized intracellularly. We also analyzed the distribution of two other proteins, i.e. Caveolin-1 (Cav-1), a protein known to be associated to the internal leaflet of the plasma membrane [37, 38], and Tf-R, a transmembrane protein that cycles between the plasma membrane and intracellular compartments [39]. As shown in Figure 3(A), in the same cell extracts used for the detection and distribution of *MmNEU3*-HA, Cav-1 was detected only in the non-biotinylated fraction, as expected, confirming that under these experimental conditions intracellularly localized proteins are not accessible to biotin. Conversely, Tf-R was detected in both the biotinylated and non-biotinylated fractions, as expected. Superimposable results were obtained also using HeLa cells (data not shown). Based on this evidence we can reasonably assume that the portion of *MmNEU3*-HA that is bound to the plasma membrane is accessible to biotinylation, hence exposed to the extracellular environment. In other words, the enzyme is associated to the external leaflet of the plasma membrane. In order to confirm this finding, non permeabilized COS-7 and HeLa cells expressing *MmNEU3*-HA were processed for indirect immunofluorescence using, at the same time, anti-HA antibodies and, as control, anti-PDI antibodies. Under these experimental conditions no labelling for the intracellular endoplasmic reticulum marker PDI was observed at all (data not shown), demonstrating the integrity of the plasma membrane, while a specific labelling for *MmNEU3*-HA was detected (Figures 3B and 3C). This result further strengthens the notion that sialidase NEU3 is associated to the external leaflet of the plasma membrane.

NEU3 is internalized from the cell surface to the recycling compartment.

As described above, we could demonstrate that sialidase NEU3 localizes to the external leaflet of the plasma membrane and in the endosomal compartment. We then tried to directly assess the propensity of the protein to move from the surface to the cell interior. For this purpose, cultured COS-7 cells transfected with *MmNEU3*-HA were incubated for 3 h in presence of anti-HA

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antibody. Cells were then fixed, permeabilized and the distribution of the *Mm*NEU3-HA/anti-HA antibody immuno-complexes was analyzed and compared to Tf-R distribution. As shown in Figure 4(A), in transfected cells a significant labelling for NEU3 could be detected at the plasma membrane but also in vesicular structures that are supposed to be located intracellularly. Co-localization between the anti-HA antibody signal and Tf-R (Figure 4B) is observed in the juxtannuclear region of the cell (Figure 4C), suggesting that the *Mm*NEU3-HA/anti-HA antibody immunocomplexes were internalized from the cell surface and localize to the recycling endosomal compartment. The evidence that the anti-HA antibody is internalized and localizes intracellularly is given by the direct vertical confocal sections shown in Figure 4(A' – C'). Indeed, a significant labelling for *Mm*NEU3-HA both at the surface and in intracellular structures that concentrate at the juxtannuclear region of the cell is evidenced. Notably, these structures co-localize with the intracellular labelling for Tf-R. Taken together these results clearly indicate that NEU3 internalizes from the plasma membrane and that internalized molecules reach the recycling endosomal compartment.

Sialidase NEU3 is a peripheral membrane-associated protein.

The aminoacid sequence of *Mm*NEU3 was analyzed for the prediction of transmembrane domain(s) that would anchor the protein to the lipid bilayer. Only TMpred program (see Experimental) predicts the presence of a single hydrophobic aminoacid stretch, spanning between residues A178 and S203. If we assume this stretch as a transmembrane portion of the enzyme, the protein would additionally feature an intracellular and an extracellular domain. This structural model is unlikely because, in this configuration, putative aminoacid residues essential for the catalytic activity would be located at opposite sides of the membrane, that would be clearly inconsistent with catalytic activity [2]. Moreover, based on the crystal structure of NEU2 [29] and considering the high homology between the two proteins, a common β -propeller fold for the human NEU3 was recently suggested [30]. Bioinformatic analysis of *Mm*NEU3 aminoacid sequence excludes also the possibility that the protein may associate to membranes as a monotopic protein [40]. Finally, sequence analysis does not reveal the presence of any aminoacid motifs for post-translational modifications that would anchor the protein to the lipid bilayer, such as GPI-, fatty acyl- or prenyl-linkages. On this basis, we took into consideration the possibility that sialidase NEU3 would associate to cellular membranes as a peripheral protein. For this purpose, total cell membranes obtained from HeLa and COS-7 cells expressing *Mm*NEU3-HA were incubated for 30 min in presence of 0.1 M sodium carbonate at pH 11.5 and then soluble and membrane fractions were obtained by ultracentrifugation [41-43]. The resulting fractions were then adjusted to pH 7.5 and

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analyzed for their enzyme content by Western-blot and sialidase activity. As control, total cell membranes were incubated for the same time period in 0.01 M Tris/HCl pH 7.5 and processed as above. As expected, incubation of membranes under control conditions, had no significant effect on the association of *Mm*NEU3-HA to the membranes (Figure 5) and the sialidase activity was almost completely recovered in the particulate fraction (82.5-87.3%, depending on the substrate used) (see Table 1). The small amount of *Mm*NEU3-HA activity in the soluble fraction may derive by a spontaneous release of the protein, as a consequence of membrane manipulation. On the contrary, a remarkable, almost complete, solubilization of *Mm*NEU3-HA was achieved when membranes were incubated under alkaline conditions, irrespective to the cell line taken in consideration (Figure 5). Under these experimental conditions, the peripheral membrane protein EEA1 was completely recovered in the soluble fraction, as expected [44]. Noteworthy, carbonate treatment preserved the lipid structure without affecting the association of integral proteins to the lipid bilayer, as confirmed by the analysis of the repartition of Cav-1 in the same cell extracts. In fact, Cav-1 remained always associated to the membranous fractions.

In order to investigate whether alkaline extraction of *Mm*NEU3-HA could be somehow ascribed to the over-expression of the heterologous protein, solubilization of the human endogenous protein *Hs*NEU3 was analyzed in non transfected (Mock) HeLa cells. For this purpose, we took advantage of a rabbit anti-*Hs*NEU3 antiserum that specifically recognizes the human protein in immunoblotting experiments [45]. The endogenous enzyme showed a roughly superimposable behaviour compared to *Mm*NEU3-HA, with a complete extraction of the protein only after alkaline treatment (Figure 5). However, using both the artificial 4MU-NeuAc and the [³H]GD1a ganglioside as substrates, almost no sialidase activity could be detected in the particulate and in the soluble fractions after 30 min carbonate treatment, although samples were adjusted to pH 7.5 immediately after ultracentrifugation (Table 1). Indeed, the enzymatic activity measured after carbonate extraction was markedly lower compared to control experimental conditions (3-7%, when assayed with [³H]GD1a). Loss of activity might be ascribed either to the presence of carbonate in the enzyme assay mixture or to exposure of the protein to the alkaline pH. We therefore determined the sialidase activity of total membranes by adding buffered-carbonate (see Experimental), at the appropriate final concentration, to the enzymatic assay mixture before the addition of the enzyme source. A small reduction in sialidase activity was observed as compared to control conditions (Figure 6), indicating that the presence of carbonate per se in the reaction mixture is compatible with the catalytic activity. We then subjected total membranes to alkaline pH treatment for different time intervals and, after adjustment to pH 7.5, we tested the sialidase activity. As shown in Figure 6, already after 1 min exposure to alkaline pH, more than 90% of the enzymatic activity was lost.

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By considering that the enzymatic activity was determined without separation of the soluble and membranous fractions, we can conclude that loss of catalytic activity can be ascribed to exposure to alkaline pH. To gain further information about the mechanism of NEU3 membrane anchoring, HeLa cells expressing *Mm*NEU3-HA were subjected to Triton X-114 extraction followed by aqueous/detergent phase separation, and the distribution of the protein was analyzed by Western-blot. These experimental conditions allow the separation of hydrophilic peripheral membrane proteins from transmembrane, GPI-anchored and fatty acyl- or prenyl-anchored membrane proteins [35, 46]. After phase separation, we observed a significant enrichment or a complete segregation of *Mm*NEU3-HA in the aqueous phase (Figure 7). The same results were obtained for the endogenous *Hs*NEU3 in HeLa cells. On the other hand, Cav-1 that is associated to membranes by a hydrophobic domain and palmitoylations [38], segregates exclusively in the detergent phase. As in the case of carbonate extraction, we were not able to recover any activity either in the single or in the reconstituted fractions after Triton X-114 phase separation, regardless of the substrate used in the assay. In addition to these findings, treatment of membranes derived from transfected cells with high ionic strength (1 M KCl) or with divalent cation chelator (25 mM EGTA) did not result in any solubilization of sialidase NEU3 (data not shown).

Overall, these findings demonstrate that: (i) NEU3 behaves as a hydrophilic peripheral protein; (ii) its biological activity is highly sensitive to alkaline pH; (iii) the interaction of the protein with the membrane does not seem to depend either on direct or calcium-mediated electrostatic interactions with phospholipids.

*Membrane association and subcellular localization of sialidase NEU3***DISCUSSION**

The plasma membrane-associated sialidase NEU3 is known to be highly active toward gangliosidic substrates [3, 5, 6, 34] and to be involved in several biological processes, possibly through the modulation of sialic acid content at the cell surface. Sialidase NEU3 is present in lipid rafts and co-fractionates with the lipid raft marker Cav-1 [9, 10]. These organized subsets of the membrane represent specialized areas where cell-to-cell interactions and signal transduction events take place [13, 26]. Up-regulation of sialidase NEU3 has been correlated to apoptosis suppression in human colon carcinoma [46], in renal carcinoma [25], as well as in other tumor cell lines [28]. In addition, it has been recently demonstrated that silencing of NEU3 results in the activation of apoptosis mechanisms in tumor derived cells [48]. Also, sialidase NEU3 plays an important role in normal cellular processes like axonal growth and regeneration [27], as well as axonal fate in unpolarized neurons [48]. Transfection and over-expression of sialidase NEU3 induces dramatic changes in the ganglioside composition of transfected cells and in neighbouring non-transfected cells [6], supporting the hypothesis that the protein is associated to the external leaflet of the plasma membrane.

Here we provide the first direct demonstration that sialidase NEU3 is topologically associated to the outer leaflet of the plasma membrane, where its natural gangliosidic substrates are present. In fact, after cell surface protein biotinylation, sialidase NEU3 is recovered in the biotinylated fraction. However, we also found that most sialidase NEU3 was not accessible to biotin, suggesting that the protein is present in intracellular compartments too. We confirmed this hypothesis by indirect immunofluorescence experiments coupled to laser confocal analysis where we observed a diffused cytoplasmic labelling for sialidase NEU3, with a specific localization in vesicular structures. The presence of sialidase NEU3 in intracellular structures was already observed by Yamaguchi K. et al. [8], but no precise topological identification was given. We could demonstrate that a subset of the intracellular labelling corresponds to the endosomal compartment since intracellular NEU3-positive structures were found to co-localize with EEA1 and the Tf-R. These results were independent from the cell type taken into consideration (HeLa and COS-7 cells), and from the expression level of the protein, indicating that the presence of sialidase NEU3 in the endosomal compartment is not a consequence of any possible impairment of intracellular trafficking and distribution of the protein, due to transfection. Possibly, sialidase NEU3 is present in different cellular pools in a dynamic equilibrium with each other and such a possibility is strongly suggested by the specific internalization of exogenously administrated antibody recognizing the HA epitope of the transfected protein. Interestingly, internalized antibody co-localize with Tf-R at the recycling endosomal

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compartment, further indicating a possible dynamic equilibrium between these two pools of protein. It has been recently demonstrated that sialidase NEU3 dynamically accumulates at peripheral areas of the plasma membrane upon stimulation of starved cells with EGF [8]. Under these experimental conditions, sialidase NEU3 co-localizes with Rac-1, a small GTPase protein that is involved in cell motility events. This indicates that sialidase NEU3 undergoes a specific recruitment to membrane ruffles upon external stimuli. Nothing is known concerning the possibility that sialidase NEU3 may traffic between the plasma membrane and intracellular compartments and whether this event is dependent on the presence of extracellular factors. Our finding that at steady-state the protein is present both at the plasma membrane and in the recycling endosomal compartment and that NEU3 internalizes from the plasma membrane, strongly supports such a possibility. The molecular mechanisms by which sialidase NEU3 is internalized and factors that may regulate this phenomenon are under investigation.

Moreover, the mechanism of sialidase NEU3 anchorage to the lipid bilayer was studied. The bioinformatic analysis of *Mm*NEU3 aminoacid sequence performed, excludes the possibility that the protein is a member of the GPI-anchored protein family or is a fatty acylated or prenylated protein because none of the consensus sequences necessary for these post-translational modifications are found. This evidence adds to the already established lack of transmembrane domains in sialidase NEU3 [2]. Therefore, we considered the possibility that sialidase NEU3 would associate to the lipid bilayer as a peripheral membrane protein. These are hydrophilic proteins that associate to membranes usually by ionic and/or hydrogen interactions between the protein itself and either phospholipids (protein-lipid interactions) or with integral membrane proteins (protein-protein interactions). In any case, peripheral membrane proteins can be released by different treatments such as extreme pH, high ionic strength and chaotropic agents, without disruption of the lipid bilayer organization [50]. Extracted peripheral proteins can be then isolated from intrinsic membrane proteins by ultracentrifugation. Under control conditions sialidase NEU3 remained associated to the particulate fraction and fully maintained its catalytic activity. Alkaline treatment [41] resulted in the extraction of the peripheral membrane protein EEA1 [44], as well as of both the murine and human sialidases NEU3, as assessed by Western-blot analysis. The same treatment did not affect at all the association to membranes of Cav-1, an integral membrane protein. We were not able to recover appreciable activity in the soluble and particulate fractions obtained after carbonate extraction, although the incubating conditions for the enzyme assay were optimal. Loss of activity can not be ascribed either to carbonate itself or to the separation between the soluble and the particulate fractions. Presumably, the enzyme is highly sensitive to exposure to alkaline pH, becoming then inactive.

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In order to further support the hydrophilic nature of NEU3 we analyzed the partitioning of the protein between the aqueous and detergent phases obtained after protein extraction in the presence of Triton X-114, followed by temperature-induced phase separation. By this method, hydrophilic proteins partition in the aqueous phase, while proteins that associate to biological membranes via fatty acyl- or prenyl-chains, GPI-anchors or transmembrane domains distribute into the detergent phase [35, 46]. Triton X-114 treatment allows the enrichment of both the murine and the human sialidase NEU3 in the aqueous phase, thus demonstrating the hydrophilic nature of the two proteins. However, also in this case, phase separation results in a complete lack of NEU3 enzymatic activity. A possible explanation can reside in the segregation of the enzyme in the aqueous phase and a putative cofactor, important for catalytic activity, in the detergent phase, although upon pooling the two phases the enzymatic activity could not be recovered. Possibly, the lack of activity in the reconstituted system can be ascribed to the Triton X-114 insolubility at temperatures higher than 0°C. In addition, when membranes were incubated in presence of high salt concentrations or treated with a divalent cation chelator (data not shown), sialidase NEU3 was not released from the lipid bilayer. As a whole, these observations clearly demonstrate that NEU3 is a hydrophilic peripheral membrane protein.

In summary, here we give the first direct demonstration that sialidase NEU3 is a peripheral membrane protein that resides on the external leaflet of the plasma membrane. Moreover, the protein is localized also in intracellular compartments that are in relation with the endocytic/recycling pathway. Presence of the protein in these two different cellular compartments suggests a possible dynamic equilibrium between the different pools of NEU3. This hypothesis is suggested by the evidence that NEU3 can internalize from the cell surface and then localizes to the recycling compartment. Finally, given the hydrophilic nature of the protein and the phospholipids-independent mechanism of association to membrane, it is possible that NEU3 may interact with an integral protein partner. The latter may represent also the driving force for the recycling of NEU3 in relation to extracellular stimuli, thus regulating the presence of sialidase NEU3 on the cell surface. The latter aspect may be critical in relation to the role of NEU3 and gangliosides in cell growth and malignant transformation.

*Membrane association and subcellular localization of sialidase NEU3***ACKNOWLEDGMENTS**

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*Membrane association and subcellular localization of sialidase NEU3***Table 1. Repartition of sialidase activity.**

Total NEU3 enzymatic activity in particulate (P) and soluble (S) fractions obtained from total cell membranes (T) of transfected COS-7 cells (*MmNEU3*-HA) was assayed under control and alkaline (Carbonate extraction) conditions. Values, representative of three independent experiments, are expressed as total activity recovered in each fraction.

Mock: COS-7 cells transfected with vector alone.

	Mock	<i>MmNEU3</i> -HA	Control		Carbonate extraction	
	T	T	P	S	P	S
4MU-NeuAc (nmol/h)	15.7 ± 1.8	125.7 ± 20.8	110.7 ± 14.1	8.1 ± 2.8	0.4 ± 0.2	0.3 ± 0.2
[³ H]GD1a (μmol/h)	73.5 ± 7.8	1090.0 ± 185.3	900.0 ± 117.0	160.0 ± 56.0	30.0 ± 14.4	10.0 ± 6.7

*Membrane association and subcellular localization of sialidase NEU3***LEGENDS TO FIGURE**

Figure 1: Sialidase NEU3 localizes both at the plasma membrane and intracellularly. COS-7 (A) and HeLa (D) cells transfected with *MmNEU3*-HA were fixed and, after permeabilization, subjected to indirect immunofluorescence using anti-HA antibody. Transfectants were then analyzed by laser confocal microscopy. Details of the plasma membrane (B and E) and intracellular labelling (C and F) are given for both cell types. Single confocal planes are reported. Bars: 10 μ m.

Figure 2: Sialidase NEU3 colocalizes with endosomal markers. COS-7 cells were transfected with *MmNEU3*-HA and subjected to immunofluorescence for co-localization experiments. Subcellular distribution of *MmNEU3*-HA was related to EEA1 (top panel), Tf-R (middle panel) and LBPA (bottom panel), markers of the endocytic/recycling pathway. Single confocal planes are shown in figure. Bars: 10 μ m.

Figure 3: Sialidase NEU3 is associated with the extracellular leaflet of the plasma membrane. (A) Cell surface proteins of COS-7 cells transfected with *MmNEU3*-HA were selectively biotinylated and isolated by avidin-biotin affinity chromatography. Equal volumes of the total cell extract (Total), avidin-unbound (Intracellular) and avidin-bound (Cell surface) fractions were analyzed by immunoblotting in order to reveal *MmNEU3*-HA and the endogenous markers Cav-1 and Tf-R. Non-permeabilized HeLa (B) and COS-7 (C) cells transfected with *MmNEU3*-HA were subjected to indirect immunofluorescence using anti-HA antibody. Projections of sequential confocal planes (0.37 μ m distance each plane) acquired through the cell thickness are shown. Bars: 10 μ m.

Figure 4: Cell surface sialidase NEU3 internalizes to the recycling endosomal compartment. *MmNEU3*-HA transfected COS-7 cells were subjected to anti-HA antibody uptake for 3 h, processed for indirect immunofluorescence and analyzed by laser confocal microscopy as described in the Experimental section. Subcellular distribution of *MmNEU3*-HA/anti-HA antibody immunocomplexes (A, A') was related to Tf-R staining (B, B'). Nuclei are evidenced by DAPI staining. Merge is given in (C, C'). The white lines in A-C represent the plane through which the corresponding direct vertical confocal sections(A'-C') were acquired. Bars: 10 μ m.

Figure 5: Sialidase NEU3 is a peripheral membrane-associated protein. A crude preparation of cell membranes from COS-7 and HeLa cells transfected with *MmNEU3*-HA were extracted with

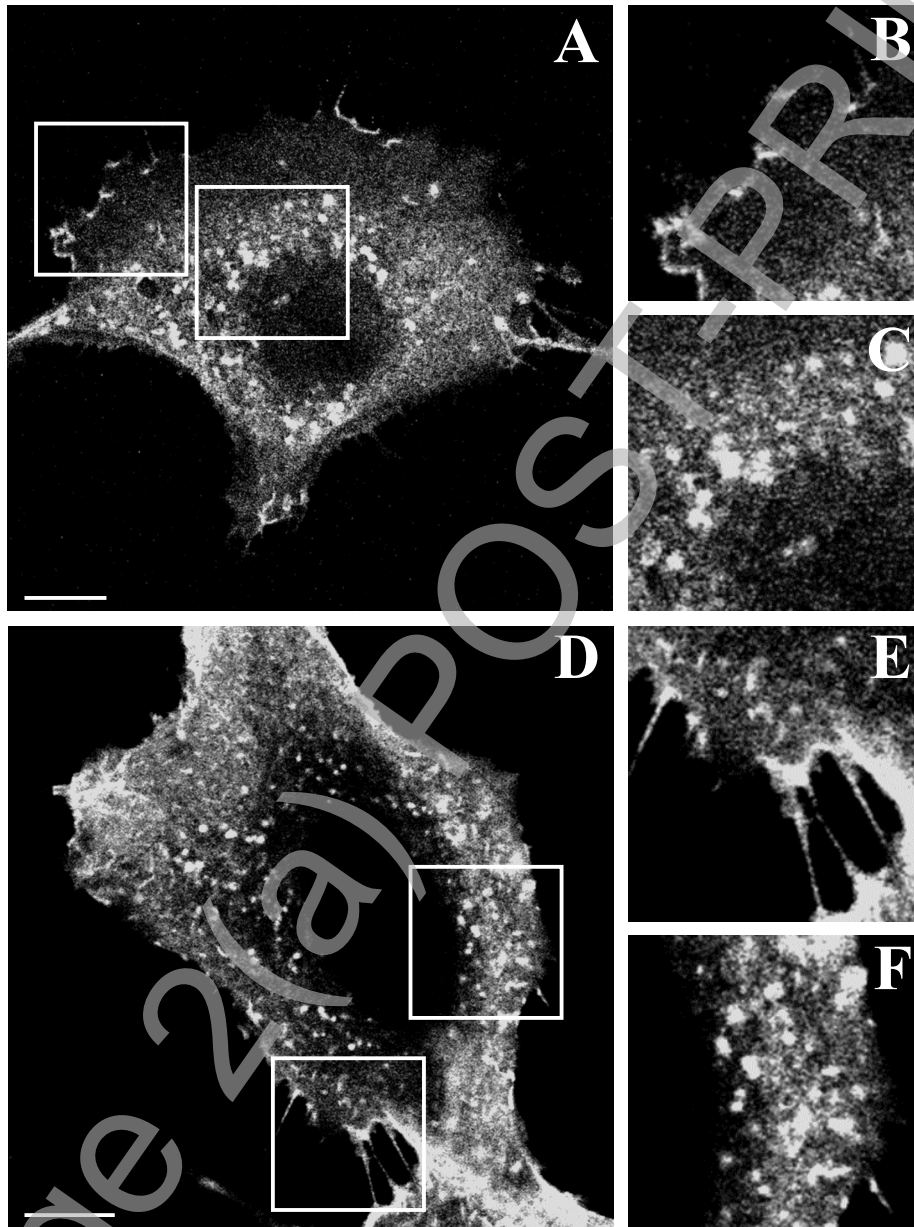
Membrane association and subcellular localization of sialidase NEU3

Tris buffer (Control) or with sodium carbonate (Carbonate extraction). After treatment, membranes were collected by ultracentrifugation and equal volumes corresponding to the starting crude cell membranes (T), membranes (P) and soluble (S) fractions were analyzed by Western-blot using anti-HA, anti-*Hs*NEU3, anti-EEA1 and anti-Cav-1 antibodies.

Figure 6: NEU3 enzymatic activity is highly sensitive to alkaline pH. NEU3 enzymatic activity toward the artificial substrate 4MU-NeuAc was assayed using total cell membranes in presence of buffered-carbonate in the reaction mixture (Carbonate +) or following exposure to alkaline pH for different time periods (0.5 to 30 min). Values are given as percentage of recovered activity related to control conditions. Data are representative of two independent experiments.

Figure 7: Sialidase NEU3 is a hydrophilic protein. Total cell extracts derived from HeLa cells transfected with *Mm*NEU3-HA were extracted and subjected to phase separation with Triton X-114. Equal aliquots of the aqueous (Aq) and detergent (De) phases were then analyzed by Western-blot using anti-HA, anti-*Hs*NEU3, anti-Cav-1 and anti-PDI antibodies.

Figure 1



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Figure 2

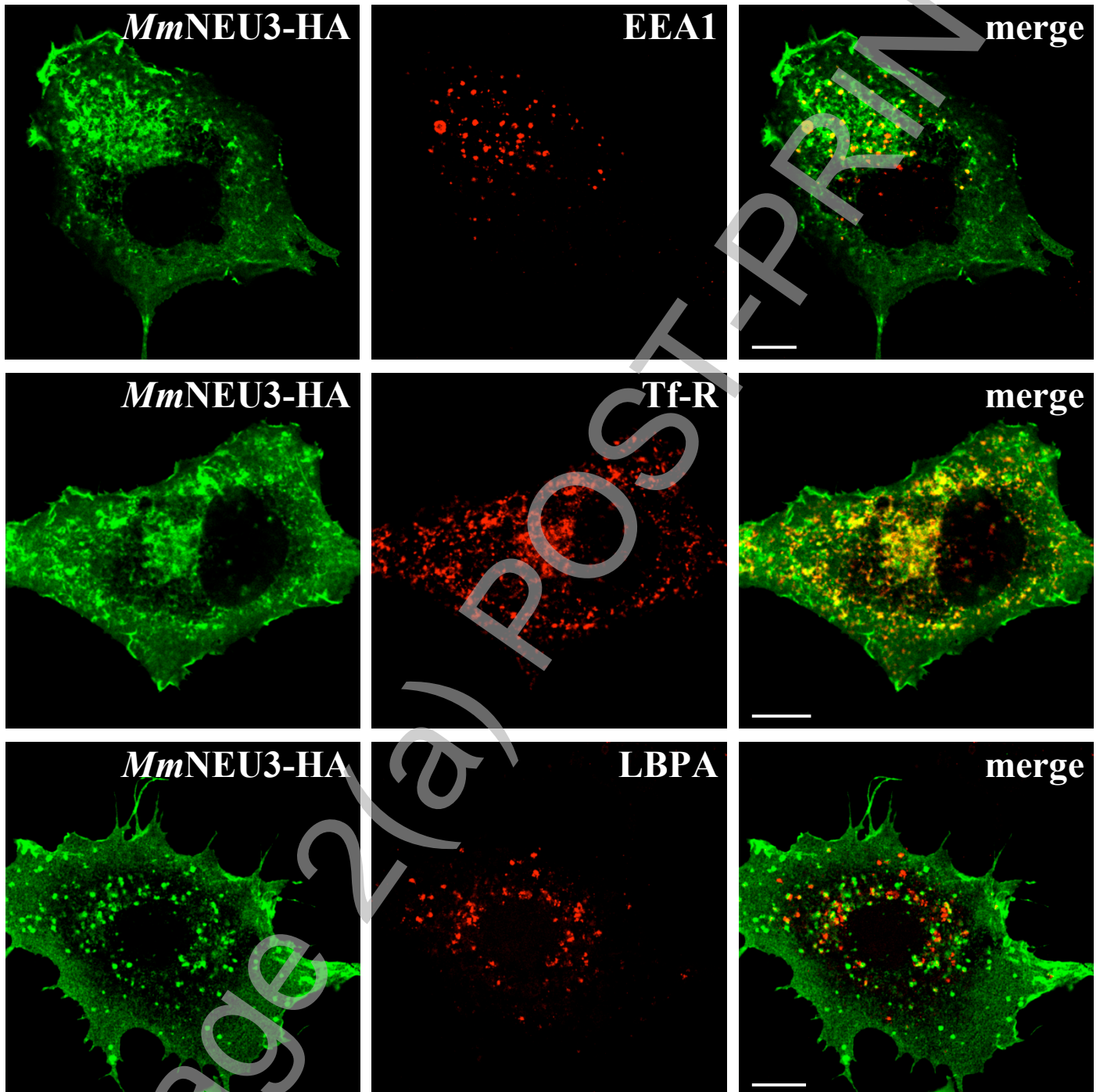
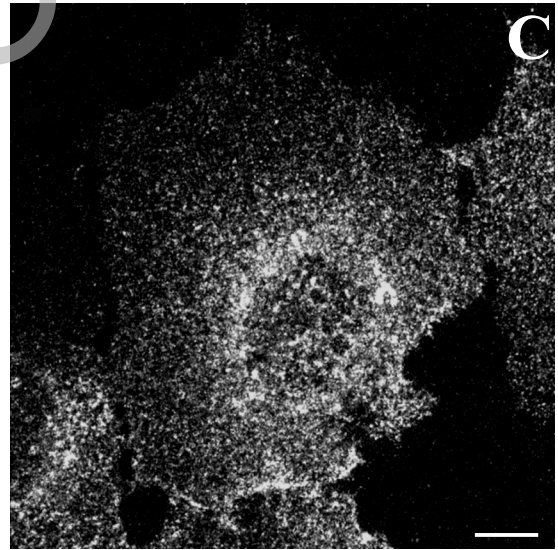
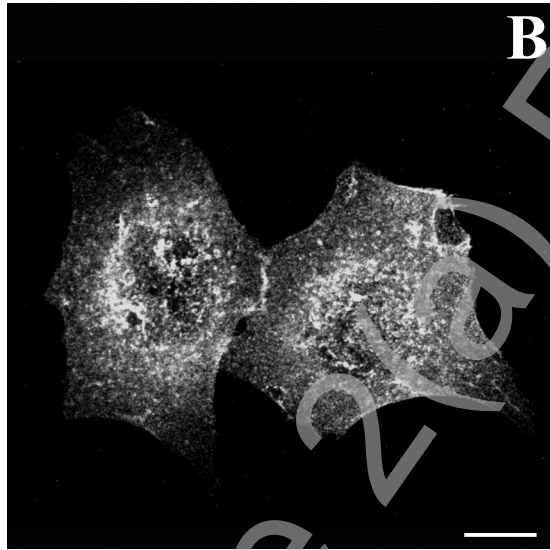
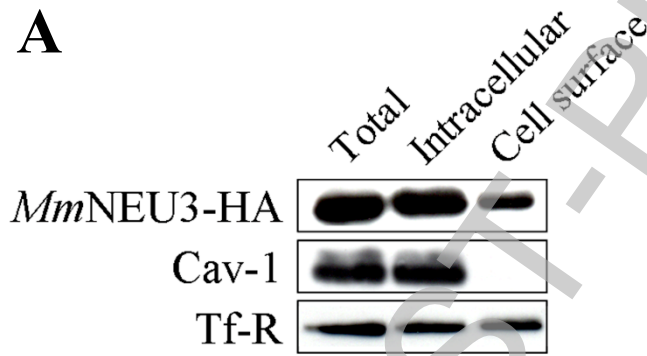
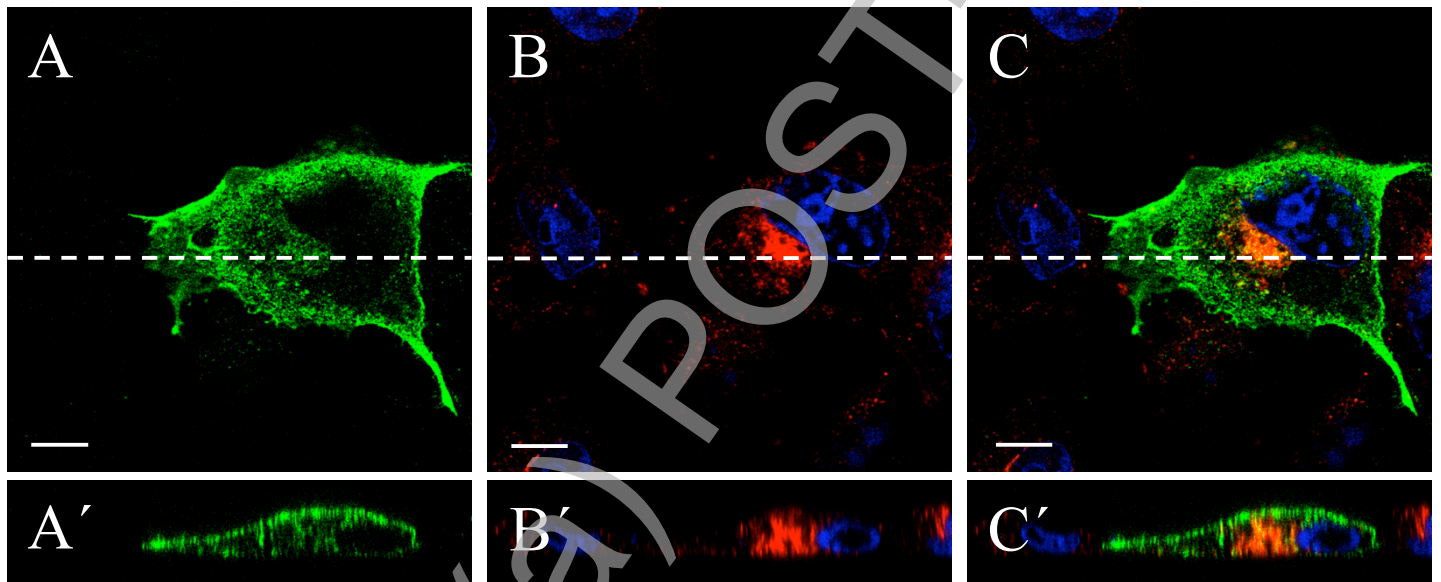


Figure 3



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Figure 4



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Figure 5

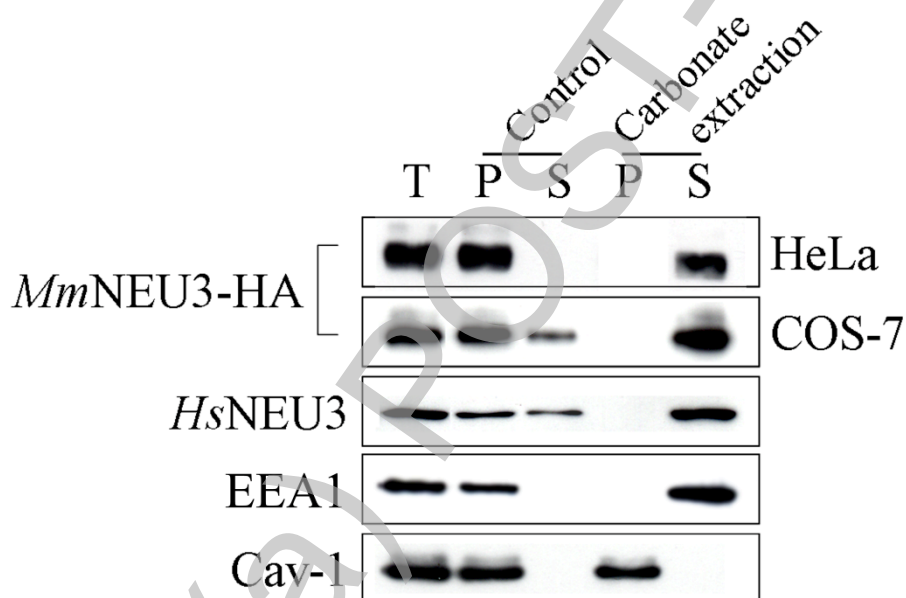
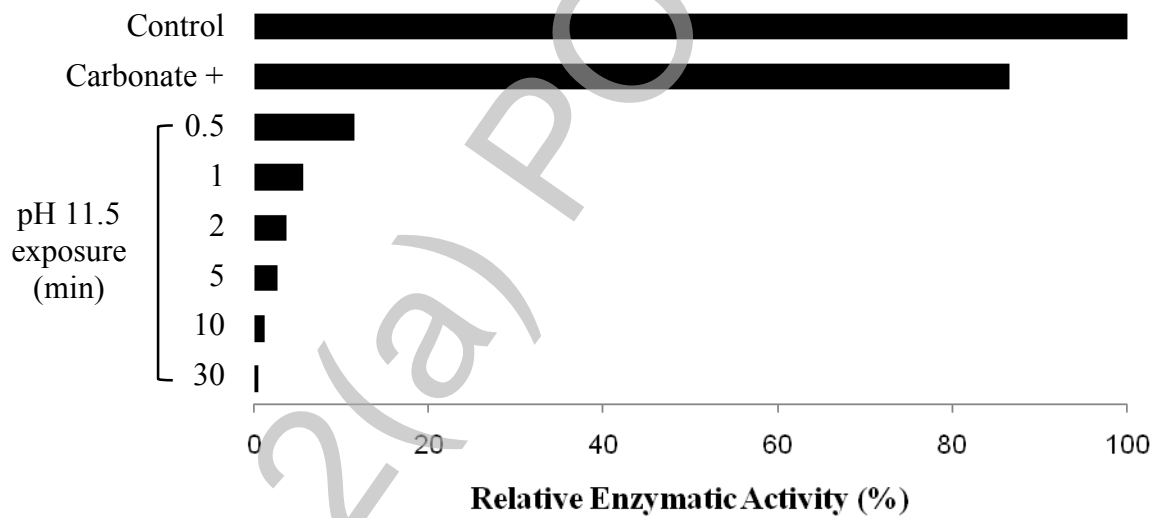


Figure 6



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Figure 7

