Human sialidase NEU4 long and short are extrinsic proteins bound to outer mitochondrial membrane and the endoplasmic reticulum, respectively

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Received on April 29, 2009; revised on September 11, 2009; accepted on September 27, 2009

Sialidases are widely distributed glycohydrolytic enzymes removing sialic acid residues from glycoconjugates. In mammals, several sialidases with different subcellular localizations and biochemical features have been described. NEU4, the most recently identified member of the human sialidase family, is found in two forms, NEU4 long and NEU4 short, differing in the presence of a 12-amino-acid sequence at the N-terminus. Contradictory data are present in the literature about the subcellular distribution of these enzymes, their membrane anchoring mechanism being still unclear. In this work, we investigate the human NEU4 long and NEU4 short membrane anchoring mechanism and their subcellular localization. Protein extraction with Triton X-114 and sodium carbonate and cross-linking experiments demonstrate that both forms of NEU4 are extrinsic membrane proteins, anchored via protein–protein interactions. Moreover, through confocal microscopy and subcellular fractionation, we show that the long form localizes in mitochondria, while the short form is also associated with the endoplasmic reticulum. Finally, mitochondria subfractionation experiments suggest that NEU4 long is bound to the outer mitochondrial membrane.

Keywords: endoplasmic reticulum/long and short isoforms/mitochondrial membrane/peripheral membrane protein/sialidase NEU4

Introduction

Sialidases (EC 3.2.1.18), hydrolases cleaving sialic acid residues from sialylated glycoconjugates, are widely distributed in nature, from microorganisms to mammals (Saito and Yu 1995). Since 1993 several mammalian sialidases have been cloned (Monti et al. 2002): a lysosomal form (NEU1), a cytosolic form (NEU2), a plasma membrane-associated form (NEU3), and another form, also bound to intracellular membranes, called NEU4 (Monti et al. 2004). These sialidases show important similarities in their primary structures, such as the F(Y)RIP motif, Asp boxes, as well as some common aminoacids constituting the catalytic site and a β-propeller structure, with the catalytic site lying in a deep cleft on one side of the molecule (Crennell et al. 1993; Taylor 1996). However, they also show different biochemical properties, as regards substrate specificity and subcellular localization. The three-dimensional structure of human sialidase NEU2 has recently been solved, showing that the enzyme possesses the well-known β-propeller 3D structure already described in viral, microbial, and tripanosomal sialidases (Chavas et al. 2005).

NEU4, the fourth member of the sialidase family in mammals, was identified by searching sequence databases for entries showing similarities to the human cytosolic sialidase NEU2 (Monti et al. 2004). Its activity on different sialoglycoconjugates, including the artificial substrate 4MU-NeuAc, is optimal at very acidic pH (3.2). Different authors have investigated NEU4 subcellular localization. Monti and colleagues demonstrated the association of NEU4 with the inner cellular membranes (Monti et al. 2004), through Western blot analysis and immunofluorescence staining. More recently, Seyrantepe et al. (2004) showed that human NEU4 is targeted to the lysosomes. On the other hand, Yamaguchi et al. (2005) isolated a cDNA fragment coding for two NEU4 isoforms which differ for the presence, in the long form, of an additional 12-amino-acids N-terminal sequence. These authors also showed that the two isoforms were not distinguishable in substrate specificity, but exhibited different subcellular localizations, the long form being targeted mainly to mitochondria and the short form to intracellular membranes.

This work was undertaken with the aim of accurately assessing the subcellular localization of both forms of NEU4 and of providing more details on its membrane anchoring mechanism. Analysis of the NEU4 primary structure rules out the possibility of it being a transmembrane protein since the only potential transmembrane domain is incompatible with the β-propeller sialidase structure; moreover, no membrane anchoring motifs, such as GPI anchors, palmitoylation, or myristoylation sites, are present on the NEU4 aminoacidic sequence, leaving the mechanism through which NEU4 binds to membranes still an open question. To this purpose we expressed both long (HsNEU4 long) and short (HsNEU4 short) forms of human NEU4 in COS-7 cells, both carrying a C-terminal c-myc tag. Solubilization experiments, carried out with Triton X-114 and sodium carbonate, showed that both NEU4 long and NEU4 short are extrinsic membrane proteins; cross-linking experiments also strongly suggested an association with the membrane through protein–protein interactions. Moreover, subcellular localization studies, performed through confocal microscopy and subcellular...
fractionation, showed that NEU4 long resides in the outer mitochondrial membrane, while the short form is bound to the endoplasmic reticulum.

**Results**

*Trigon X-114 phase separation shows that both NEU4 long and NEU4 short are hydrophilic proteins*

Transmembrane regions prediction methods, such as TMPred and TMHMM, suggest the existence of a potential transmembrane domain in the NEU4 primary structure. However, the presence of such a domain is not compatible with the typical sialidase β-propeller three-dimensional structure, yielded by homology modeling (Magesh et al. 2006). On the other hand, no membrane anchoring motifs, such as GPI anchors, palmitoylation, or myristoylation sites, are present on the NEU4 aminoacidic sequence. In order to gain insight into the mechanism through which NEU4 long and NEU4 short bind to the membranes, we undertook protein extraction with Trigon X-114 followed by temperature-induced phase separation, as previously performed for sialidase NEU3 (Zanchetti et al. 2007). This detergent allows protein solubilization with phase-separation of hydrophilic from amphiphilic membrane proteins. Crude extracts, obtained from COS-7 cells transiently expressing either HsNEU4 long or short form as fusion proteins carrying a C-terminal c-myc epitope, were initially treated with Trigon X-114 followed by SDS–PAGE and analyzed by Western blotting. Both NEU4 forms were detected using an anti-c-myc antibody. To check separation, antibodies directed against hydrophilic protein disulfide isomerase (PDI) and integral membrane protein Caveolin-1 (Cav-1) were used.

In order to assess whether NEU4 is a peripheral membrane protein, the membrane fractions obtained from COS-7 cells expressing either HsNEU4 long or HsNEU4 short were brought to pH 11.5 with sodium carbonate and incubated for 30 min on ice. A subsequent ultracentrifugation yielded a soluble fraction and a membrane fraction, which were brought to pH 7.5 and subjected to SDS–PAGE and analyzed by Western blotting. NEU4 was detected using an anti-c-myc antibody. To assess the accuracy of extraction, antibodies directed against peripheral membrane protein early endosomal antigen 1 (EEA1) and integral membrane protein Caveolin-1 (Cav-1) were used. Relative quantification of the NEU4 protein level in each fraction compared with input sample. Densitometric analysis was performed using NIH Image-based software Scion Image (Scion Corporation, Frederick, MD USA). Quantification data are representative of three independent experiments. Sialidase activity determined with 4MU-NeuAc under control condition. Values are means ± SD of three independent experiments.
addition, early endosome antigen 1 (EEA1) and Caveolin-1 (Cav-1) were used as controls for peripheral and intrinsic membrane proteins, respectively. As expected, both the long and the short form of HsNEU4 were found in the particulate fraction of untreated samples, as confirmed by sialidase activity assay using 4MU-NeuAc as a substrate (Figure 2C). Partial recovery of both NEU4 long and NEU4 short in the soluble fraction in control samples is due to minor protein release during collection and manipulation of membranes samples. Both long and short forms of NEU4 were partially solubilized by sodium carbonate treatment. A densitometric analysis of the bands showed that about 30% and 50% of HsNEU4 short and HsNEU4 long, respectively, were solubilized upon carbonate treatment (Figure 2B). As expected, after treatment with sodium carbonate, the peripheral membrane protein EEA1 was completely recovered in the soluble fraction, while Cav-1, an integral membrane protein, was not solubilized at all, thus demonstrating that carbonate treatment extracts peripheral proteins without affecting membrane integrity. After sodium carbonate treatment, no appreciable sialidase activity could be recorded in any fraction (data not shown), presumably due to alkaline denaturation or inactivation of the enzyme, as previously reported for sialidase NEU3 (Zanchetti et al. 2007). In addition, treatment of the membrane fraction prepared from COS-7 expressing either HsNEU4 long or HsNEU4 short with a high ionic strength buffer, containing 1.5 M NaCl, did not cause any NEU4 release (data not shown), suggesting that this sialidase is a peripheral protein strongly associated with membranes.

Reversible cross-linking shows that HsNEU4 long and HsNEU4 short are anchored to membranes through protein–protein interactions

Since our data showed that both forms of HsNEU4 are extrinsic membrane proteins, we performed cross-linking experiments in order to assess whether these sialidases might be anchored to the membrane via protein–protein interactions. The lack of any motif for membrane anchoring through prenylation, acylation, or GPI on NEU4 aminoacidic sequence suggested protein–protein interactions as the most likely membrane associating mechanism. Paraformaldehyde (PFA) is known to form covalent bonds between chemical groups not further apart than 2 Å, which can readily be reversed by heat treatment at 95°C. COS-7 cells transiently expressing either HsNEU4 long or HsNEU4 short were supplemented in the medium with PFA, as reported in Material and methods. Crude cell extracts were then subjected to SDS–PAGE and Western blotting in order to assess the presence of NEU4 complexes. Both HsNEU4 forms and their complexes were detected with an anti-c-myc antibody. Initially, COS-7 cells expressing either long or short form of HsNEU4 were treated for different times with various concentrations of PFA (data not shown). These preliminary tests showed that 20 min incubation with 0.25% (w/v) PFA was sufficient to obtain cross-linking of both HsNEU4 long and HsNEU4 short to adjacent membrane proteins; longer incubation times or higher PFA concentrations resulted in smeared electrophoretic bands, indicative of a specific cross-linking. As shown in Figure 3, after PFA treatment, NEU4 was detected only as a high Mr complex (higher than 250 kDa), which hardly entered into the running gel. Cross-linking reversion, obtained by incubation at 95°C for 20 min, led to the disappearance of the NEU4 complex and to the reappearance of bands corresponding to HsNEU4 long and HsNEU4 short molecular masses. This experiment confirmed that both forms of NEU4 interact with other proteins likely involved in their anchorage to membranes.

Confocal microscopy and subcellular fractionation show that the long form of HsNEU4 localizes in mitochondria, while the short form is bound to the endoplasmic reticulum

The existence of contradictory data in the literature (Monti et al. 2004; Seyrantepe et al. 2004; Yamaguchi et al. 2005) about NEU4 subcellular localization prompted us to further investigate this issue, both through confocal microscopy and subcellular fractionation studies. Colocalization experiments were carried out in COS-7 cells, transiently expressing either HsNEU4 long or HsNEU4 short as fusion proteins carrying a C-terminal c-myc epitope. Markers of different cellular compartments, such as cytochrome c (cyt c) for mitochondria, lysosome-associated membrane protein-1 (LAMP-1) for lysosomes, and Calnexin (Cnx) for endoplasmic reticulum, were also used. Twenty-four hours after transfection, cells were fixed, permeabilized, and analyzed by confocal microscopy. Results, reported in Figure 4, showed that HsNEU4 long colocalizes with the mitochondrial marker cyt c (Figure 4A, panel F), as reported by Yamaguchi et al. (2005), but not with the lysosomal marker LAMP-1 (Figure 4B, panel F), as claimed by Seyrantepe et al. (2004). Moreover, while colocalization of HsNEU4 short with either of these markers could not be observed (Figure 4A, panel C and B, panel C), superimposition with a Cnx-diffused fluorescent signal, shown in Figure 4C, panel C, suggested that HsNEU4 short can be bound to the endoplasmic reticulum. Colocalization of HsNEU4 long or HsNEU4 short with the subcellular markers was confirmed by Z-stack analyses, performed on confocal microscopy images (data not shown).

In order to check the possibility that the observed subcellular localization of both HsNEU4 forms might be an artifact due to protein transient over-expression, HsNEU4 subcellular localization was evaluated at different post-transfection times. No differences in subcellular localization of both HsNEU4 forms were observed when fixing and analyzing the cells 5, 10, 24, and 36 h after transfection (Supplementary data, Figure S1). HsNEU4 short appeared to be diffusely localized inside the cells at all times, its signal only slightly superimposing with cyt c. On the
Studies on human NEU4 isoforms subcellular localization

Fig. 4. Subcellular localization of HsNEU4 long and HsNEU4 short determined by indirect immunofluorescence staining. COS-7 cells were transiently transfected with HsNEU4 long or short and subjected to immunofluorescence staining and confocal microscopy analysis. (A) To analyze mitochondrial localization, COS-7 cells were double-stained with an anti-c-myc antibody, for detection of NEU4 short (panel A) or long (panel D), and an anti-cytochrome c (cyt c) antibody (panels B and E) as a mitochondrial marker. Overlay images are shown in panels C and F. Scale bars: 20 μm (panel A) and 25 μm (panel D). (B) To analyze lysosomal localization, COS-7 cells were double-stained with an anti-c-myc antibody, for detection of NEU4 short (panel A) or long (panel D), and an anti-LAMP-1 antibody (panels B and E) as a lysosomal marker. Overlay images are shown in panels C and F. Scale bars: 30 μm (panel A) and 15 μm (panel D). (C) COS-7 cells were double-stained with an anti-c-myc antibody for detection of NEU4 short (panel A) and an anti-Calnexin (Cnx) antibody (panel B) as an ER marker. Overlay image is shown in panel C. Scale bar: 25 μm.

Other hand, HsNEU4 long exhibited the already observed complete colocalization with the mitochondrial marker. Moreover, a total and partial mitochondrial colocalization of HsNEU4 long and HsNEU4 short, respectively, was also observed in HeLa cells, demonstrating that this subcellular distribution is not restricted to a particular cell type (Supplementary data, Figure S2).

Finally, to assess the difference in subcellular localization between the short and long form of HsNEU4, we performed cotransfection experiments with both c-myc-tagged HsNEU4 long and HA-tagged HsNEU4 short expressing vectors. Results are shown in Figure 5: HsNEU4 short showed a diffused intracellular label both in COS-7 and in HeLa cells, while a more localized distribution, closely mirroring the one observed in single transfection experiments, was found for HsNEU4 long in both cell types. The overlay confirms the results obtained by separate transfections, showing only a partial colocalization for HsNEU4 long and HsNEU4 short.

Results obtained by confocal microscopy were confirmed in subcellular fractionation experiments, shown in Figure 6, yielding a mitochondrial, a microsomal, and a cytosolic fraction. HsNEU4 long was found only in the mitochondrial fraction, while the short form was found also in the microsomal fraction, enriched in endoplasmic reticulum membranes. As expected, none of the two proteins was found in the cytosolic fraction. Detection of COX IV, Cnx, and 3-phosphoglycerate kinase (PGK) confirmed fractionation efficiency. In order to quantify the relative abundance of HsNEU4 long and HsNEU4 short...
in mitochondria, these organelles were purified from crude extracts transiently expressing each form of HsNEU4, as described in Material and methods. This procedure allowed us to obtain a highly purified fraction containing heavy mitochondria; only 3.5% of total lysosomes were found in this fraction, as confirmed by activity assay of the lysosomal α-mannosidase (Figure 7C). Western blot analysis performed with an anti-c-myc antibody showed that only HsNEU4 long is abundantly recovered in the mitochondrial fraction (Figure 7A). A densitometric analysis showed that 66% of HsNEU4 long is found in mitochondria, while only 13% of HsNEU4 short is recovered in this fraction (Figure 7B). The partial recovery of HsNEU4 long is accounted for by the fact that most of the small mitochondria are normally lost in the supernatant during the centrifugation. The inner mitochondrial membrane complex COX IV was used as a loading control for the mitochondrial fraction and to normalize densitometric data. The apparently high content of HsNEU4 short in the mitochondrial fraction shown in Figure 6 is due to the fact that the same quantities of total proteins of all subcellular fractions were loaded onto the gel. However, when the same volumes of total extract and purified mitochondria were evaluated for both the short and the long form, as done in the experiment reported in Figure 7, results clearly showed that most HsNEU4 long is found in mitochondria, while only a small fraction of HsNEU4 short localizes in these organelles. The different amount of mitochondria analyzed in these two experiments was also indicated by intensity of the COX IV band, which is higher in Figure 6 than that in Figure 7.

Submitochondrial fractionation shows that HsNEU4 long is bound to the outer mitochondrial membrane

To further analyze the submitochondrial localization of HsNEU4 long, heavy mitochondria from COS-7 cells expressing this sialidase were purified as described above and then lysed with 2% CHAPS, yielding a pellet containing the mitochondrial membranes and a supernatant representing the soluble fraction. These samples were subsequently subjected to SDS–PAGE, followed by Western blotting with an anti-c-myc antibody. Results, reported in Figure 8A, showed that the HsNEU4 long form is bound to mitochondrial membranes. The presence of VDAC1, a porin which is found inside the outer mitochondrial membrane, demonstrated that mitochondria are purified undamaged, carrying both outer and inner membranes. Moreover, sialidase activity performed on the same mitochondrial fractions showed that about 95% of the total enzyme activity is membrane associated (Figure 8B).

In order to assess in which of the two mitochondrial membranes the HsNEU4 long is located, isolated intact mitochondria were subjected to osmotic shock (OS) and treated with trypsin at different concentrations. This treatment partially removes the outer mitochondrial membrane, as previously demonstrated (Noma et al. 2001). Crude extracts obtained from whole mitochondria (−OS) and mitoplasts (+OS) were subjected to SDS–PAGE followed by Western blotting (Figure 8C and D). The peripheral outer membrane protein TOMM22, the peripheral inner membrane protein TIMM50, and the mitochondrial matrix enzyme SOD2 were used as controls. As expected for a peripheral outer membrane protein, Figure 8D shows that there is a decrease in intensity for the TOMM22 band already in the presence of 10 μg/mL trypsin in intact mitochondria (−OS) as well as in mitoplasts (+OS). Moreover, a fragment is also detectable in the case of TOMM22 as a result of a partial trypsin digestion. HsNEU4 long was proteolyzed in the same manner in intact (−OS) as well as in osmotically shocked (+OS) mitochondria, highlighting a behavior similar to TOMM22.

On the other hand, peripheral inner membrane protein TIMM50, due to its localization, appeared to be less susceptible to trypsin treatment in intact mitochondria, showing a decrease in band intensity only at 25 μg/mL trypsin. When trypsin treatment was performed on mitoplasts, TIMM50 became more accessible to the protease and was found to be already degraded at 10 μg/mL trypsin. Moreover, in agreement with its localization in the matrix, SOD2 was found to be unaffected by trypsin digestion in all conditions. Finally, as shown in Figure 8C, both TOMM22 and HsNEU4 long showed the same behavior in being partially released during osmotic shock and recovered in
Discussion

Although all human sialidases share a high primary structure similarity (ranging from 42 to 70%), some (the lysosomal sialidase NEU1 complex and the cytosolic sialidase NEU2) are soluble, while others (NEU4 and NEU3) are membrane associated. However, no apparent structural differences account for their different subcellular localizations and solubilities. No membrane binding motives, such as GPI anchors, mistroylation, or palmitoylation sites, are evident from NEU4 and NEU3 primary structure analysis. Moreover, a potential transmembrane domain, predicted in the NEU4 primary structure by TMPred and TMHMM servers, cannot fit into the β-propeller three-dimensional structure, yielded by homology modeling (Magesh et al. 2006), performed using the HsNEU2 crystallographic structure as a template (Chavas et al. 2005). Therefore, the mechanism through which sialidases HsNEU3 and HsNEU4 are anchored to the membrane is still unclear. Moreover, as regards human sialidase NEU4, different and contrasting subcellular localizations had been previously suggested for its two forms (Monti et al. 2004; Seyrantepe et al. 2004; Yamaguchi et al. 2005). Yamaguchi and colleagues demonstrated mitochondrial localization for NEU4 long and intracellular membrane localization for NEU4 short (Yamaguchi et al. 2005). On the other hand, Seyrantepe et al. (2004) claimed that NEU4 is a lysosomal enzyme and recently showed that mice deficient in NEU4 exhibit abnormal ganglioside catabolism and lysosomal storage (Seyrantepe et al. 2008).

We therefore undertook this work with the aim of elucidating membrane anchoring and subcellular localization of NEU4 long and NEU4 short and of gaining some insight into the function of this sialidase.

In the first part of this work, we showed that NEU4 is recovered in the aqueous phase after treatment with Triton X-114, clearly ruling out the possibility of it being an integral membrane protein. This result indirectly confirms that HsNEU4 folds into a β-propeller structure, which disrupts the only possible transmembrane domain. Moreover, NEU4 complete solubilization in the aqueous phase also rules out any possible interaction with membrane lipids, in accordance with primary structure analysis, suggesting that membrane proteins are involved in NEU4 anchoring. This is well in accordance with NEU4 partial solubilization obtained after alkaline treatment with sodium carbonate, that shows it to be, at least in part, an extrinsic membrane protein, as already demonstrated for HsNEU3 (Zanchetti et al. 2007). However its interaction with the membrane is apparently stronger than that of the EEA1 protein, which is readily and completely solubilized by carbonate treatment. Reversible cross-linking experiments with PFA strongly suggest that both forms of HsNEU4 are associated with the membrane through protein–protein interactions. All these experiments yielded the same results for both NEU4 long and NEU4 short, showing that both forms interact with the membrane through the same anchoring mechanism, despite their different subcellular localizations. This in turn shows that the first 12 aminoacids, which are lacking in NEU4 short, are not responsible for membrane anchoring, although they might play a role in cellular sorting.

The confocal microscopy studies on COS-7 cells transiently transfected with HsNEU4 long, reported in this work, clearly show that this enzyme colocalizes with mitochondria, as previously suggested by Yamaguchi et al. (2005). Instead, in contrast to what reported by Seyrantepe et al. (2004, 2008), no appreciable colocalization with lysosomes was observed in our experiments. Using the same experimental approach, we also showed that HsNEU4 short is found in intracellular membranes, but does not colocalize with lysosomes. A diffused intracellular fluorescence, well superimposing with Calnexin fluorescence strongly suggests that this enzyme is located in the endoplasmic reticulum, as previously suggested (Monti et al. 2004; Yamaguchi et al. 2005). The different subcellular localizations of the two forms are not restricted to a single cellular type, as shown by experiments performed on HeLa cells. Moreover, it not due to an over-expression artifact, as demonstrated by the fact that it does not change when both NEU4 forms are coexpressed, nor

![Fig. 8. Submitochondrial localization of the long form of HsNEU4.](https://academic.oup.com/glycob/article-abstract/20/2/148/1988064/153)
when each of them is expressed at lower levels, after shorter post-transfection times.

Our subcellular fractionation experiments confirmed confocal microscopy results: HsNEU4 long was found in the mitochondrial fraction, while HsNEU4 short was also detected in the microsomal fraction. None of these two sialidases was detected in the cytosolic fraction. In order to remove contaminating lysosomes as much as possible, Western blottings were performed also on isolated mitochondria. The employed fractionation procedure has been reported to yield a highly purified mitochondrial fraction and control enzyme activity assays showed the presence of only 3.5% contaminant lysosomes. Thus, although the HsNEU4 long presence in lysosomes cannot be completely ruled out, its predominant localization is beyond any doubt in mitochondria. Our data are therefore in contrast with those of Seyrantepe et al. (2004, 2008), who found a predominantly lysosomal localization of NEU4, although their activity assays performed after subfractionation of transfected COS-7 cells also showed a partial colocalization with mitochondrial and microsomal fractions. These authors also found (Seyrantepe et al. 2008) that transfection of cells from sialidosis patients with NEU4 expression vectors decreased lysosomal storage, yielding a 25% of transfected cells with normal lysosomes. Moreover they observed only 30% reduction of sialidase activity in lysosomes from NEU4 knock-out mice and a lysosomal storage phenotype only in lungs and spleen cells. Although these results may suggest a role for NEU4 in lysosomal degradation of gangliosides, the lack of NEU1 involvement in the process cannot be easily explained. In fact, NEU4 knock-out mice still possess a functional lysosomal NEU1 complex, actively degrading sialic acid containing compounds. Moreover, mutations of this enzyme only have been demonstrated beyond any reasonable doubt to cause sialidosis (de Geest et al. 2002).

Although our data suggest that HsNEU4 short is found primarily in the endoplasmic reticulum, a partial localization at the mitochondrial level is also apparent; the first 12 aminoacids seem likely to be involved in mitochondrial sorting, as already suggested (Yamaguchi et al. 2005), although a longer tract, approximately 30 aminoacids from the N-terminus, would be required for this localization, according to the prediction programs MITOPROT and signalP. On the whole, it can be said that the sequence required for correct mitochondrial localization is probably longer than 12 aminoacids, but the lack of the first 12 aminoacids is sufficient to impair translocation to mitochondria. When isolated mitochondria were subfractionated into a soluble fraction and a membranes containing pellet, HsNEU4 long was found only in the pellet, confirming its features of the membrane bound enzyme. In addition, the application of a further mitochondrial subfractionation provided evidence for HsNEU4 long localization in the outer mitochondrial membrane since this enzyme behaves exactly as the outer mitochondrial membrane protein TOMM22. When mitoplasts were prepared through osmotic shock removing the outer mitochondrial membrane, both proteins were partially solubilized. Moreover, the fact that they were degraded by trypsin to the same extent in whole mitochondria and in mitoplasts suggests that this sialidase is tightly anchored to the membrane and only partially accessible to trypsin. In contrast, a different behavior was observed in the case of the inner mitochondrial membrane protein TIMM50, which, unlike fully accessible TOMM22, was found to be susceptible to trypsin treatment only when the outer membrane was partially destroyed after osmotic shock. Our data are consistent with those obtained by Yamaguchi et al. (2005) in Percoll density gradient centrifugation, where about 70% of sialidase activity was recovered in the outer mitochondrial membrane fraction. Cross-linking experiments, performed in this work, will be the basis for a future purification and characterization of membrane proteins interacting with NEU4.

Another issue still to be elucidated is the role of NEU4 sialidase. It seems likely that this lowly expressed, selectively located sialidase must have a specific function, different from the main degradative role of the lysosomal sialidase NEU1. A role in signal transduction connected to apoptosis has been already proposed for mitochondrial NEU4 (Yamaguchi et al. 2005), as a modulator of GD3 levels. Moreover, Hasegawa et al. (2007) demonstrated that in SH-SY 5Y cell lines HsNEU4 long expression was decreased prior to catechol metabolite-induced apoptosis, that ganglioside GD3 was targeted to mitochondria during apoptosis, and that an inhibitor of glucosylceramide synthase was able to partially recover cell viability. The same research group found that NEU4 expression was markedly decreased in colon cancer, while it was subjected to an early upregulation during apoptosis (Yamanami et al. 2007). More recently, a paper has been published showing that mouse Neu4 plays an important regulatory role in neurite formation, likely through desialylation of glycoproteins (Shiozaki et al. 2009). All these data suggest a role in apoptosis for NEU4, through its action on GD3 ganglioside, as well as in neurite differentiation.

Still obscure is the role of the short form of this enzyme. An accurate characterization of the kinetic properties of both the short and the long form of the enzyme, when affecting gangliosidic and glycoproteic substrates, will be necessary to shed light on this point.

Material and methods

Antibodies

Mouse anti-c-myc mAb and rabbit anti-Caveolin-1 pAb, for Western blotting experiments, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-PDI mAb was from Assay Designs/Stressgen Bioreagents (Ann Arbor, MI) and mouse anti-EEA1 mAb from BD Biosciences (San Jose, CA). Rabbit anti-Calnexin, anti-VDAC1/porin, anti-COX IV and anti-Superoxide Dismutase 2 pAbs, goat anti-TIMM50 pAb, and mouse anti-TOMM22 [1C9-2] mAb were obtained from Abcam (Cambridge, UK). Mouse anti-PGK mAb was purchased from Molecular Probes (Invitrogen, Eugene, OR). Goat anti-mouse and rabbit anti-goat IgG HRP-conjugated antibodies were from Calbiochem (San Diego, CA). A goat anti-rabbit IgG HRP-conjugated antibody was from Bio- rad Laboratories (Hercules, CA). For immunofluorescence staining, mouse anti-HA mAb was from Santa Cruz Biotechnology, rabbit anti-c-myc pAb from Sigma (St. Louis, MO) and mouse anticytochrome c mAb from Promega Corporation (Madison, WI). Mouse anti-LAMP1 and anti-Calnexin mAbs were from BD Biosciences (San Jose, CA). Donkey anti-rabbit Cy3- and anti-mouse Cy2-conjugated antibodies were purchased from Jackson.
cDNA encoding the long form of HsNEU4 was amplified by PCR using oligonucleotide primers HNEU4-EcoRI-F (5'-cDNA encoding the long form of HsNEU4 was amplified by PCR using oligonucleotide primers HNEU4-EcoRI-F (5'-GG AATT CATGAGCTGACGCTCCC-3') and HNEU4-XbaI-R (5'-GTC TTAGGAGGGCGAGCACTCCC-3'), Pfu Turbo DNA-polymerase (Stratagene, La Jolla, CA) and pCDNA3x-long (Invitrogen Corporation, Carlsbad, CA) as a template. The resulting PCR product was subcloned into pCDNA3.1/myc- His expression vector (Invitrogen) to obtain HsNEU4 long fused in C-terminal with c-myc epitope and a polyhistidine tag, to be used for recognition purposes. HsNEU4 short cDNA was generated by PCR deletion of the sequence encoding the additional 12-amino-acid residues at N-terminal of NEU4 long form. Mutagenesis was performed using a Quick-Change Site-directed Mutagenesis Kit (Stratagene), according to the manufacturer’s guidelines; specific primers were used, together with pCDNA3.1/myc- His-HsNEU4 long or pCDNA3x(+)-HA-HsNEU4 long plasmid as a template, in order to obtain HsNEU4 short fused in C-terminal with c-myc epitope and a polyhistidine tag or in N-terminal with HA epitope, respectively. HsNEU4 long and HsNEU4 short sequences were confirmed by automated sequencing using vector and gene-specific primers (T7 and BGH for plasmid and NEU4-INT 5' - CGCCGCGCGCTCTGCTG-3' for NEU4 insert).

Cell cultures and transfection

COS-7 and HeLa cells were cultured using DMEM supplemented with 10% (v/v) FBS, 4 mM L-glutamine, 100 μg/mL penicillin, and 100 μg/mL streptomycin and maintained at 37°C in a humidified 5% CO2 incubator. Cells, cultured in 100 mm cell culture dishes (seeded at 6 × 10^5 cells/dish) or onto glass coverslips (seeded at 2.5 × 10^4 cells/coverslip), were transiently transfected with NEU4 expressing vectors in a serum-free medium (Fujiki et al. 1982). Briefly, 36 h after transfection, COS-7 cells were washed twice with cold PBS, harvested by scraping, and then collected by centrifugation at 800 g for 10 min at 4°C. Cells were suspended in ice-cold 10 mM Tris-HCl, pH 7.5, containing a proteases inhibitor cocktail (Roche) and sonicated at the minimum setting for 5 s. After centrifugation at 800 g for 10 min at 4°C, the supernatant (crude extract) was centrifuged at 100,000 g for 1 h at 4°C to collect total cell membranes. The pellet was resuspended in the lysis buffer and then split into identical aliquots. To obtain peripheral protein extraction, membrane samples were then treated with an equal volume either of ice-cold 0.2 M Na₂CO₃, pH 12.0, or 10 mM Tris-HCl, pH 7.5, 3 M NaCl or lysis buffer alone, as a control, and incubated for 30 min on ice. After centrifugation at 100,000 g, pellets were resuspended in the appropriate buffer to yield the membrane fractions, while the supernatants represented the soluble fractions. Samples containing sodium carbonate were quickly brought to pH 7.5 by the addition of acetic acid. Finally, soluble and membrane fractions were adjusted to the same final volume and then subjected to sialidase activity assay and Western blotting for detection of HsNEU4 and endogenous protein markers, Caveolin-1 (as an integral membrane protein) and PDI (as a soluble protein).

Cross-linking with paraformaldehyde

Paraformaldehyde cross-linking was performed in transfected cells as described (Vasilescu et al. 2004). Briefly, 24 h after transfection, COS-7 cells were treated with 0.25–1.0% (w/v) paraformaldehyde (PFA) in PBS for 5–60 min at 37°C. The cross-linking reaction was quenched with glycine to a final concentration of 125 mM, for 5 min at room temperature. Cells harvested by scraping were collected at 800 g for 10 min at 4°C, washed twice with PBS and resuspended in the lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 5 mM EDTA) containing the Roche proteases inhibitor cocktail. After an incubation of 30 min on ice, cell lysate was centrifuged at 18,000 g, to pellet cell debris. Prior to SDS-PAGE, cell extract was heated in Sample Buffer for 10 min at 65°C for complexes analysis or boiled for 20 min at 95°C to reverse the formaldehyde cross-links. Aliquots of PFA-treated cell extracts, before and after subjecting them to cross-link reversal condition, were

ImmuResearch Laboratories (West Grove, PA). Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin were obtained from Lonza (Basel, Switzerland). All other reagents were purchased from Sigma.

**Vectors**

**Sodium carbonate extraction**

Sodium carbonate extractions were performed as described (Fujiki et al. 1982). Briefly, 36 h after transfection, COS-7 cells were washed twice with cold PBS, harvested by scraping, and then collected by centrifugation at 800 g for 10 min at 4°C. Cells were suspended in ice-cold 10 mM Tris-HCl, pH 7.5, containing a proteases inhibitor cocktail (Roche) and sonicated at the minimum setting for 5 s. After centrifugation at 800 g for 10 min at 4°C, the supernatant (crude extract) was centrifuged at 100,000 g for 1 h at 4°C to collect total cell membranes. The pellet was resuspended in the lysis buffer and then split into identical aliquots. To obtain peripheral protein extraction, membrane samples were then treated with an equal volume either of ice-cold 0.2 M Na₂CO₃, pH 12.0, or 10 mM Tris-HCl, pH 7.5, 3 M NaCl or lysis buffer alone, as a control, and incubated for 30 min on ice. After centrifugation at 100,000 g, pellets were resuspended in the appropriate buffer to yield the membrane fractions, while the supernatants represented the soluble fractions. Samples containing sodium carbonate were quickly brought to pH 7.5 by the addition of acetic acid. Finally, soluble and membrane fractions were adjusted to the same final volume and then subjected to sialidase activity assay and Western blotting for detection of HsNEU4 and endogenous protein markers, Caveolin-1 (as an integral membrane protein) and PDI (as a soluble protein).

**Triton X-114 phase separation**

Membrane solubilization with Triton X-114 was performed as described (Bordier 1981). Briefly, 24 h after transfection, COS-7 cells were washed twice with cold PBS, harvested by scraping and collected at 800 g for 10 min at 4°C. Cells were lysed by 5 s probe sonication (Bandelin Sonoplus 2070 sonicator) in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, supplemented with proteases inhibitor cocktail (Roche), and then centrifuged at 800 g for 10 min at 4°C. The resulting crude extract was diluted in 100 μL of the above buffer to yield a final protein concentration of 1.0 mg/mL. Protein extraction was performed by an addition to the sample of a corresponding volume of 2% (v/v) precondensed Triton X-114 in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, followed by incubation for 1 h on ice. Detergent-extracted samples (200 μL) were then layered onto a cushion of 6% (w/v) sucrose, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.06% (v/v) Triton X-114 (300 μL), incubated for 3 min at 30°C, and centrifuged at 300 g for 3 min at room temperature. After centrifugation, the upper aqueous phase was removed and treated again with 1% (v/v) fresh Triton X-114. A second phase separation was then performed as above using the same sucrose cushion. Finally, the detergent and aqueous phases were adjusted to the same final volume with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl. Aliquots of the starting sample and separated phases were subjected to sialidase activity assay and analyzed by Western blotting for detection of HsNEU4 and endogenous protein markers, Caveolin-1 (as an integral membrane protein) and PDI (as a soluble protein).
analyzed by Western blotting with an anti-c-myc antibody for detection of HsNEU4.

Confocal immunofluorescence microscopy
For indirect immunofluorescence staining, COS-7 and HeLa cells were cultured onto glass-coverslips and transfected with NEU4 expressing vectors; 24 h after transfection, cells were briefly washed in PBS, fixed with 3% (w/v) paraformaldehyde (PFA) in PBS for 20 min at room temperature or in methanol for 5 min at −20°C. PFA reaction was quenched by treatment with 50 mM NH4Cl in PBS for 30 min. Fixed cells were washed three times with PBS and then permeabilized with 0.3% Saponin (w/v) in PBS (PBS-Sap) for 20 min and double-stained at room temperature with anti-c-myc and anti-cytochrome c, anti-LAMP1, or anti-Calnexin antibodies at appropriate dilutions in PBS-Sap for 1 h. After incubation, cells were washed three times in the same buffer and then double-stained with Cy2- and Cy3-conjugated secondary antibodies as above. After washes with PBS-Sap and PBS, coverslips were mounted using DakoCytomation Fluorescent Mounting Medium (DAKO Denmark A/S, Glostrup, Denmark). Slides were examined with a Leica Mod. TCS-SP2 (Leica Microsystems, Bannockburn, IL) confocal microscopy and images were processed with Leica Confocal software (LCS.EXE) and Adobe Photoshop software.

Subcellular fractionation
Thirty-six hours after transfection, COS-7 cells were washed twice with cold PBS and lysed on a plate using a fractionation buffer (20 mM HEPES, pH 7.4, 250 mM sucrose, 1.5 mM MgCl2, 1 mM EDTA, and 1 mM EGTA), containing a proteases inhibitor cocktail (Roche) and 1 mM DTT. Cell lysate was then passed through a 25 G needle 10 times, leaved for 15 min on ice. Mitochondria extraction and fractionation
Mitochondria were removed and pellets were resuspended in a buffer containing 50 mM NH4Cl in PBS for 30 min. Mitoplasts were recovered by centrifugation at 4,000 × g and then resuspended in 10 mM Heps/KOH, pH 7.4, 220 mM mannitol, 70 mM sucrose. As a control, whole mitochondria were incubated in an isotonic buffer (10 mM Heps/KOH, pH 7.4, 220 mM mannitol, 70 mM sucrose) for 30 min on ice and centrifuged as described above. Samples of mitochondria and mitoplasts were treated with trypsin at a final concentration of 0.10, and 25 μg/mL for 20 min on ice. Reactions were stopped by adding PMSF at a final concentration of 0.4 mg/mL and a proteases inhibitor cocktail (Roche). After incubation for 5 min on ice, the samples were centrifuged at 12,000 × g for 10 min at 4°C, and the pellets were analyzed by Western blotting for detection of HsNEU4 and mitochondrial markers, TOMM22 (outer membrane), TIMM50 (inner membrane), and SOD2 (matrix).

SDS–PAGE and Western blotting
Protein concentration of samples was determined by the Bradford assay (Bradford 1976) using Coomassie Plus – the Better Bradford™ Assay Kit (Pierce). Protein samples were separated by 12% SDS–PAGE and transferred to a PVDF Immobilon-P (Millipore, Billerica, MA) membrane. Membranes were blocked with 5% (w/v) dried milk in PBS for 30 min at room temperature and then incubated overnight at 4°C with appropriate dilutions of antibodies in 1% (w/v) dried milk in PBS (anti-c-myc, anti-EEA1, anti-Cav-1, and anti-PDI antibodies) or 5% (w/v) bovine serum albumin (BSA) in PBS (for anti-Cnx, anti-PGK, anti-VDAC1, anti-SOD2, anti-COX IV, anti-TOMM22, and anti-TIMM50 antibodies). After three washes (10 min each) with PBS containing 0.1% (v/v) Tween 20 (PBS-T), membranes were treated for 1 h at room temperature with an HRP-conjugated secondary antibody diluted in 1% (w/v) dried milk in PBS-T (for anti-mouse antibody) or 5% (w/v) dried milk in PBS (for antirabbit and anti-goat antibodies). After three washes in PBS-T, detection was performed using an ECL plus detection system (Millipore, Billerica, MA). Protein levels were quantified by densitometry of immunoblots using the NIH Image-based software Scion Image (Scion Corporation, Frederick, MD).

Sialidase activity assay
NEU4 enzymatic activity was determined with 4MU-NeuAc (Sigma) as a substrate (Monti et al. 2004). Briefly, reactions were set up in triplicate using 30 μg of total proteins in the 25 mM Na citrate/phosphate buffer, pH 3.2, 0.1 mM 4MU-NeuAc, 6 mg/mL BSA, in a final volume of 100 μL. After incubation for 30 min at 37°C, reactions were stopped by adding 1.5 mL of 0.2 M glycine/NaOH, pH 10.8. The amount of sialic acid
released was evaluated by spectrophotometric measurement of the 4-methylumbellifereone released.

Supplementary Data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Funding
Ministero dell’Istruzione, dell’Università e della Ricerca (MIUR) (grant 2006 to G.T., E.M., and P.F.) and Fondazione Cariplo (grant 2006 to E.M.).

Acknowledgements
We thank Dr. Gabriele Zanchetti (University of Brescia) for his technical support in the solubilization experiments, and Professor Roberto Bresciani (University of Brescia) and Dr. Anna Maria Villa (University of Milan-Bicocca) for guidance in the confocal microscopy experiments.

Conflict of interest statement
None declared.

Abbreviations
4MU-NeuAc, 4-methylumbelliferyl-N-acetyl-α-D-neuraminic acid; BSA, bovine serum albumin; Cav-1, Caveolin-1; CHAPS, 3-[3-Cholamidopropyl]dimethylammonio]-1-propanesulfonate; Cnx, Calnexin; COX IV, cytochrome c oxidase; cyt c, cytochrome c; DAKO, DakoCytomation Fluorescent Mounting Medium; DMEM, Dulbecco’s Modified Eagle’s Medium; DTT, dithiorethiol; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EEA1, early endosomal antigen 1; EGTA, ethylene glycol tetaacetic acid; FBS, fetal bovine serum; GPI, glycosylphosphatidylinositol; HRP, horseradish peroxidase; HsNEU1, homo sapiens sialidase NEU1; HsNEU2, homo sapiens sialidase NEU2; HsNEU3, homo sapiens sialidase NEU3; HsNEU4, homo sapiens sialidase NEU4; LAMP-1, lysosome-associated membrane protein 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate buffered saline; PBS-Sap, 0.3% saponin in PBS; PBS-T, PBS containing 0.1% Tween 20; PDI, protein disulfide-isomerase; PFA, paraformaldehyde; PGK, 3-phosphoglycerate kinase; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SOD2, superoxide dismutase 2; TIMM50, translocase of inner mitochondrial membrane, subunit 50; TOMM22, translocase of outer mitochondrial membrane, subunit 22; VDAC1/Perin, voltage-dependent anion channel.

References