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Identification of Lysosomal Sialidase NEU1 and Plasma Membrane Sialidase NEU3 in Human Erythrocytes

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ABSTRACT

The sialylation level of molecules, sialoglycoproteins and gangliosides, protruding from plasma membranes regulates multiple facets of erythrocyte function, from interaction with endothelium to cell lifespan. Our results demonstrate that: (a) Both sialidases NEU1 and NEU3 are present on erythrocyte plasma membrane; (b) NEU1 is kept on the plasma membrane in absence of the protective protein/cathepsin A (PPCA); (c) NEU1 and NEU3 are retained on the plasma membrane, as peripheral proteins, associated to the external leaflet and released by alkaline treatments; (d) NEU1 and NEU3 are segregated in Triton X-100 detergent-resistant membrane domains (DRMs); (e) NEU3 shows activity also at neutral pH; and (f) NEU1 and NEU3 are progressively lost during erythrocyte life. Interestingly, sialidase activity released from erythrocyte membranes after an alkaline treatment preserves its functionality and recognizes sialoglycoproteins and gangliosides. On the other hand, the weak anchorage of sialidases to the plasma membrane and their loss during erythrocyte life could be a tool to preserve the cellular sialic acid content in order to avoid the early ageing of erythrocyte and processes of cell aggregation in the capillaries. J. Cell. Biochem. 114: 204–211, 2013. © 2012 Wiley Periodicals, Inc.

KEY WORDS: SIALIDASE; ERYTHROCYTE; SIALOGLYCOCONJUGATES; PERIPHERAL PROTEINS

The remodelling of cell plasma membrane sialoglycoconjugates could deeply alter cell signalling. In particular, the plasma membrane represents a crucial site for the desialylation of key sialylated substrates, such as gangliosides and sialoglycoproteins, which participate in cell–cell and cell–extracellular matrix interactions, as well as in the regulation of receptors and, consequently, of intracellular signaling pathways [Hakomori, 1990]. The plasma membrane associated sialidase, NEU3, holds a strategic role in these events owing to its strict substrate specificity for gangliosides [Monti et al., 2002], and to its association also to Triton X-100 detergent-resistant membrane domains (DRMs), that host receptors and signal molecules [Wang et al., 2002].

Recently, it has been demonstrated that NEU3 is not the unique sialidase present on the plasma membrane. In fact, from the lysosomes where it is mainly restricted as a complex with β galactosidase and the protective protein/cathepsin A (PPCA), sialidase move to the plasma membrane in several cell types, that is, T lymphocytes [Nan et al., 2007], macrophages [Liang et al., 2006], and myofibroblasts [Hinek et al., 2006], accomplishing here important physiological tasks. In immune cells, this event is instrumental to engulf bacteria, to produce cytokines [Liang et al., 2006], and to maintain Toll-like receptors in a functional state [Amith et al., 2009]. Moreover, the surface-residing NEU1 has been shown to aid elastogenesis, particularly in the lungs and aorta and to down-regulate the mitogenic responses to platelet-derived growth factor (PDGF) and insulin-like growth factor-2 (IGF-2), through the desialylation of their correspondent receptors [Hinek et al., 2008]. Therefore, the evidence is emerging of a remodeling of plasma membrane sialoglycoconjugates promoted by NEU3 but also by NEU1.

Like in other cells, the control of membrane sialoglycoconjugate composition and the degree of sialylation is vital for erythrocytes.

Francesca D'Avila and Cristina Tringali contributed equally to this work.

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Gangliosides modulate Ca^{2+} ATPase activity [Duan et al., 2006], support the formation of exosomes [de Gassart et al., 2003], and together with sialoglycoproteins form a net of protruding charged molecules which regulate the interactions with the endothelium and other cells and prompt the formation of antigens which indelibly mark senescent erythrocytes [Bratosin et al., 1995]. The occurrence of sialidase activities in human erythrocyte membranes has been documented. Venerando et al. [1997] identified in these membranes two enzymatic activities, one showing an optimal pH in the acidic range (4.2–4.7) ("acidic sialidase"); the other one with an optimal neutral pH (7.2) ("neutral sialidase"). The origin of the human erythrocyte acidic and neutral sialidases, particularly their possible relationship to NEU1 and NEU3, are not known.

On these bases, we better investigated these aspects of human erythrocyte sialidase biology. The results obtained demonstrated that: (a) NEU1 and NEU3 are present on erythrocyte membrane; (b) NEU1 does not form the typical lysosome complex on the plasma membrane since PPCA is not present; (c) NEU1and NEU3 are retained on the plasma membrane, as a peripheral proteins, associated to the external leaflet; and (d) NEU1 and NEU3 are segregated in Triton X-100 detergent-resistant domains.

MATERIALS AND METHODS

MATERIAL

Gangliosides GD1a and GM3 were purified from bovine brain [Tettamanti et al., 1973]. Anti-NEU1 antibody was kindly provided by Dr. A. D'Azzo (St. Jude Children's Research Hospital, Memphis, TN); anti-β-actin (Sigma–Aldrich, St Louis, MO), anti-LAMP-1 (BD Biosciences, San Jose, CA); anti-NEU3, anti-cathepsin A, anti-flotillin 1 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were employed. The human chronic myeloid leukemic cell line K562 was purchased from ECACC (European Collection of Cell Culture, Sigma–Aldrich); human fibroblasts were kindly provided by Dr. M. Sampaolesi.

ISOLATION OF ERYTHROCYTES FROM HUMAN BLOOD SAMPLES AND AGE-BASED FRACTIONING

The study was performed in accordance with the principles contained in the Declaration of Helsinki as revised in 2001. Following informed consent, blood samples (6–8 ml) provided by Avis (Blood donors Italian volunteer association, Milan, Italy) were collected in heparinized tubes from healthy adult donors, and processed within 1 h from collection. Erythrocytes were isolated according to the method of Beutler et al. [1977].

Erythrocytes were fractioned into young, average-aged (two bands) and old erythrocytes by Percoll discontinuous density gradient, as described by Tringali et al. [2001].

White ghosts (WG) were prepared by hypotonic lysis, following the method of Steck and Kant [1974]. Resealed membranes were prepared according to Steck et al. [1970], as modified by Venerando et al. [1997].

MEMBRANE PROTEIN EXTRACTION TREATMENTS

To solubilize erythrocyte membrane proteins, four different treatments were used, according to previous experience [Fujiki et al., 1982]: (a) PBS (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl pH 7.4); (b) PBS containing 1 M NaCl; (c) PBS containing 1.2% Triton X-100; and (d) 0.1 M $NaHCO_3$ buffer pH 9.0. After the incubation time (5 min, at 4°C for $NaHCO_3$ treatment or 30 min, at 4°C for other treatments), the samples were centrifuged at 100,000*g*, for, at 4°C, on TL100 Optima Ultracentrifuge (Beckman Coulter, Fullerton, CA) in order to separate solubilized proteins (supernatant) from membrane associated proteins (pellet).

2D ELECTROPHORESIS

Protein samples (100 μg) from erythrocyte membranes were desalted using 2D Sample Prep for Soluble Proteins (Thermo Fisher Scientific, Rockford, IL). Desalted protein samples were diluted in a rehydration solution (7 M urea, 2 M thiourea, 2% (w/v) ASB14 (amidosulfobetaine detergent), 100 mM DTT, 0.2% ampholytes), and applied to 7 cm ReadyStrip pH 3–10 IPG (immobilized pH gradient) strips (Bio-Rad, Richmond, VA) by passive rehydration overnight. Isoelectric focusing was conducted in a Protean IEF System (Bio-Rad) at 20°C, following the manufacturer's protocol (Bio-Rad). Before the second-dimension separation, IPG strips were first incubated in the equilibration buffer I (0.05 M Tris/HCl pH 8.8, 6 M urea, 2% (w/v) SDS, 30% glycerol, 2% (w/v) DTT) for 15 min, at room temperature, and, then, in the equilibration buffer II (0.05 M Tris/HCl pH 8.8, 6 M urea, 2% (w/v) SDS, 20% glycerol, 2.5% (w/v) iodoacetamide) for 15 min, at room temperature. Finally, IPG strips were linked to a 12% SDS–PAGE gel through solution. SDS–PAGE electrophoresis and Western blot were carried out by standard procedure.

WESTERN BLOT ANALYSIS

Sixty microgram protein samples (from erythrocyte unsealed and resealed membranes, supernatant and pellets obtained after membrane protein extraction treatments) were separated on 12% SDS–PAGE and subsequently electrotransferred onto a PVDF membrane (Amersham Pharmacia Bioscience, Buckinghamshire, England). Membranes were blocked with 5% (w/v) dried milk in TBS (20 mM Tris/HCl pH 7.4, 0.15 M NaCl) for 1 h, at room temperature. After three washes (5 min each) with TBS-T (TBS containing 0.1% (v/v) Tween 20 (Sigma–Aldrich)), at room temperature, the membranes were incubated with primary antibodies diluted in TBS-T plus 5% dried milk, overnight at 4°C. The following antibodies were used: polyclonal anti-NEU1, kindly provided by Dr. A. D'Azzo (St. Jude Children's Research Hospital); monoclonal anti-NEU3 (MBL, Woburn, MA); monoclonal anti-β-actin (Sigma–Aldrich); polyclonal anti-cathepsin A; monoclonal anti-LAMP-1 (BD Biosciences); monoclonal anti-flotillin 1 (MBL, Woburn, MA). After three washes in TBS-T, membranes were treated for 1 h at room temperature with horseradish peroxidase conjugated IgG diluted in TBS-T plus 5% dried milk. Detection was carried out with SuperSignal West Dura kit (Thermo Fisher Scientific) and densitometric analysis was performed using the Quantity One software (Bio-Rad).

CELL SURFACE PROTEIN BIOTINYLATION

Membrane external leaflet-associated proteins of erythrocytes and resealed membranes (2.5 mg of proteins) were biotinylated and

separated from internal leaflet-associated proteins using the Cell Surface Protein Isolation Kit (Thermo Fisher Scientific) following the enclosed protocol.

SIALIDASE ACTIVITY ASSAY

Sialidase activity was assayed toward 4-methylumbellyferyl-*N*-acetylneuraminic acid (4-MU-NeuAc) (Sigma–Aldrich) according to Tringali et al. [2001]. Sialidase activity toward gangliosides GD1a, GM3, and GM1 and the sialoglycoproteins fetuin and transferrin was assayed resuspending 20–60 μg of sample proteins in a mixture containing 50 mM sodium-citrate/phosphate buffer at pH 4.7, 0.6 mM GM3 or 1.8 mM GD1a or 1.8 mM GM1 (with or without 0.04% Triton X-100), or 1.8 mg fetuin or transferrin. Mixtures were incubated at 37°C, for 1–3 h; the reaction was stopped at −20°C. Sialic acid released was determined according to Caimi et al. [1979].

Enzyme activity was expressed as units (U); 1 U representing the enzyme amount that liberates 1 μmol of product min/mg protein.

ISOLATION OF DETERGENT RESISTANT MEMBRANES

DMRs were isolated from erythrocytes following the method of Wickstrom et al. [2003]. Briefly, 5×10^6 of erythrocytes were incubated in 1 ml of lysis buffer (25 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, pH 7.4) supplemented with protease inhibitors, for 20 min, on ice. The cell lysate was mixed with two volumes of 60% OptiPrep and loaded at the bottom of the gradient. An OptiPrep gradient (40–30–5%) in lysis buffer was layered over the lysate and centrifuged at 170,000*g*, for 4 h, at 4°C. A first 1.2 ml fraction and seven following 1.5 ml fractions were collected from the top of the tube. DRMs were identified [Wickstrom et al., 2003] in fraction two from the top.

STATISTICAL ANALYSES

Values are presented as the mean ± SD. Statistical analyses were performed using the Student's *t*-test or ANOVA test.

RESULTS

NEU1AND NEU3 SIALIDASES ARE PRESENT ON HUMAN WG

As shown in Figure 1A and as expected [Venerando et al., 1997], the sialidase activity of WG assessed toward 4-MU-NeuAc, in the pH range 3.7–7.7, displayed two peaks of maximal activity: a main broad one at pH 4.7 (10.83 ± 1.3 μU/mg protein), and a second smaller one at pH 7.2 (2.66 ± 0.3 μU/mg protein), corresponding to the acidic and neutral sialidases, respectively. In order to clarify the genetic origin of these enzymatic activities, an immunoblotting analysis was performed, employing anti-NEU1 and anti-NEU3 antibodies. The presence of NEU2 and NEU4 sialidases was not investigated because, on the basis of our previous results obtained analyzing sialidase expression during erythroid cell differentiation toward erythrocytes, these enzymes are not synthesized [Tringali et al., 2007]. mRNA related to NEU2 was identified at the end of the erythroid differentiation process, but the corresponding protein was never detected in both eryhroid cells and mature erythrocytes [Tringali et al., 2007]. As shown in Figure 1B, NEU3 was detected on WG. It showed a pI of 6.7, assessed by 2D electrophoresis, very similar to the theoretical pI reported for the enzyme (data not shown). Surprisingly, also NEU1 appeared to be present on WG (Fig. 1B). The anti-NEU1 antibody reacted with a 46 kDa protein and, a more abundant, 44 kDa protein. As positive control, total extract from K562 cells, known to carry NEU1, was assayed in parallel with WG: in both cases the two bands of 44 and 46 kDa were present. To further confirm the presence of NEU1, WG were also analyzed by 2D electrophoresis. This further demonstrated that NEU1 was present on WG with an isoelectric point (pI) of 6.0 (Fig. 1C), identical to that of NEU1 present in K562 cells (data not shown).

It is known that in the lysosomes, sialidase NEU1is strictly associated with other proteins, in particular PPCA, which protects its activity [Potier et al., 1990]. Remarkably, on WG, PPCA was undetectable demonstrating the absence of the typical lysosomal complex (Fig. 1D). Instead, the lysosomal associated membrane protein-1 (LAMP-1) was revealed on WG (Fig. 1E). Noteworthy, erythrocyte LAMP-1 appeared to be present as two forms of 110 and 100 kDa, instead of a single protein of 110 kDa (as shown in the control cells, human fibroblasts), possibly because of a different glycosylation profile [Fukuda, 1991].

NEU1 AND NEU3 BOND TO ERYTHROCYTE MEMBRANE

WG were submitted to different membrane extraction treatments: PBS pH 7.4, PBS pH 7.4 containing 1 M NaCl, PBS pH 7.4 containing 1.2% Triton X-100, 0.1 M $NaHCO_3$ buffer pH 9. Protein content activity at pH 4.7 and pH 7.2 toward 4-MU-NeuAc were determined in each supernatant and pellet obtained after each treatment and compared to that of starting (Fig. 2A). After incubation in PBS pH 7.4, 8% of proteins were solubilized but both the acidic and neutral sialidase activities were entirely found in the pellet (Fig. 2B). A quite similar result was obtained by treating WG with PBS pH 7.4 containing 1 M NaCl, which extracted 11% of proteins (Fig. 2C). Notably, our previous results demonstrated that this procedure allowed the release of other glycohydrolases localized on erythrocyte plasma membrane (α-D-mannosidase and α-D-fucosidase) [Goi et al., 2000]. PBS pH 7.4 containing the non-ionic detergent Triton X-100 extracted 20% of proteins but once again failed to release acidic sialidase activity. Instead, neutral activity was partially extracted (about 17%), the specific activity of the solubilized enzyme being 3.2 ± 0.8 μU/mg protein (Fig. 2D). The treatment of WG with 0.1 M $NaHCO_3$ pH 9 solubilized 9% of proteins and caused a marked release of the acidic sialidase activity (specific activity 55.5 ± 1.1 μU/mg protein) (Fig. 2E). Also, 12% of the neutral sialidase activity was released by the same treatment (specific activity 4 ± 0.2 μU/mg protein) (Fig. 2E). Incubation time longer than 5 min was demonstrated to induce a partial loss of catalytic activity (from 15% to 75%). WG were also treated with alkaline buffers with pHs higher than 9:0.1 M $NaHCO_3$ pH 10 and 11 released the 19% and 40% of proteins, respectively, but led to a loss of specific activity of released sialidases (Fig. 3A,B). Alkaline buffers with pHs lower than 9 (0.1 M Tris/HCl at pH 8.5, 8, 7.4) were less efficient to release sialidase activities (Fig. 3A,B).

The enzymatic properties and substrate specificity of the $NaHCO_3$ solubilized sialidase activity was assayed toward gangliosides and sialoglycoproteins, at pH 4.7. The highest activity was exhibited on ganglioside GM3 (540 ± 3.5 μU/mg protein), followed by fetuin, 4-MU-NeuAc, and ganglioside GD1a (135 ± 2.9 μU/mg;

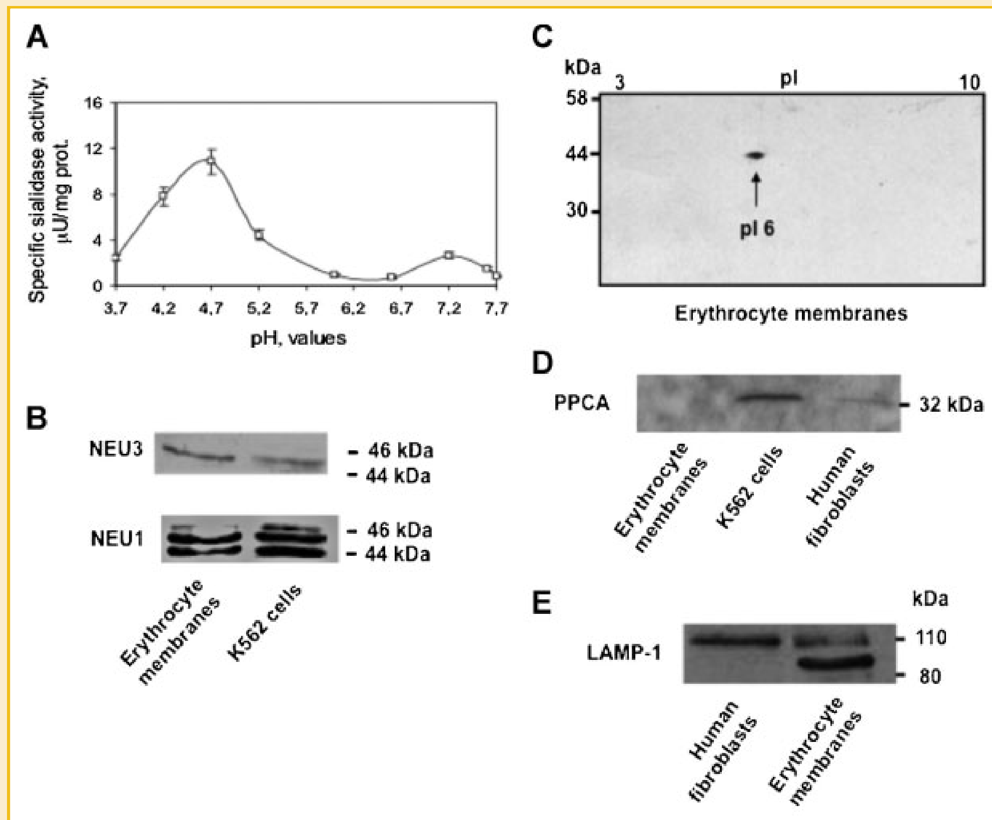

Fig. 1. Presence of sialidases NEU1 and NEU3 on WG. A: Sialidase activity curve over a pH range of 3.7–7.7, obtained incubating WG with 4-MU-NeuAc. The data are mean values of 6 experiments ± SD. B: Western blot of WG, total extracts from K562 cells (as positive control) stained with anti-NEU1 and anti-NEU3 antibodies. C: 2D electrophoresis and Western blot of WG stained with anti-NEU1 antibody. D: Western blot of WG, and total extracts from K562 cells and human fibroblasts (as positive control) using anti-PPCA antibody. E: Western blot of WG, and total extracts from human fibroblasts (as positive control), using antibody recognizing LAMP-1.

55.5 ± 1.1 μU/mg; 41 ± 2.3 μU/mg protein, respectively). No activity was recorded toward ganglioside GM1 and α2-6 sialoglycoproteins (transferrin), as expected [Monti et al., 2002]. Instead, the addition of Triton X-100 when using ganglioside substrates appeared to inhibit sialidase activity released by $NaHCO_3$ (Fig. 3C). Triton X-100 is known to increase the activity of NEU3 sialidase toward gangliosides [Papini et al., 2004]; therefore, on the basis of Triton X-100 inhibition and of the recorded activity toward sialoglycoproteins, it can be supposed that the enzymatic activity released by $NaHCO_3$ could be mainly related to NEU1. Moreover, to further corroborate this hypothesis, Zanchetti et al. [2007] previously demonstrated that NEU3 is inactive when it is cut off from the plasma membrane.

Immunoblotting analysis demonstrated that NEU1 was not solubilized by PBS pH 7.4, PBS pH 7.4 + 1 M NaCl, and PBS pH 7.4 + 1.2% Triton X-100 (Fig. 2B–D). Instead, Western blot proved the presence of NEU1 in the supernatant following treatment with $NaHCO_3$ (60% release of the enzyme) (Fig. 2E). Conversely, the presence of NEU3 was recorded in the supernatants recovered after treatments performed with PBS pH 7.4 + 1.2% Triton X-100 and $NaHCO_3$ (70% and 80% release, respectively). These results indicate that NEU1 and NEU3 behave as peripheral membrane-associated proteins. Moreover, neutral enzymatic activity seemed to be related to NEU3, the only enzyme that could be selectively released by Triton X-100.

The release of NEU1 and NEU3 from erythrocyte membrane could be an event occurring also in vivo. NEU1 and NEU3 appeared to be physiologically lost during erythrocyte aging as their content decreased from young to senescent erythrocytes (−53% and −60%, respectively) (Fig. 3D). In particular, the loss of both enzymes appeared to occur during the middle phase of erythrocyte life, as the decrease of both NEU1 and NEU3 is already evident in the average-age erythrocyte population.

NEU1 AND NEU3 ARE ASSOCIATED TO THE EXTERNAL LEAFLET OF THE ERYTHROCYTE PLASMA MEMBRANE

In order to investigate whether sialidases NEU1 and NEU3 are anchored to the external or inner leaflet of the erythrocyte membrane, cell surface protein biotinylation was performed employing erythrocytes, and resealed membrane vesicles. β-actin was employed to confirm that intracellular proteins were not biotinylated. Unfortunately, during the biotinylation step, a portion of treated erythrocytes broke leading to the partial biotin-labeling of β-actin. Anyhow, most of NEU1 and NEU3 were found among the biotinylated proteins. Nevertheless, in order to avoid experimental troubles due to erythrocyte fragility, the cell surface protein

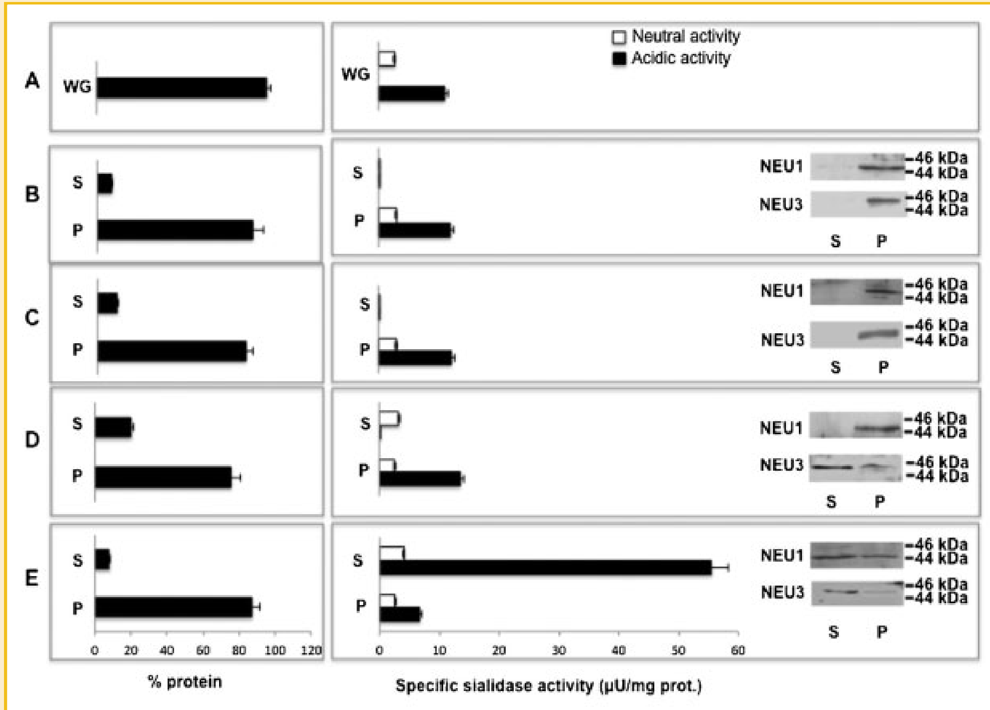

Fig. 2. Solubilization of sialidase activities from WG. Protein content, acidic and neutral sialidase specific activities toward 4-MU-NeuAc, and Western blot analysis of (A) WG and of the supernatant (S) and pellet (P) obtained after treatment of erythrocyte membranes with (B) PBS pH 7.4, (C) PBS containing 1 M NaCl pH 7.4, (D) PBS containing 1.2% Triton X-100, (E) 0.1 M $NaHCO_3$buffer pH 9. The data are mean values of seven experiments ± SD.

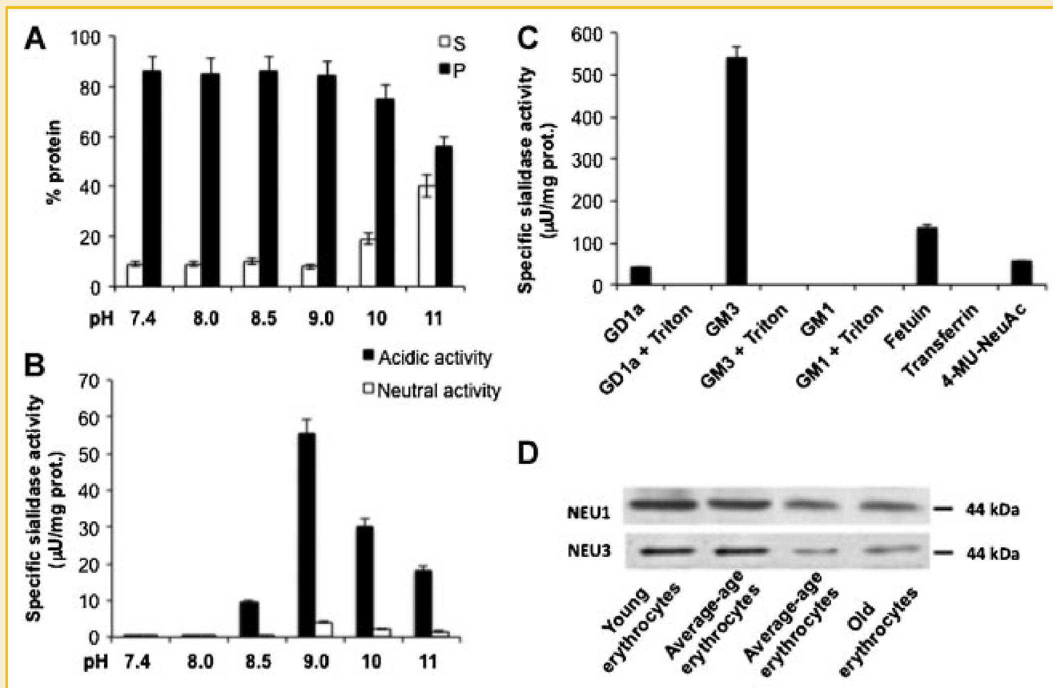

Fig. 3. Characterization of sialidase release induced by $NaHCO_3$buffer. A: Percentage of released proteins after alkaline buffer treatments. B: Acidic and neutral sialidase specific activities released by alkaline buffers. C: Substrate specificity of sialidase activity released with 0.1 M $NaHCO_3$ buffer pH 9, assayed at pH 4.7. D: Western blot analysis of NEU1and NEU3 content in white ghosts obtained from young, average-age, and old erythrocytes. Equal loading of total proteins was performed in each lane.

biotinylation was also performed using resealed membrane vesicles. These vesicles are known to maintain the outer/inner side position of the erythrocyte membrane. The analysis of the biotinylated and non-biotinylated proteins revealed that resealed ghosts did not break during the biotinylation procedure, as β-actin was mainly detected in the non-biotinylated protein fraction, and confirmed that NEU1 and NEU3 were biotynilated. Therefore, it can be concluded that both the enzymes are essentially associated to the membrane external leaflet (Fig. 4A).

NEU1 AND NEU3 LOCALIZE INTO DRMs ON THE HUMAN ERYTHROCYTE MEMBRANES

To verify whether sialidases NEU1 and NEU3 were associated to DRMs, an OptiPrep gradient was employed. Eight fractions were collected and analyzed for NEU1, NEU3, flotillin-1 (DRMs marker) content, and sialidase activity toward 4-MU-NeuAc. Both NEU1 and NEU3 largely co-fractionated with fraction 2, correspondent to DRMs, as confirmed by the presence of flotillin 1 (Fig. 4B). Moreover, two sialidase activities with pH optimum at 4.7 (13.6 ± 0.9 U/mg protein) and at pH 7.2 (14.6 ± 1.1 μU/mg protein) were detected in the same fraction. Instead, in the other fractions of the gradient we did not find the presence of NEU1 and NEU3, as protein or as catalytic activity.

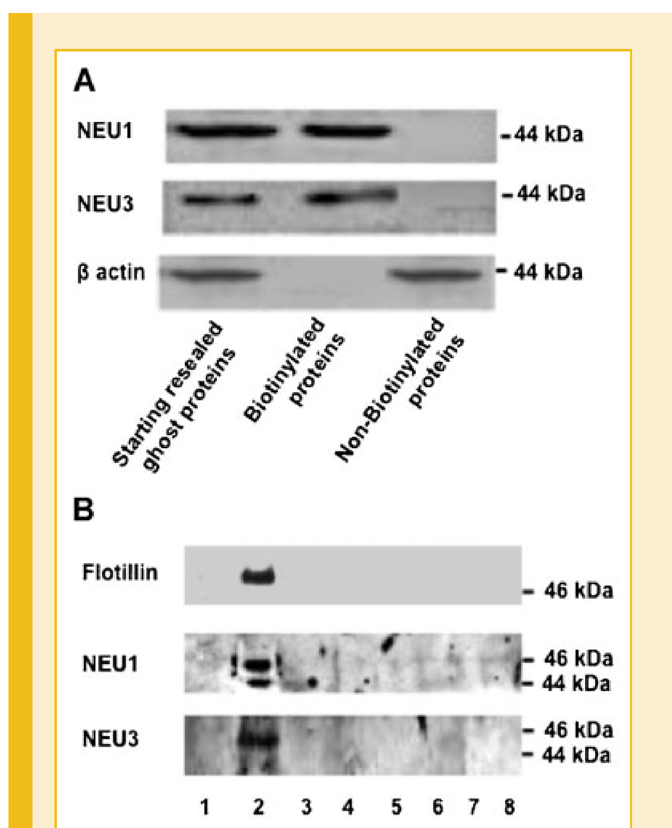

Fig. 4. Localization of NEU1 and NEU3 in erythrocyte membranes. A: Western blot analysis of biotinylated and non-biotinylated proteins isolated after biotinylation of resealed ghosts probed with anti-NEU1, anti-NEU3, and anti-β actin antibodies. β actin was employed as control for internal leaflet proteins. B: Western blot analysis of NEU1 and NEU3 content in fractions isolated through OptiPrep gradient. Flottilin 1 was employed as DRMs marker.

DISCUSSION

Sialidase considered for a long time a typical lysosomal enzyme, primarily involved in catabolic routes [Monti et al., 2002]. However, recent evidence widened this viewpoint documenting novel important roles of NEU1 in cell functionality. In some cell types, NEU1 is able to move from lysosomes to the plasma membrane during selected events, such as immune activation or differentiation in lymphocytes and macrophages [Liang et al., 2006], and elastogenesis in fibroblasts [Hinek et al., 2008]. The translocation of NEU1 enriches the plasma membrane of a second sialidase, in addition to NEU3, allowing the surface rapid remodeling of sialoglycoconjugates barely recognizable by NEU3 [Monti et al., 2002], such as sialoglycoproteins. TOLL-like receptor, CD44, integrins, PDGF, and IGF-1 receptors were recognized to be substrates of NEU1 in plasma membranes [Miyagi and Yamaguchi, 2012].

In this report, we demonstrated that NEU1 is present on the plasma membrane of human erythrocytes as well as the classical plasma membrane sialidase NEU3. In particular, while the neutral sialidase activity previously detected on human erythrocytes appeared to be entirely ascribable to NEU3, the acidic one could be related to both NEU3 and NEU1. NEU3 showed characteristics similar to that previously recorded in other cell types: it is mainly an external peripheral protein, as demonstrated by its release with $NaHCO_3$ buffer, even if it could be supposed that it is partially inserted as an integral protein in the leaflets because of its partial release with Triton X-100. Moreover, NEU3 segregated in DRMs.

In erythrocytes, we demonstrated that also NEU1 is localized in DMRs and protrudes toward the extracellular environment. It is likely that also in other cells the translocation of NEU1 to the plasma membrane could involve DMRs, because of its activity on receptors localized in these areas [Miyagi and Yamaguchi, 2012]. Noteworthy, we did not identify PPCA in erythrocytes; therefore the constitution of the typical protein complex which protects NEU1 in the lysosome [Potier et al., 1990] does not seem to be necessary to preserve the functionality of the sialidase on erythrocyte surface. It has been hypothesized that glycosylation of NEU1 could compensate PPCA deficiency [Miyagi and Yamaguchi, 2012]; actually, the presence of two forms of NEU1 in erythrocyte strongly suggests that they could differ in glycosylation. Moreover, NEU1 appeared to be linked to erythrocyte membrane through ionic and/or hydrogen interactions with phospholipids or integral membrane proteins, through charged amino acids, particularly arginine [Bonten et al., 2009], exposed on NEU1surface. These links could be cracked by mild alkaline conditions (pH 9), with no damage to the membrane organization. NEU1recovered in $NaHCO_3$ soluble fraction appeared to preserve its catalytic activity and efficiently recognized α2-3 sialoglycoproteins with an optimal pH at 4.7, instead of NEU3 that possibly lost its catalytic activity, as observed previously [Zanchetti et al., 2007]. After all, it is likely that the attachment of NEU1 to the erythrocyte membrane may undergo breaking during erythrocyte life span.

Notably, we detected a minor content of NEU1 and, also of NEU3, in average-age and old erythrocytes, in comparison to young erythrocytes; because of its capability to preserve catalytic activity, released NEU1 could correspond to the sialidase activity identifiable in the serum [Schauer et al., 1976].

The mechanisms responsible for the transfer ofNEU1 from lysosomes to the plasma membrane seemingly occur during the phases of erythroblast life that prelude the ejection of intracellular organelles. The presence of LAMP-1 on the erythrocyte plasma membrane may be the consequence and the proof of lysosomal exocytosis taking place during the process of lysosome degradation in erythroblasts [Yogalingam et al., 2008]. Therefore, it can be conceived that pieces of lysosomal membranes can be integrated into erythrocyte plasma membrane [Huynh et al., 2004] and enrich it of their enzyme and protein content. Indeed, other glycohydrolases of lysosomal origin were previously identified on erythrocytes plasma membrane, that is, β hexosaminidase [Massaccesi et al., 2007]. Of course, this "rescue" pathway likely involves only selected lysosomal proteins; in fact, we failed to find the presence on the erythrocyte plasma membrane of another protein abundant in lysosomes, LAMP-2. Recruitment of NEU1 on the plasma membrane may enable erythrocytesto actively participate in membrane adhesion and cell-to-cell interaction phenomena through the modulation of membrane sialoglycoconjugates, especially glycoproteins [Hakomori, 1990; Kato et al., 2006]. As the external microenvironment, its action could not be confined to the cells to which it belongs but also to sialoglycoconjugates, including receptors and integrins, exposed by other cells or molecules that come in contact with erythrocytes. Notably, vascular endothelia are highly sialylated and their treatment with exogenous sialidase increased leukocyte adhesion and migration across the endothelium [Cross et al., 2012].

In addition, our data demonstrated that NEU1 and NEU3 were progressively lost during erythrocyte life. This event could be essential to avoid aggregation of senescent erythrocytes.

Erythrocyte aggregation is one of the main determinants influencing blood circulation and is associated with cardiovascular risk factors such as hypertension, hyperlipoproteinemia, and with clinical situations including myocardial ischemia and thromboembolic states [Hadengue et al., 1998]. It was demonstrated that membrane sialic acid content hinders erythrocyte aggregation, conferring a negative charge of cell surface that causes the repulsion of erythrocytes [Hadengue et al., 1998]. During ageing, erythrocytes lost about 25% of sialic acid [Tringali et al., 2007], and, accordingly, senescent erythrocytes showed an increased dextran-induced aggregation [Hadengue et al., 1998]. Pathological conditions associated with an early decrease in erythrocyte sialic acid content, such as hypercholesterolemia, show also an increased aggregation of young and middle-aged erythrocytes [Hadengue et al., 1998]. Therefore, it could be hypothesized that the release of both sialidases during the life of the erythrocyte could be a valid mechanism to prevent an early decrease in erythrocyte sialic acid content that may influences the rheological properties of blood by increasing the adhesive energy of erythrocyte aggregate. On the other hand, the loss of biosynthetic pathways in erythrocyte makes the formation of new sialoglyconjugates impossible; therefore, the release of sialidases appears to be the unique way to avoid an early erythrocyte ageing.

In this perspective, the presence of NEU1 and NEU3 on erythrocyte plasma membrane seems to be involved to determine interaction and aggregation processes between erythrocytes and between erythrocytes and other blood or endothelial cells, with important implications in several pathological conditions caused by altered adhesiveness.

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REFERENCES

Amith SR, Jayanth P, Franchuk S, Siddiqui S, Seyrantepe V, Gee K, Basta S, Beyaert R, Pshezhetsky AV, Szewczuk MR. 2009. Dependence of pathogen molecule-induced Toll-like receptor activation and cell function on Neu1 sialidase. Glycoconj J 26:1197–1212.

Beutler E, Blume KG, Kaplan JC, Lohr GW, Ramot B, Valentine WN. 1977. International committee for standardization in haematology: Recommended methods for red-cell enzyme analysis. Br J Haematol 35:331–340.

Bonten EJ, Campos Y, Zaitsev V, Nourse A, Waddell B, Lewis W, Taylor G, d'Azzo A. 2009. Heterodimerization of the sialidase NEU1 with the chaperone PPCA prevents its premature oligomerization. J Biol Chem 284:28430–28441.

Bratosin D, Mazurier J, Debray H, Lecocq M, Boilly B, Alonso C, Moisei M, Motas C, Montreuil J. 1995. Flow cytofluorimetric analysis of young and senescent human erythrocytes probed with lectins. Evidence that sialic acids control their life span. Glycoconj J 12:258–267.

Caimi L, Lombardo A, Preti A, Wiesmann U, Tettamanti G. 1979. Optimal conditions for the assay of fibroblast neuraminidase with different natural substrates. Biochim Biophys Acta 571:137–146.

Cross AS, Hyun SW, Miranda-Ribera A, Feng C, Liu A, Nguyen C, Zhang L, Luzina IG, Atamas SP, Twaddell WS, Guang W, Lillehoj EP, Puche AC, Huang W, Wang LX, Passaniti A, Goldblum SE. 2012. NEU1 and NEU3 sialidase activity expressed in human lung microvascular endothelia. NEU1 restrains endothelial cell migration whereas NEU3 does not. J Biol Chem 287:15966–15980.

de Gassart A, Geminard C, Fevrier B, Raposo G, Vidal M. 2003. Lipid raft-associated protein sorting in exosomes. Blood 102:4336–4344.

Duan J, Zhang J, Zhao Y, Yang F, Zhang X. 2006. Ganglioside GM2 modulates the erythrocyte Ca2+-ATPase through its binding to the calmodulin-binding domain and its "receptor". Arch Biochem Biophys 454:155–159.

Fujiki Y, Hubbard AL, Fowler S, Lazarow PB. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. J Cell Biol 93:97–102.

Fukuda M. 1991. Lysosomal membrane glycoproteins: Structure, biosynthesis, and intracellular trafficking. J Biol Chem 266:21327–21330.

Goi G, Bairati C, Massaccesi L, Lovagnini A, Lombardo A, Tettamanti G. 2000. Membrane anchoring and surface distribution of glycohydrolases of human erythrocyte membranes. FEBS Lett 473:89–94.

Hadengue AL, Del-Pino M, Simon A, Levenson J. 1998. Erythrocyte disaggregation shear stress, sialic acid, and cell aging in humans. Hypertension 32:324–330.

Hakomori S. 1990. Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions. J Biol Chem 265:18713–18716.

Hinek A, Pshezhetsky AV, von Itzstein M, Starcher B. 2006. Lysosomal sialidase (neuraminidase-1) is targeted to the cell surface in a multiprotein complex that facilitates elastic fiber assembly. J Biol Chem 281:3698–3710.

Hinek A, Bodnaruk TD, Bunda S, Wang Y, Liu K. 2008. Neuraminidase-1, a subunit of the cell surface elastin receptor, desialylates and functionally inactivates adjacent receptors interacting with the mitogenic growth factors PDGF-BB and IGF-2. Am J Pathol 173:1042–1056.

Huynh C, Roth D, Ward DM, Kaplan J, Andrews NW. 2004. Defective lysosomal exocytosis and plasma membrane repair in Chediak-Higashi/beige cells. Proc Natl Acad Sci USA 101:16795–16800.

Kato K, Shiga K, Yamaguchi K, Hata K, Kobayashi T, Miyazaki K, Saijo S, Miyagi T. 2006. Plasma-membrane-associated sialidase (NEU3) differentially regulates integrin-mediated cell proliferation through laminin- and fibronectin-derived signalling. Biochem J 394:647–656.

Liang F, Seyrantepe V, Landry K, Ahmad R, Ahmad A, Stamatos NM, Pshezhetsky AV. 2006. Monocyte differentiation up-regulates the expression of the lysosomal sialidase, Neu1, and triggers its targeting to the plasma membrane via major histocompatibility complex class II-positive compartments. J Biol Chem 281:27526–27538.

Massaccesi L, Lombardo A, Venerando B, Tettamanti G, Goi G. 2007. Isoenzyme pattern and partial characterization of hexosaminidases in the membrane and cytosol of human erythrocytes. Clin Biochem 40:467–477.

Miyagi T, Yamaguchi K. 2012. Mammalian sialidases: Physiological and pathological roles in cellular functions. Glycobiology 22:880–896.

Monti E, Preti A, Venerando B, Borsani G. 2002. Recent development in mammalian sialidase molecular biology. Neurochem Res 27:649–663.

Nan X, Carubelli I, Stamatos NM. 2007. Sialidase expression in activated human T lymphocytes influences production of IFN-gamma. J Leukoc Biol 81:284–296.

Papini N, Anastasia L, Tringali C, Croci G, Bresciani R, Yamaguchi K, Miyagi T, Preti A, Prinetti A, Prioni S, Sonnino S, Tettamanti G, Venerando B, Monti E. 2004. The plasma membrane-associated sialidase MmNEU3 modifies the ganglioside pattern of adjacent cells supporting its involvement in cell-to-cell interactions. J Biol Chem 279:16989–16995.

Potier M, Michaud L, Tranchemontagne J, Thauvette L. 1990. Structure of the lysosomal neuraminidase-beta-galactosidase-carboxypeptidase multienzymic complex. Biochem J 267:197–202.

Schauer R, Veh RW, Wember M. 1976. Demonstration of neuraminidase activity in human blood serum and human milk using a modified, radioactively labelled alpha1-glycoprotein as substrate. Hoppe Seylers Z Physiol Chem 357:559–566.

Steck TL, Kant JA. 1974. Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes. Methods Enzymol 31:172–180.

Steck TL, Weinstein RS, Straus JH, Wallach DF. 1970. Inside-out red cell membrane vesicles: Preparation and purification. Science 168:255–257.

Tettamanti G, Bonali F, Marchesini S, Zambotti V. 1973. A new procedure for the extraction, purification and fractionation of brain gangliosides. Biochim Biophys Acta 296:160–170.

Tringali C, Fiorilli A, Venerando B, Tettamanti G. 2001. Different behavior of ghost-linked acidic and neutral sialidases during human erythrocyte ageing. Glycoconj J 18:407–418.

Tringali C, Anastasia L, Papini N, Bianchi A, Ronzoni L, Cappellini MD, Monti E, Tettamanti G, Venerando B. 2007. Modification of sialidase levels and sialoglycoconjugate pattern during erythroid and erytroleukemic cell differentiation. Glycoconj J 24:67–79.

Venerando B, Fiorilli A, Croci GL, Tettamanti G. 1997. Presence in human erythrocyte membranes of a novel form of sialidase acting optimally at neutral pH. Blood 90:2047–2056.

Wang Y, Yamaguchi K, Wada T, Hata K, Zhao X, Fujimoto T, Miyagi T. 2002. A close association of the ganglioside-specific sialidase Neu3 with caveolin in membrane microdomains. J Biol Chem 277:26252–26259.

Wickstrom SA, Alitalo K, Keski-Oja J. 2003. Endostatin associates with lipid rafts and induces reorganization of the actin cytoskeleton via down-regulation of RhoA activity. J Biol Chem 278:37895–37901.

Yogalingam G, Bonten EJ, van de Vlekkert D, Hu H, Moshiach S, Connell SA, d'Azzo A. 2008. Neuraminidase 1 is a negative regulator of lysosomal exocytosis. Dev Cell 15:74–86.

Zanchetti G, Colombi P, Manzoni M, Anastasia L, Caimi L, Borsani G, Venerando B, Tettamanti G, Preti A, Monti E, Bresciani R. 2007. Sialidase NEU3 is a peripheral membrane protein localized on the cell surface and in endosomal structures. Biochem J 408:211–219.