Glycoconjugate Journal (1993) **10**:64–71

Human erythrocyte sialidase is linked to the plasma membrane by a glycosylphosphatidylinositol anchor and partly located on the outer surface

ANNA CHIARINI, AMELIA FIORILLI, LUCIA DI FRANCESCO, BRUNO VENERANDO and GUIDO TETTAMANTI*

Department of Medical Chemistry and Biochemistry, The Medical School, University of Milan, Via Saldini 50, 20133 Milan, Italy

Received 8 September 1992

Treatment of human erythrocyte ghosts with phosphatidylinositol-phospholipase C (PIPLC) from *Bacillus cereus* liberated the ghost-linked sialidase. Maximal release of sialidase (about 70% of total) was achieved by incubating ghosts at 37 °C for 60 min, at pH 6.0, with PIPLC (PIPLC total units/ghost protein ratio, 4.5 each time) added at the beginning of incubation and every 15 min (four subsequent additions). Liberated sialidase was fully resistant to at least four cycles of rapid freezing and thawing and to storage at 4 °C for at least 48 h. The liberated enzyme had an optimal activity at pH 4.2, degraded ganglioside GD1a better than methylumbelliferyl *N*-acetylneuraminic acid (about fourfold), and gave a K_m value of 2.56×10^{-4} M and an apparent V_{max} of 2.22 mU per mg protein on GD1a. Treatment of intact erythrocytes with PIPLC (PIPLC total units/erythrocyte protein ratio, 8), under conditions where haemolysis was practically negligible, caused liberation of 10–12% of membrane linked sialidase, indicating that the enzyme is, at least in part, located on the outer surface of the erythrocyte membrane. It is concluded that the erythrocyte membrane sialidase is anchored by a glycosylphosphatidylinositol structure sensitive to PIPLC action, and is partly located on the outer surface.

Keywords: erythrocyte, sialidase, glycosylphosphatidylinositol anchor, exoenzymes

Abbreviations: PLC, phospholipase C; PIPLC, phospholipase C acting selectively on phosphatidylinositol; NeuAc, *N*-acetylneuraminic acid; MU, 4-methylumbelliferone; PBS, Dulbecco's phosphate buffer saline solution. Gangliosides were coded according to Svennerholm [42] and the IUPAC-IUB recommendations [43].

The outer surface of the erythrocyte membrane carries large amounts of sialic acid, linked mainly to glycoproteins and partly to glycolipids (gangliosides). The presence of sialic acid seems to play a role in the maintenance of erythrocyte viability and survival in circulating blood [1–3]. In fact, removal of sialic acid by treatment with exogenous sialidases facilitates erythrocyte phagocytosis [4, 5], suggesting that cellular ageing may also depend on the cell surface sialic acid content [6, 7].

Animal cells contain possibly more than a single sialidase, the enzyme that splits off sialic acid from sialoglycoconjugates [8]. These enzymes are generally present at different subcellular levels, the plasma membrane, the lysosome and the cytosol [9–13]. Contrary to other cells, erythrocytes from different animals appear to possess only the plasma membrane bound sialidase [14–16]. This enzyme was partially purified, characterized, and shown to be able to affect different sialoglycoconjugates, including gangliosides [15, 16]. Particularly, the sialidase purified from human erythrocytes is able to release sialic acid from intact erythrocytes, decreasing their electrophoretic mobility [15].

Important questions regarding the erythrocyte sialidase remain to be answered. A primary question is whether the enzyme is located in the external surface, or in the inner surface or imbedded within the plasma membrane. Another question is which way the enzyme is linked to the membrane. A third is whether the enzyme is capable of affecting the endogenous sialoglycoconjugates that are present in the same membrane.

The present work contributes to solving some of the above problems. We provide evidence that the sialidase of human erythrocytes is linked to the membrane by a glycosylphosphatidylinositol anchor, and is located, at least in part, on the external surface of the membrane.

* To whom correspondence should be addressed.

Materials and methods

Materials

Commercial chemicals were of the highest grade available. Phospholipase C (PLC) from *Bacillus cereus* (grade II, 800 U mg^{-1}), a source of PLC reported [17] to hydrolyse the substrate phosphatidylinositol more selectively than phosphatidylcholine and phosphatidylserine (PIPLC), from Boehringer Mannheim (Germany); 4-methylumbelliferyl α-*N*-acetyl-D-neuraminic acid (MU-NeuAc) and 4-methylumbelliferone (MU), from Koch-Light (Colbrook, UK); precoated silica gel thin-layer plates (HPTLC, Kiesel gel 60; 250 μm thickness; 10 cm × 20 cm), and 2,3-dichloro-5,6-dicyanobenzoquinone from Merck GmbH (Darmstadt, Germany); *Vibrio cholerae* sialidase from Behringwerke (Marburg, Germany); NeuAc, crystalline bovine serum albumin, human haemoglobin, Triton X-100 and sodium deoxycholate from Sigma Chemical Co. (St. Louis, MO, USA); sodium [^{3}H]borohydride (6,500 Ci mol^{-1}) from Amersham International (Amersham, UK). Water was doubly distilled in a glass apparatus and used to prepare the different solutions.

Preparation of gangliosides and ganglioside micelles

Gangliosides GM1 and GD1a were prepared from calf brain [18] and structurally analysed as described by Sonnino *et al.* [19]. Both were ^{3}H-labelled at C-3 of the sphingosine by the 2,3-dichloro-5,6-dicyanobenzoquinone/sodium [^{3}H]-borohydride method of Ghidoni *et al.* [20] and the *erythro* forms, separated and purified [21], were employed. The radiochemical purity of [^{3}H]GM1 and [^{3}H]GD1a was better than 99%, and the specific radioactivities were 1.25 and 0.96 Ci $mmol^{-1}$, respectively. ^{3}H-Labelled gangliosides were stored at −20 °C in n-propanol–water (4:1, by vol). Micellar dispersions of gangliosides, used as substrates for sialidase, were obtained as described [13].

Preparation of erythrocytes and their ghosts

After informed consent was obtained, venous blood was drawn from healthy adult donors, anticoagulated with heparin, and used immediately. Erythrocytes were separated from plasma by low speed centrifugation (1000 × **g**, 4 min), and washed several times with PBS. During preparation, attention was paid to the careful removal by aspiration of the buffy coat from the surface of the pellet [16]. Ghost membranes were prepared [16] by rapidly and thoroughly mixing 1 ml of packed cells with 20 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, and stirring for 10 min. The ghosts were pelleted by centrifugation at 10000 × **g** for 15 min; after removal of the supernatant, the tubes were tipped and rotated on their axis so that the loosely precipitated ghosts slid away from a small compact button containing intact erythrocytes and contaminating leukocytes. The washing procedure was repeated three times. All work was conducted at 0–4 °C.

Release of sialidase from ghosts by treatment with PIPLC

Treatment of ghosts with PIPLC was carried out in two different ways. In the first method the ghosts specimen (5.5 mg of protein) was homogenized with a loose Teflon–glass homogenizer (2 min, 200 rev min^{-1}) with 1.6 ml of 50 mM Tris-sodium maleate buffer containing 5 mM EDTA, reported to inhibit selectively the enzyme activity on glycerophospholipids not containing inositol [22] and having a given pH (5.2–7.4). PIPLC (8–100 U) was then added and the assay mixture, brought to 2.4 ml with the above buffer, was incubated at 37 °C for an established period of time (5–40 min). At the end of the incubation, the mixture was centrifuged at 150000 × **g** for 10 min. The pellet was resuspended in the same buffer, with an additional aliquot of PIPLC (equal to that used in the previous treatment), and incubated at 37 °C as previously (5–40 min). The resulting pellet was treated three further times under the same conditions. Each of the supernatants obtained, as well as the starting material and the final pellet, were assayed for protein, sialidase and acetylcholinesterase, used as a reference enzyme known to be liberated from erythrocyte ghosts by treatment with PIPLC [23, 24]. In the second method, the first incubation with PIPLC was carried out as described above. At the end of the established period of incubation an additional aliquot of PIPLC (equal to that used in the first treatment) was added to the same incubation mixture, and incubation allowed to continue for the same time employed in the first treatment. The addition of PIPLC and prolongation of incubation were repeated twice again. At the end of incubation the mixture was centrifuged at 150000 × **g** for 10 min, and the supernatant and final pellet analysed. In both types of experiment a blank incubation without PIPLC was run concomitantly.

Treatment of intact erythrocytes with PIPLC

Washed erythrocytes were resuspended as a haematocrit of 20% in 50 mM Tris-sodium maleate buffer, pH 6.0, containing 0.25 M sucrose [25], added with PIPLC (15–128 U) and incubated at 37 °C for an established period of time (10–120 min) with gentle shaking. At the end of incubation the mixture was centrifuged at 2000 × **g** for 3 min and the supernatant further centrifuged at 150000 × **g** for 10 min. The final supernatant was analysed for acetylcholinesterase, sialidase, total proteins and haemoglobin (as a marker of haemolysis). According to Futerman *et al.* [26], the starting amounts of acetylcholinesterase and sialidase were considered those measured on the ghosts obtained from the same quantity of erythrocytes; therefore the percentage of release of the two enzymes was referred to the enzymes content in the corresponding ghost preparation. The percentage of haemolysis was calculated as the percentage of haemoglobin released from erythrocytes over the total haemoglobin content [25].

Enzyme assays

Membrane-bound and PIPLC-released sialidase were routinely determined by the fluorimetric method described by Marchesini *et al.* [27], using MU-NeuAc as substrate. In some experiments sialidase was assayed by a radiochemical method [28] using [^{3}H]GD1a as substrate. According to this procedure residual [^{3}H]GD1a and formed [^{3}H]GM1 were separated by HPTLC and quantified by radiochromatoscanning using a Berthold automatic TLC linear analyser, model MB 2832, equipped with an Apple II Europlus computer. In the radiochemical assay 250 000 disintegrations min^{-1} of [^{3}H]GD1a were employed (corresponding to 0.1 nmol of [^{3}H]GD1a in the incubation mixture). With both assay procedures the optimal conditions (pH, incubation time, substrate concentration, ionic strength, etc.) were verified by preliminary experiments. Acetylcholinesterase activity was determined by the method of Ellman *et al.* [29]. Enzyme activities were expressed as units (U), that is, the amount of enzyme that liberates 1 μmol of product per min at 37 °C, under optimal conditions.

Characterization of the product of GD1a hydrolysis catalysed by PIPLC liberated-sialidase

The recognition and identification of GM1 formed from GD1a by the action of PIPLC-liberated sialidase were accomplished by (a) HPTLC chromatography in comparison with labelled and unlabelled GM1, (b) treatment with *V. cholerae* sialidase, (c) direct chemical analysis, and (d) spectral proton nuclear magnetic resonance (^{1}H-NMR) analysis (Bruker 300 NMR spectrometer), following previously described procedures [30, 31]. For this purpose, incubation mixtures containing 0.2 mg (as protein) of PIPLC-liberated sialidase were incubated with GD1a under optimal conditions for 4 h at 37 °C, and GM1 was extracted and isolated from the incubation mixture as reported [18].

Other methods

Protein content was determined by the method of Lowry *et al.* [32], using crystalline bovine serum albumin as the standard; when Tris-maleate buffer was present, the Coomassie Brilliant Blue method [33] was used. Haemoglobin was determined, and haemolysis expressed, as described by Taguchi *et al.* [25], using human haemoglobin as standard. Ganglioside-bound sialic acid was determined as described by Svennerholm [34], using pure *N*-acetylneuraminic acid as standard.

Results

Release of sialidase from human erythrocyte ghosts by treatment with PIPLC

Human erythrocyte ghosts were found to release bound sialidase upon treatment with PIPLC. As the results (Table 1) obtained using the first experimental approach described under Materials and methods, show four subsequent 15 min treatments of ghosts with 25 U of PIPLC (enzyme/ghost protein ratio of 4.5; as total U per mg protein each time), at pH 6.0, caused an overall release of about 60% of ghost-bound sialidase. A fifth treatment with PIPLC did not succeed in liberating any further sialidase activity.

Table 1. Release of sialidase and acetylcholinesterase from human erythrocyte ghosts by treatment with PIPLC as described in the Materials and methods section, based on five successive incubations of 5.5 mg ghosts (as protein) with 25 U PIPLC at pH 6.0 for 15 min (37 °C). After each treatment, the incubation mixture was centrifuged (150 000 × **g**, 10 min) and the pellet was suspended with the incubation buffer and, after addition of PIPLC, incubated again. The five supernatants and final pellet were analysed. The data are the mean values of five experiments ±SD values.

	Protein		*Sialidase*			*Acetylcholinesterase*		
	Total (mg)	*Recovery* (%)	*Specific activity* (μU per mg protein)	*Total activity* (μU)	*Recovery* (%)	*Specific activity* (U per mg protein)	*Total activity* (U)	*Recovery* (%)
Ghost	5.5	100	24.3	133.6	100	7.52	41.36	100
1st Supernatant	0.80 ± 0.05	14.5	55.5	44.4 ± 3.1	33.2	2.32	1.86	4.50
2nd Supernatant	0.15 ± 0.01	2.7	144.0	21.6 ± 1.3	16.2	3.75	0.56	1.35
3rd Supernatant	0.072 ± 0.006	1.3	149.7	10.8 ± 0.8	8.1	2.11	0.15	0.36
4th Supernatant	0.018 ± 0.002	0.3	156.7	2.8 ± 0.18	2.1	n.d.	n.d.	n.d.
5th Supernatant	n.d.	–	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	1.04	18.8	–	79.6	59.6	–	2.57	6.21
Final pellet	3.60 ± 0.22	65.4	12.0	43.2	32.3	6.97	25.10	60.7
Total	4.64	84.2	–	122.8	91.9	–	27.67	66.91

n.d. = not detectable.

Table 2. Release of sialidase and acetylcholinesterase from human erythrocyte ghosts by treatment with PIPLC as described in the Materials and methods section, based on four successive incubations of 25 U PIPLC to the incubation mixture at pH 6.0 containing 5.5 mg ghosts as protein. After each addition, the mixture was incubated at 37 °C for 15 min, with a total period of incubation of 60 min. At the end of incubation the mixture was centrifuged (150 000 × **g**, 10 min) a supernatant and a pellet being obtained. The data are the mean values of five experiments ±SD values.

	Protein		*Sialidase*			*Acetylcholinesterase*		
	Total (mg)	*Recovery* (%)	*Specific activity* (μU per mg protein)	*Total activity* (μU)	*Recovery* (%)	*Specific activity* (μU per mg protein)	*Total activity* (U)	*Recovery* (%)
Ghost	5.5	100	24.3	133.6	100	7.52	41.36	100
Supernatant	1.0 ± 0.07	18.2	91.8 ± 7	91.8 ± 7	68.7	2.63 ± 0.12	2.63 ± 0.12	6.3
Pellet	4.15 ± 0.3	75.4	9.3 ± 0.7	38.6 ± 2.7	28.9	6.81 ± 0.36	28.26 ± 1.6	68.3
Total recovery	5.15	93.6		130.4	97.6		30.89	74.6

Under the same conditions, the reference enzyme acetylcholinesterase showed a modest release (6.2%), as expected [24]. According to the second experimental approach (Table 2), 25 U aliquots of PIPLC were successively added to the same incubation mixture four times, with 15 min periods of incubation each time: under these conditions the total release of sialidase was 68.7% and that of acetylcholinesterase, 6.3%. This was the highest percentage of sialidase release that could be obtained. Released sialidase had a markedly higher (almost fourfold) specific activity compared with the ghost-linked enzyme, indicating some selectivity in the action of PIPLC. Data on the optimal conditions for sialidase and acetylcholinesterase release by treatment with PIPLC, obtained adopting the second experimental approach, are reported in Fig. 1. As shown, the optimal release of sialidase was obtained at pH 6.0, with a ratio between PIPLC and ghost protein (total U mg^{-1}) of 18, and a total incubation period of 60 min. It is interesting to note that the pH optimum for acetylcholinesterase release was 5.2, different from that of sialidase.

Experiments carried out in the absence of EDTA showed that the release of sialidase occurred to the same extent as in the presence of EDTA; also, the addition of 5 mM $ZnCl_2$ to the incubation mixture completely blocked the release of sialidase.

Some characteristics of PIPLC-released sialidase were studied, using the supernatant obtained by the second experimental approach as the enzyme source. The optimal pH for the enzyme acting on MU-NeuAc, as well as on GD1a, was 4.2, a value identical to that of the ghost-linked sialidase (Fig. 2). The kinetics of the ghost-linked and PIPLC-liberated sialidase on MU-NeuAc and GD1a are represented in Figs 3 and 4, respectively. In all cases the $V/[S]$ relationships obeyed regular Michaelis–Menten kinetics. With MU-NeuAc the apparent K_m value was 2.0×10^{-4} M, for both the ghost-linked and liberated sialidases; with GD1a the K_m values were 2.56×10^{-4} M and 5.0×10^{-4} M for the ghost-linked and liberated sialidase, respectively. The apparent V_{max} values were higher for the liberated than the membrane-bound sialidase: 0.14 versus 0.034 mU per mg protein with MU-NeuAc, and 2.22

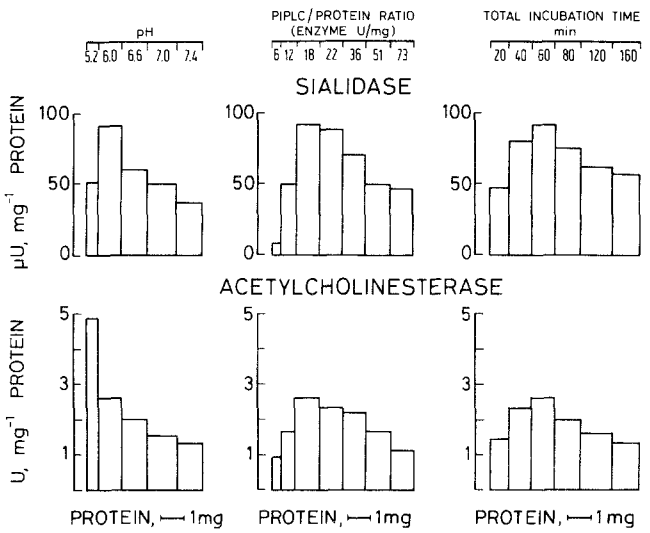

Figure 1. Effect of pH, incubation time, and ratio between PIPLC and ghost protein on the extent of liberation of sialidase and acetylcholinesterase from human erythrocyte ghosts on treatment with PIPLC. The area of each column (enzyme specific activity × protein) represents the total amount of enzyme released. The data reported refer to experiments where there were four additions of PIPLC, each time with the same amount of enzyme and with the same period of incubation. The ratio between PIPLC and ghost protein is expressed as total units of enzyme added over each mg of protein (U mg^{-1}). The incubation time reported is the total period of incubation. Sialidase was assayed using MU-NeuAc as substrate. The data reported are the mean values of five experiments using 5.5 mg ghost protein. The SD values were less than 7% of the mean values.

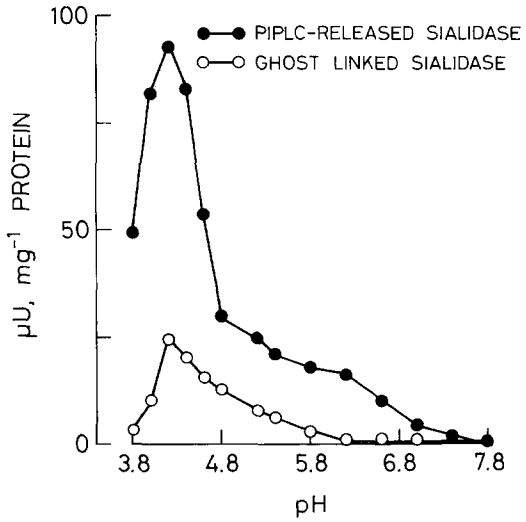

Figure 2. Effect of pH on the sialidase activity contained in human erythrocyte ghost and released upon ghost treatment with PIPLC. Enzyme protein: 100 μg for the ghost-linked; 57 μg for the released enzyme. The preparation of released sialidase was that obtained by incubating the reaction mixture at pH 6.0, for a total period of 60 min, with four subsequent additions of PIPLC (PIPLC/ghost protein ratio 4.5 each time) at 0 time and after 15, 30, and 45 min of incubation.

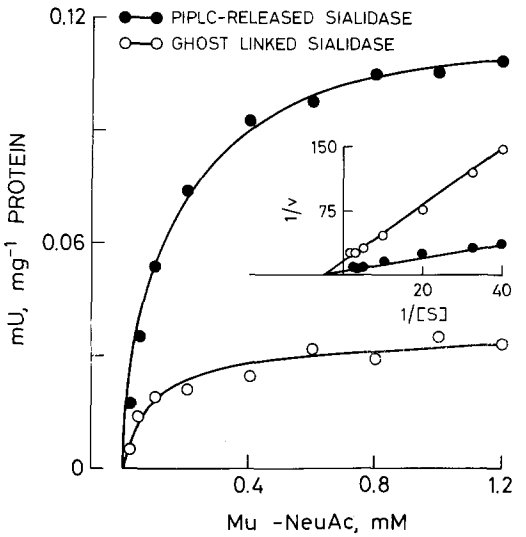

Figure 3. Kinetics of human erythrocyte sialidase action on MU-NeuAc. The sialidase contained in the erythrocyte ghost and released upon ghost treatment with PIPLC were employed. The assay conditions were as for Fig. 1. The optimal pH was 4.2. Enzyme protein: 100 μg for the ghost linked enzyme; 37.6 μg for the released enzyme.

versus 0.52 mU per mg protein with GD1a. The K_m/V_{max} values were 5.9×10^{-3} and 1.4×10^{-3} with MU-NeuAc and 4.9×10^{-4} and 2.2×10^{-4} with GD1a, for the ghost-linked and the liberated sialidase, respectively. Using MU-NeuAc as substrate, the activity of the PIPLC-liberated sialidase proceeded linearly with protein content from 15 μg

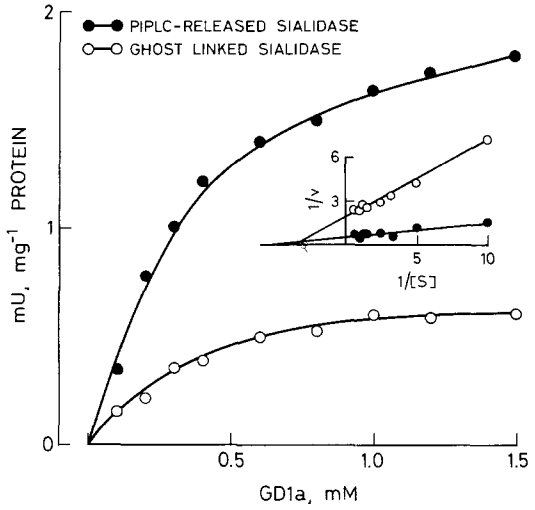

Figure 4. Kinetics of human erythrocyte sialidase action on ganglioside GD1a. The sialidase contained in the erythrocyte ghosts and released upon ghost treatment with PIPLC were employed. The preparation of released sialidase was as for Fig. 3. Enzyme protein: ghost linked enzyme 270 μg; released enzyme 7.9 μg.

to at least 120 μg, and linearly with incubation time up to 60 min. The same released enzyme maintained full activity after at least four cycles of rapid freezing and thawing, and did not lose activity upon storage at 4 °C for 48 h. Instead, the enzyme lost activity almost completely during ammonium sulfate treatment and subsequent dialysis against isotonic or hypotonic buffers (pH 6.0–7.0).

Upon prolonged treatment (4 h) with PIPLC-released sialidase the only recognized product of GD1a hydrolysis was a compound (Fig. 5) having the same chromatographic behaviour of GM1. The same compound was characterized by chemical, enzymatic and ^{1}H-NMR analysis as GM1. No sialidase activity was observed on GM1 regardless of the presence or not of various amounts of detergents (Triton X-100; sodium deoxycholate) and the duration (up to 4 h) of incubation.

Membrane topology of sialidase: release of sialidase from intact erythrocyte by treatment with PIPLC

The possible location of sialidase on the outer surface of erythrocyte membrane was explored by measuring the release of sialidase from intact erythrocytes upon treatment with PIPLC. Concomitantly, the degree of erythrocyte integrity was assessed by determining haemolysis. All this was based on the assumption [24, 35, 36] that PIPLC attacks the membrane surface(s) but does not penetrate into the membrane. As shown in Fig. 6, sialidase was progressively released from human intact erythrocytes upon incubation (20 min, 37 °C) with increasing amounts of PIPLC. Under the experimental conditions (16 mg erythrocytes, as protein, corresponding to 5.5 mg ghost protein) a maximal release

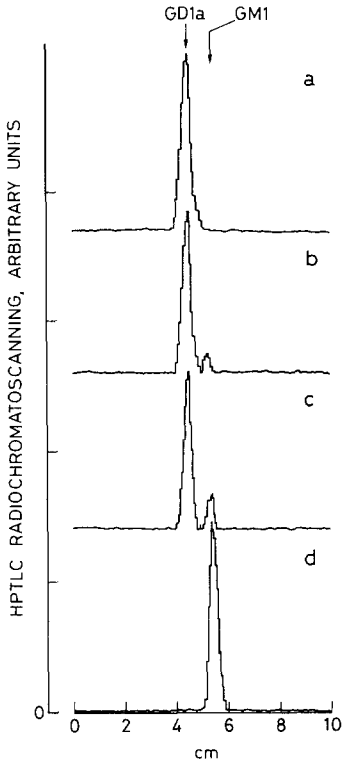

Figure 5. Identification by HPTLC radiochromatoscanning of the product(s) of GD1a hydrolysis by PIPLC-released sialidase. The incubation mixture was as for Fig. 4, and contained 250000 disintegrations min^{-1} [^{3}H]GD1a. (a) [^{3}H]GD1a at 0 time incubation; (b) [^{3}H]GD1a after 20 min incubation with sialidase; (c) [^{3}H]GD1a after 60 min incubation with sialidase; (d) standard [^{3}H]GM1. For details see the Materials and method section.

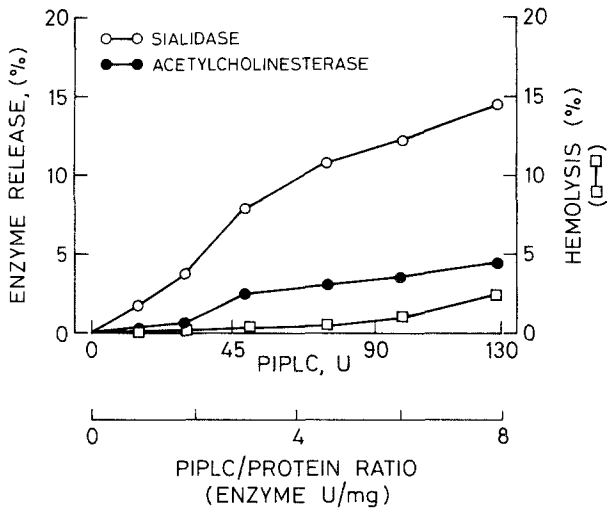

Figure 6. Release of sialidase and acetylcholinesterase from intact human erythrocytes by treatment with PIPLC. Concurrent haemolysis was assessed by measurement of liberated haemoglobin. Sixteen milligrams of erythrocytes (as protein) were employed and incubation was carried out at 37 °C for 20 min under the conditions reported in the Materials and method section. The percentage enzyme release was referred to the enzyme content measured on the ghost prepared from the same amount of erythrocytes. The data reported are the mean values of five experiments, SD values never exceeding ±8% of the mean values.

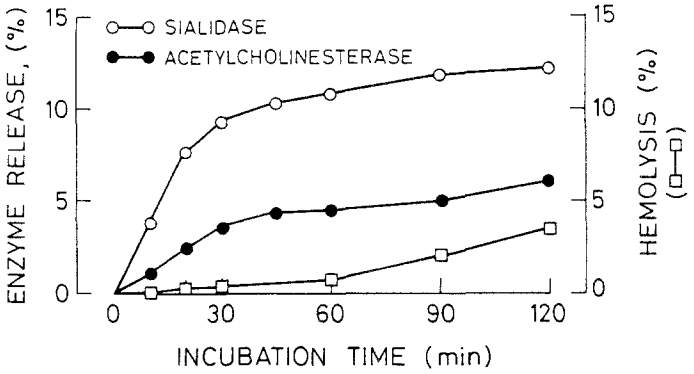

Figure 7. Time course of sialidase and acetylcholinesterase release from intact human erythrocytes by treatment with PIPLC. Concurrent haemolysis was assessed by measurement of liberated haemoglobin. Sixteen milligrams of erythrocytes (as protein) were employed and incubations carried out at 37 °C in the presence of 48 U PIPLC under the conditions reported in the Materials and methods section. The percentage enzyme release was established as for Fig. 6. The data reported are the mean values of five experiments, the SD values never exceeding ±8% of the mean values.

of about 15% was obtained with a PIPLC/erythrocytes (total U/total protein) ratio of 8. Concomitantly, acetylcholinesterase was also liberated, to a maximum of 4.4%. Haemolysis was practically negligible (less than 0.5%) till a PIPLC/erythrocyte ratio of 5, increasing to 2.5% at a ratio of 8. Using a PIPLC/erythrocyte ratio of 5 (Fig. 7), the release of sialidase proceeded linearly and with the highest rate till 20 min, then after 60 min a release of 11%, with a maximal release of 12% being achieved at 120 min of incubation. The release of acetylcholinesterase showed a similar trend with a maximum of 6% at 120 min of incubation. Haemolysis remained practically negligible till 60 min of incubation and reached a value of 3.5% at 120 min. Owing to the occurrence of haemolysis in the conditions of more marked action of PIPLC on intact erythrocytes, no attempts were made to obtain higher percentage values of liberation of sialidase than those given in Figs 6 and 7.

Discussion

It is known that several enzymes, like alkaline phosphatase, 5′-nucleotidase and acetylcholinesterase are linked to the plasma membranes of different cells by a glycosylphosphatidylinositol anchor, and therefore may be released by treatment with PIPLC [23, 26, 35, 37, 38]. Recently, Chiarini *et al.* [39] demonstrated that also the plasma membrane linked sialidase from pig brain could be released by PIPLC. With the present work we showed that the sialidase carried

by human erythrocyte ghosts can be released by treatment with PIPLC from *Bacillus cereus*, indicating that the enzyme is linked to the membrane by a glycosylphosphatidylinositol anchor. In agreement with this conclusion are the observations that addition of $ZnCl_2$, which inhibits PIPLC [40] completely blocked sialidase liberation, whereas the presence of EDTA, which inhibits the phospholipase activity on glycerophospholipids not containing inositol [22], did not affect sialidase release. The maximal sialidase release, obtained by the simple procedure described in the text, is about 70%, a high degree of liberation, similar to that reported for alkaline phosphatase and acetylcholinesterase in some tissues or cells [38]. The fact that the release of sialidase appears to be incomplete is not surprising. In fact it is known [23, 35, 38, 41] that enzymes such as 5′-nucleotidase and alkaline phosphatase are linked to the membrane by the glycosylphosphatidylinositol structure in addition to other anchoring systems, and that not necessarily all the enzyme linked by a PIPLC-sensitive anchor is accessible to PIPLC action. Therefore, it may be suggested that the sialidase of human erythrocyte ghosts is mostly, but not totally, linked via a glycosylphosphatidylinositol anchor, or/and that not all the enzyme anchored by this system is available to PIPLC-assisted hydrolysis. The fact is therefore not surprising that the plasma membrane bound sialidase from human erythrocytes undergoes a 70% release upon PIPLC treatment against the 24% exhibited by the pig brain enzyme [39]. In fact, acetylcholinesterase also is released by the action of PIPLC at different degrees, depending on the tissue [26, 38], and on the animal source (up to 100% in sheep and horse erythrocytes [24] only 6% in human erythrocytes, see the present work).

The liberated sialidase appeared to be relatively stable: this feature contrasts with the extremely unstable nature of the same enzyme solubilized from erythrocyte ghosts by detergent (sodium deoxycholate, Triton X-100) treatment [16], suggesting that the chemical characteristics of the sialidase detached from the erythrocyte membrane by detergent dispersion is different from those of the enzyme liberated by PIPLC treatment. Of course, the observed stability of the PIPLC liberated enzyme may greatly facilitate attempts towards its purification.

Interestingly, liberated sialidase expressed a much higher activity (about 15-fold) on ganglioside GD1a than on the artificial substrate MU-NeuAc, in agreement with previous findings [16], and its action on ganglioside did not require the presence of detergents. Contrary to the findings of Sagawa *et al.* [16], we found no evidence of any activity of the liberated sialidase on ganglioside GM1. Should the ability of erythrocyte sialidase to affect ganglioside GM1 be linked to the chemical difference between the PIPLC-released enzyme and that isolated from the ghosts by detergent dispersion, a deeper study on the structure–function relationship of the sialidase activity on ganglioside would be required. The action of PIPLC released-sialidase on ganglioside GM3, the major ganglioside in erythrocytes, presented some pecularities, and is currently under investigation in our laboratory.

A further remarkable finding obtained in this study is that the PIPLC releasable sialidase of human erythrocyte is located, at least in part, on the outer surface of the erythrocyte membrane. At least 10–12% of the linked enzyme was released from erythrocytes by PIPLC treatment, under conditions where integrity of erythrocytes was preserved. This indicates that the enzyme resides on the outer membrane surface, which is the only one available to PIPLC. Of course, it cannot be excluded that a higher proportion of the enzyme has the same location. The fact that a sialidase activity is present in the outer surface of the erythrocyte membranes means that this enzyme and its potential natural substrates (sialoglycoproteins, sialoglycoconjugates) have the same topology. This raises the question whether the enzme is capable of attacking these substrates under physiological conditions. On the other hand, the erythrocyte sialidase isolated and purified from human erythrocytes is able (*in vitro*) to release sialic acid from intact erythrocytes [15]. Presumably, the PIPLC liberated sialidase has the same ability. It is not known whether a PIPLC capable of releasing sialidase is present in the erythrocytes and, if so, which conditions trigger the enzyme to start functioning.

In conclusion, the present experimental evidence gives a new impetus to research aimed at ascertaining the details of sialidase–sialoglycoconjugates interactions occurring at the erythrocyte surface, and stimulates interest on the possible role of erythrocyte sialidase in regulating the sialic acid content on the erythrocyte surface and, hence, erythrocyte survival and viability.

Acknowledgements

This work was supported in part by a grant from the Italian Research Council (CNR), Target Project 'Biotechnology and Bioinstrumentation' (grant n°91.00052.99.115) and Strategic Project for Mezzogiorno (grant n°91.04207.ST75).

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