# Membrane anchoring and surface distribution of glycohydrolases of human erythrocyte membranes

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**Abstract The membrane anchoring of the following glycohydrolases of human erythrocyte plasma membranes was investigated: α- and β-D-glucosidase, α- and β-D-galactosidase, β-D-glucuronidase, *N*-acetyl-β-D-glucosaminidase, α-D-mannosidase, and α-L-fucosidase. Optimized fluorimetric methods for the assay of these enzymes were set up. Treatment of the ghost preparation with 1.0 mol/l (optimal concentration) NaCl caused release ranging from 4.2% of α-D-glucosidase to 70% of β-D-galactosidase; treatment with 0.4% (optimal concentration) Triton X-100 liberated 5.1% of β-D-galactosidase to 89% of α-D-glucosidase; treatment with 1.75% (optimal concentration) octylglucoside yielded solubilization from 6.3% of β-D-galactosidase to 85% of α-D-glucosidase. Treatment with phosphoinositide-specific phospholipase C caused no liberation of any of the studied glycohydrolases. These results are consistent with the notion that the above glycohydrolases are differently anchored or associated with the erythrocyte plasma membrane, and provide the methodological basis for inspecting the occurrence of these enzymes in different membrane microdomains.**



*Key words:* Human erythrocyte; Erythrocyte ghost; Glycohydrolase; Glycan phosphoinositide anchor; Membrane microdomain

## 1. Introduction

The glycohydrolases are ubiquitous enzymes, being found in lysosomes, plasma membrane, cytosol, and blood plasma [1–5]. In each subcellular location glycohydrolases may be present in different forms, as shown by *N*-acetyl-β-D-glucosaminidase and sialidase [6,7]. Glycohydrolases from lysosomes have an acidic optimal pH [8], whereas those present in plasma membranes and cytosol may have an optimal pH in the neutral range [6,9]. The insertion of glycohydrolases into the plasma membrane is assumed to occur through a transmembrane hydrophobic domain. However, examples are known of other types of anchoring [10].

Several glycohydrolases, all with an acidic pH optimum [11], were detected in the plasma membrane of human erythrocytes [11,12]. Recently, two forms of sialidase were described [6,12], one with a neutral pH optimum, the other one with an acidic pH optimum, which is released by treatment with phosphoinositide-specific phospholipase C (PIPLC), suggesting the presence of a glycanphosphoinositide (GPI) type of attachment [10]. By treatment with different detergents, it was also demonstrated that the acidic and neutral forms of sialidase likely pertain to different membrane domains [6]. There is growing evidence, arising mostly from studies on polarized cells (neurons, epithelial cells), that some proteins and lipid components of the plasma membrane are unevenly distributed on the surface and are enriched in membrane microdomains (caveolae, lipid rafts, glycosphingolipid-enriched microdomains) that can be isolated and analyzed [13–18]. It is conceived that some proteins present in microdomains are responsible for both the formation and functional performance (recognition and signalling events) of these membrane structures [16]. Although the presence of microdomains in human erythrocyte membranes was never studied as such, some data are consistent with this possibility [19–21], including the microvesiculation process occurring during human erythrocyte ageing [22]. Therefore, it seems interesting to explore whether other erythrocyte glycohydrolases, besides sialidase, have different modes of membrane anchoring, with possible involvement in different membrane microdomains, and whether these differential patterns affect the physiopathology of the erythrocyte surface. Interestingly, changes in the glycohydrolase set of human erythrocyte membranes due to diseases of infective or genetic origin have been reported [23,24].

We have investigated here the mode of attachment of the following glycohydrolases to the membrane of normal human erythrocytes: β-GlcNAc-ase, β-GlcA-ase, β-Gal-ase, α-Gal-ase, β-Glc-ase, α-Glc-ase, α-Man-ase, α-Fuc-ase. The results obtained are consistent with the notion that glycohydrolases are unevenly distributed on the erythrocyte surface and constitute the basis for more focussed investigations on the possible differential distribution of glycohydrolases in membrane microdomains.

## 2. Materials and methods

### 2.1. Materials

Commercial chemicals were of the highest available grade. The

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*Abbreviations:* β-GlcNAc-ase, *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30); β-GlcA-ase, β-D-glucuronidase (EC 3.2.1.31); β-Gal-ase, β-D-galactosidase (EC 3.2.1.23); α-Gal-ase, α-D-galactosidase (EC 3.2.1.22); β-Glc-ase, β-D-glucosidase (EC 3.2.1.21); α-Glc-ase, α-D-glucosidase (EC 3.2.1.20); α-Man-ase, α-D-mannosidase (EC 3.2.1.24); α-Fuc-ase, α-L-fucosidase (EC 3.2.1.51); AChE, acetylcholinesterase (EC 3.1.1.7); GPI, glycanphosphoinositide; MUB, methylumbelliferone


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water routinely used was freshly redistilled in a glass apparatus. 4-Methylumbelliferone (MUB), purchased from Fluka GmbH (Buchs, Switzerland), was recrystallized from ethanol three times; 4-MUB-α- and β-glycosides, used as enzyme substrates, were purchased from Melford (Suffolk, UK); bovine serum albumin, Triton X-100, octylglucoside, ethylene glycol from Sigma (St. Louis, MO, USA) and PIPLC, from *Bacillus thuringiensis*, from Oxford Glycosystems (Oxford, UK).

### 2.2. Preparation of erythrocyte membranes (ghosts)

Blood (6–7 ml per donor) was taken from 25 adult volunteer blood donors, both females and males, aged 25–55 years, from the blood bank of the Bassini Hospital (Cinisello Balsamo, Milan, Italy). All the individuals were informed of the purpose of the investigation and gave their consent. All samples were appropriately screened and were negative for HIV and hepatitis antibody. Erythrocytes were prepared from heparinized venous blood by the Leucostop 4LT-B filter (Baxter) standard procedure properly scaled down. Blood samples were centrifuged for 15 min at $3000\times g$, immediately after collection, plasma and buffy coat were aspirated from the surface of the pellet and discarded. The residual material was diluted (1:1, v/v) with phosphate buffer saline (PBS) at pH 7.4 and filtered through a Leucostop 4LT-B filter adapted and inserted into a 5 ml syringe. Under microscopic examination the filtrate was completely devoid of leukocytes and platelets, and consisted of a homogeneous preparation of erythrocytes, which were collected by centrifugation (5 min at $1200\times g$) and washed twice with PBS. Erythrocyte plasma membranes (ghosts) were prepared by the method of Steck and Kant [25]. Briefly, washed and packed erythrocytes were dispersed with 5 mmol/l PBS, pH 8.0 (1:40, v/v), and lysed by gentle mixing at 4°C for 20 min. The mixture was centrifuged (20 min at $105\,000\times g$) and the pellet, containing the ghosts, was washed twice with a PBS solution, pH 8.0 (2.5 and 1.25 mmol/l, respectively) and centrifuged as above. Before centrifugation two thirds of each suspension, obtained from the individual donors, were pooled and centrifuged. The pellet from this pooled material was employed throughout the study. Ethylene glycol at a final concentration of 30% was added to the remaining portions for storing at −40°C in order to preserve glycohydrolase activities for further investigations.

### 2.3. Enzyme assays

The studied glycohydrolases (β-GlcNAc-ase, β-GlcA-ase, β-Glc-ase, β-Gal-ase, α-Glc-ase, α-Gal-ase, α-Man-ase, α-Fuc-ase) were assayed fluorimetrically [8], using the corresponding 4-MUB-glycoside as substrate. 50 μl of ghost preparation was incubated in a final volume of 250 μl containing 25 μl of 50 mmol/l citric acid–sodium phosphate buffer (chosen as the most suitable on the basis of preliminary experiments) at the appropriate pH, and 175 μl of the specific substrate dissolved in water. The mixtures were incubated in a shaker bath at 37°C for the established period of time. The reaction was stopped and fluorescence developed by adding 750 μl of an alkaline solution (0.2 mol/l glycine–NaOH buffer, containing 0.125 mol/l NaCl, pH 10.75). The control incubation mixtures (blanks) were set up using incubation mixtures lacking the ghost sample, which was incubated separately and added immediately before stopping the reaction. Protein content was determined [26] using bovine serum albumin as standard. The enzyme activities are expressed as μU/mg protein. The analytical imprecision of the assay methods for ghost glycohydrolases was determined and expressed as the coefficient of variation. Both intra-run and day-to-day run analyses were performed, using ghost preparations stored at −40° in 30% ethylene glycol.

Sialidase and acetylcholinesterase (AChE), used as reference enzymes, were determined as described in [27] and [28], respectively.

### 2.4. Removal of non-integral, peripheral membrane-associated glycohydrolases by treatment with high ionic strength solutions

Following the general indications of Clark et al. [29], increasing amounts of NaCl (up to a final 1.5 mol/l) were added (final volume,3.6 ml) to the ghost preparations (10–12 mg protein) homogeneously suspended with 3 ml of 1.25 mmol/l PBS at pH 8.0 (equilibrated at 4°C). The mixture was gently stirred at 4°C for 20 min, and centrifuged (10 min at $150\,000\times g$) at the same temperature. The pellet was rapidly washed for a given number of times with the above NaCl containing PBS solution and centrifuged. Both the washings and the final pellet were analyzed for protein and glycohydrolase content.

### 2.5. Removal of GPI-anchored glycohydrolases by treatment with PIPLC

The pellet (containing 2.0–2.5 mg protein), obtained after ghost treatment with 1.0 mol/l NaCl (that proved to achieve optimal release of peripherally membrane-associated glycohydrolase activities, see Section 3) was homogeneously dissolved with 1 ml of acetic acid–Tris buffer (final concentration: 25 mmol/l), pH 7.4, and 200 mU of PIPLC/mg protein, and incubated at 30°C in a shaker bath for 60 min [10]. The mixture was then centrifuged as above and the pellet treated similarly several times, the supernatants and the final pellet being analyzed for protein and glycohydrolase content.

### 2.6. Solubilization of membrane-bound glycohydrolases by detergent treatment

The pellet (containing 2.0–2.5 mg protein), obtained after ghost treatment with 1.0 mol/l NaCl, was homogeneously dissolved with 1 ml PBS solution (equilibrated at 4°C), after addition of increasing concentrations of Triton X-100 or octylglucoside (up to 2% or 3%, respectively) it was gently stirred at 4°C for 20 min, then centrifuged at $150\,000\times g$ for 10 min, and the supernatant carefully poured off [30]. This treatment was repeated several more times and the final pellet and supernatants analyzed for protein and glycohydrolase content.

### 2.7. Statistical analysis

The skewness and kurtosis test [31] showed no significant difference from the normal distribution, thus parametric analysis techniques were used. Estimation of total imprecision by analysis of variance was performed following the model of Krouwer [32] and NCCLS recommendations [33].

## 3. Results

### 3.1. Assay of glycohydrolases in human erythrocyte ghosts

The experimental conditions found to be optimal for the assay for human erythrocyte plasma membranes glycohydro-

Table 1
Conditions for maximal activity and practical recommendations for the fluorimetric assay of glycohydrolases in human erythrocyte plasma membranes (ghosts)

| Enzyme | pH for maximal activity | Buffer providing maximal activity (final concentration: 0.05 mol/l) | $K_m$ value ($\times 10^{-3}$ mol/l) | Apparent saturating substrate concentration (mol/l) | Time course: linear up to (min) | Recommended time of incubation (min) | Protein range for linear *V*/*E* relationship (μg) |
|---|---|---|---|---|---|---|---|
| β-GlcNAc-ase | 3.8, 4.4, 5.6 | citrate–phosphate | 0.7, 0.6, 0.36 | 4.0 | 150 | 120 | 6–65 |
| β-GlcA-ase | 4.8 | citrate–phosphate | 2.00 | 8.0 | 90 | 45 | 1–90 |
| β-Gal-ase | 4.4 | citrate–phosphate | 0.29 | 1.6 | 180 | 180 | 20–90 |
| α-Gal-ase | 4.3 | citrate–phosphate | 0.70 | 4.0 | 180 | 180 | 20–90 |
| α-Fuc-ase | 5.0 | citrate–phosphate | 0.35 | 0.3 | 150 | 150 | 20–90 |
| α-Glc-ase | 5.8 | citrate–phosphate | 0.33 | 4.0 | 120 | 60 | 10–100 |
| β-Glc-ase | 5.0 | citrate–phosphate | 0.60 | 4.0 | 180 | 180 | 20–90 |
| α-Man-ase | 5.8 | citrate–phosphate | 1.00 | 4.0 | 180 | 180 | 20–90 |

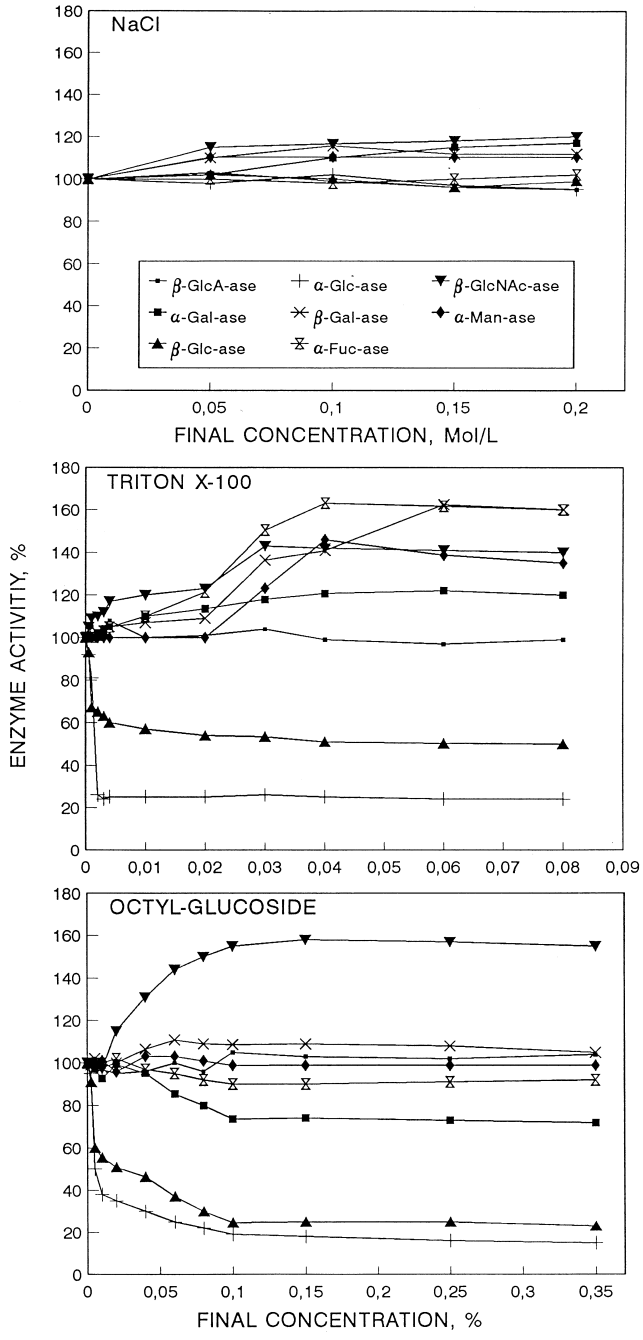


Fig. 1. Effect of the addition of increasing amounts of NaCl (A), Triton X-100 (B), and octylglucoside (C) on the activity of glycohydrolases of human erythrocyte ghosts. The final concentration of each added substance is indicated.

lases studied are reported in Table 1. As expected, all tested enzymes had an acidic pH optimum. With two exceptions, all the enzymes investigated exhibited a pH–activity curve with a single maximum; β-GlcNAc-ase had three maxima (pH 3.8, 4.4 and 5.6, the highest of which was that at pH 4.4); α-Glc-ase displayed a plateau from 5.8 to 8.0. The buffer system that proved to be best for all enzymes was citric acid–sodium phosphate, and the $K_m$ values for the corresponding MUB-glycoside ranged from a minimum of $0.29\times10^{-3}$ mol/l for β-Gal-ase to a maximum of $2.00\times10^{-3}$ mol/l for β-GlcA-ase. The minimum amount of enzyme preparation required (μg protein) ranged from 1 μg for β-GlcA-ase to 20 μg for α-Gal-ase, β-Gal-ase, α-Fuc-ase, and β-Glc-ase. The intra-run coefficient of analytical variation ranged from 1.4% for β-GlcA-ase to 6.5% for β-Glc-ase, and the day-to-day value from 2.7% for β-GlcA-ase to 7.3% for α-Glc-ase. The latter values were established using a ghost preparation dissolved in 1.25 mmol/l PBS containing 30% ethylene glycol and stored at −40°C. Under these conditions all glycohydrolases maintained full activity for at least 7 days. As shown in Fig. 1, the presence of NaCl, Triton X-100 and octylglucoside may affect the activity of the glycohydrolases used. NaCl, at 0.2 mol/l concentration, caused a 15–20% activation of β-GlcNAc-ase, β-Gal-ase, α-Gal-ase and α-Man-ase, with no effects on the other glycohydrolases. Triton X-100 caused a concentration-dependent activation, ranging from 20 to 60%, of α-Gal-ase, α-Man-ase, β-Gal-ase, β-GlcNAc-ase and α-Fuc-ase, and an inhibition up to 50 and 75% of β-Glc-ase and α-Glc-ase, respectively, with no effect on β-GlcA-ase. Octylglucoside had a similar, but more pronounced, inhibitory effect on β-Glc-ase and α-Glc-ase, and an activatory effect (60%) on β-GlcNAc-ase, a moderate inhibitory effect on α-Gal-ase, and practically no effect on the other glycohydrolases. All effects became stable and optimal around 0.08% concentration for Triton X-100 and 0.35% for octylglucoside.

### 3.2. *Removal of non-integral, peripheral membrane-associated glycohydrolases by treatment with high ionic strength solution*

Preliminary experiments showed that the highest release of glycohydrolases from erythrocyte ghosts by high ionic strength solutions was obtained with three successive treatments with NaCl, at the optimal final concentration of 1.0 mol/l. As shown in Table 2, NaCl treatment caused a release from the ghost preparation of 24% of protein and from a minimum of 4.1% of α-Glc-ase activity to a maximum of 67.1% of β-Gal-ase activity. A percent release of enzyme activity markedly higher than that of total proteins was also exhibited by β-GlcNAc-ase (42.2%), α-Man-ase (59.1%), and α-Fuc-ase (47.8%). The release of AChE was only 4% and that of sialidase null. With reference to the starting ghost preparation, in the supernatants obtained after treatment, the specific activity of β-GlcNAc-ase and α-Fuc-ase increased about two-fold, that of α-Man-ase and β-Gal-ase 2.5-, and three-fold, whereas that of α-Glc-ase decreased almost six-fold.

### 3.3. *Removal of GPI-anchored glycohydrolases by treatment with PIPLC*

The proper conditions for release of GPI-anchored proteins and enzyme activities from the pellet obtained after ghost treatment with 1.0 mol/l NaCl were set up with preliminary experiments, using as reference markers sialidase and AChE, known to be partially bound to the erythrocyte membrane by a GPI anchor [10,34]. Three successive treatments with PIPLC, under the conditions described in Section 2, succeeded in completely releasing PIPLC-sensitive enzymes. Therefore, the pooled three supernatants were used to determine the amount of liberated proteins, sialidase and AChE. PIPLC treatment released 23.7%, 78.4% and 10.2% of bound proteins, sialidase and AChE activity, respectively. These values are expected on the basis of literature data [10,23,24]. Con-

Table 2
Removal of non-integral, peripheral membrane-associated glycohydrolases by treatment with 1.0 mol/l NaCl

| Enzyme | NaCl treatment | | | | | | No treatment |
|---|---|---|---|---|---|---|---|
| | Total activity | | | Specific activity | | | Specific activity |
| | Before centrifugation | After centrifugation | | Before centrifugation | After centrifugation | | |
| | | Supernatants | Pellet | | Supernatants | Pellet | |
| β-GlcNAc-ase (pH 4.4) | 285 | 125.4 | 158.4 | 33.4 ± 2.0 | 61.5 ± 3.5 | 25 ± 1.6 | 27.6 ± 1.8 |
| α-Glc-ase | 2514 | 102.3 | 2385.0 | 294.0 ± 26.2 | 50.2 ± 4.4 | 377 ± 31.1 | 296.0 ± 25.2 |
| β-GlcA-ase | 4308 | 1038.0 | 3228.0 | 504.0 ± 15.6 | 509.0 ± 15.2 | 510 ± 15.3 | 502.0 ± 16.3 |
| α-Man-ase | 110 | 64.8 | 42.0 | 12.9 ± 0.72 | 31.7 ± 1.6 | 6.64 ± 0.4 | 11.4 ± 0.68 |
| β-Gal-ase | 66 | 44.4 | 22.2 | 7.7 ± 0.58 | 21.7 ± 1.5 | 3.5 ± 0.3 | 7.0 ± 0.55 |
| β-Glc-ase | 133 | 19.8 | 110.7 | 15.5 ± 1.5 | 9.7 ± 0.9 | 17.5 ± 1.6 | 15.3 ± 1.3 |
| α-Fuc-ase | 21 | 11.4 | 8.4 | 2.4 ± 0.11 | 5.6 ± 0.22 | 1.35 ± 0.06 | 2.5 ± 0.12 |
| α-Gal-ase | 48 | 16.5 | 30.3 | 5.6 ± 0.5 | 8.1 ± 0.7 | 4.77 ± 0.42 | 4.7 ± 0.4 |
| Sialidase (pH 4.7) | 204 | n.d. | 192.6 | 23.9 ± 2.10 | n.d. | 30.45 ± 2.9 | 24.2 ± 2.12 |
| AChE | 66 | 2.7 | 61.5 | 7.7 ± 0.68 | 1.3 ± 0.11 | 9.73 ± 0.91 | 7.6 ± 0.71 |

Starting ghost preparation: 8.55 mg, as protein. Three successive treatments with 1.0 mol/l NaCl were accomplished, obtaining three supernatants (subsequently pooled), and one final pellet. The protein content of the pooled supernatants was 2.04 mg, corresponding to 23.9% of the starting protein content. The protein content of the final pellet was 6.33 mg. Total and specific glycohydrolase activities are expressed as μU and μU/mg protein respectively. Total and specific acetylcholinesterase activities are expressed as U and U/mg protein respectively. n.d.: not detectable. The data presented are mean values, or mean values ± S.D., of five replicate determinations using the pooled ghost preparation. The assay of all glycohydrolases was performed at a final NaCl concentration of 0.2 mol/l in the assay mixture.

versely, no activity of any of the investigated glycohydrolases could be detected in the supernatants, indicating that none of these enzymes was sensitive to PIPLC treatment.

### 3.4. Solubilization of membrane-bound glycohydrolases by detergent treatment

Preliminary experiments showed that the conditions providing maximal glycohydrolase solubilization from the pellet obtained after ghost treatment with 1.0 mol/l NaCl were: (a) a final concentration of 0.4% (w/v) for Triton X-100 and 1.75% (w/v) for octylglucoside, and (b) three successive treatments at the above detergent concentrations. As shown in Table 3, Triton X-100 and octylglucoside caused the liberation of about 61% and 69% of pellet-bound proteins, respectively, with similar degrees of solubilization for α-Glc-ase (89 and 84% with the two detergents, respectively), β-GlcA-ase (61% and 69%), α-Man-ase (41 and 39%), β-Gal-ase (16 and 20%), and substantial differences for the other enzymes, especially β-Glc-ase (73 and 47% for Triton X-100 and octylglucoside, respectively). The only enzyme that was solubilized with both detergents to a markedly higher degree than total protein was α-Glc-ase, whose specific activity was substantially higher in the supernatants than in the starting pellet. β-Glc-ase was solubilized to a higher degree than total proteins with Triton X-100, but not with octylglucoside. β-Gal-ase showed the lowest degree of solubilization (16 and 20%), thus exhibiting a markedly higher specific activity in the final pellet than in the starting pellet.

Table 3
Solubilization of proteins and membrane-bound glycohydrolases by detergent treatment of the pellet obtained after ghost treatment with 1.0 mol/l NaCl

| Enzyme | Treatment with Triton X-100 | | | | | | Treatment with octylglucoside | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Before centrifugation | | After centrifugation | | | | Before centrifugation | | After centrifugation | | | |
| | Total activity | Specific activity | Total activity | | Specific activity | | Total activity | Specific activity | Total activity | | Specific activity | |
| | | | Supernatants | Pellet | Supernatants | Pellet | | | Supernatants | Pellet | Supernatants | Pellet |
| β-GlcNAc-ase | 73.9 | 35 ± 1.9 | 69.7 | 2.8 | 41 ± 2.5 | 7 ± 0.4 | 84.4 | 40 ± 2.3 | 57.8 | 24.7 | 29.5 ± 1.7 | 145 ± 8.1 |
| α-Glc-ase | 174.9 | 82.9 ± 7.1 | 164.1 | 11.2 | 96.5 ± 7.3 | 28 ± 2.2 | 119.3 | 56.5 ± 3.7 | 105.8 | 12.2 | 54 ± 3.5 | 72 ± 4.1 |
| β-GlcA-ase | 1076.0 | 510 ± 15.1 | 884.0 | 212.0 | 520 ± 14.8 | 530 ± 14.9 | 1076 | 510 ± 14.7 | 989.8 | 88.4 | 505 ± 14.5 | 520 ± 14.9 |
| α-Man-ase | 19.6 | 9.3 ± 0.62 | 21.3 | 0.1 | 12.5 ± 0.74 | 0.2 ± 0.01 | 14 | 6.64 ± 0.41 | 14.1 | 0.4 | 7.2 ± 0.51 | 2.5 ± 0.17 |
| β-Gal-ase | 11.8 | 5.6 ± 0.42 | 5.4 | 6.0 | 3.2 ± 0.23 | 15 ± 1.07 | 7.8 | 3.7 ± 0.25 | 4.5 | 3.1 | 2.3 ± 0.19 | 18 ± 1.1 |
| β-Glc-ase | 19.2 | 9.1 ± 0.82 | 16.8 | 2.8 | 9.9 ± 0.87 | 7 ± 0.62 | 8.5 | 4.0 ± 0.5 | 4.7 | 3.7 | 2.4 ± 0.22 | 22 ± 1.8 |
| α-Fuc-ase | 4.6 | 2.2 ± 0.1 | 4.3 | 0.3 | 2.55 ± 0.11 | 0.7 ± 0.03 | 2.6 | 1.2 ± 0.08 | 2.2 | 0.4 | 1.1 ± 0.07 | 2.5 ± 0.16 |
| α-Gal-ase | 12.1 | 5.7 ± 0.56 | 11.8 | 0.4 | 6.95 ± 0.63 | 1.05 ± 0.1 | 10.1 | 4.8 ± 0.45 | 8.6 | 1.5 | 4.4 ± 0.42 | 9 ± 0.86 |

Starting protein for each detergent treatment: 2.11 mg (corresponding to 74% of the ghost). Two successive treatments with 0.4% (final concentration, w/v) Triton X-100, or 1.75% (final concentration, w/v) octylglucoside were performed followed by centrifugation, according to the procedure described in Section 2. The two pooled supernatants and final pellet were analyzed. Solubilized proteins recovered in the supernatants: 1.7 and 1.96 mg with Triton X-100 and octylglucoside, respectively (corresponding to 80.6 and 92.9% of the starting proteins respectively). Proteins in the final pellet: 0.4 and 0.17 mg with Triton X-100 and octylglucoside, respectively. The data shown are the mean values ± S.D. of five replicate determinations. Total activities are expressed in μU, specific activities in μU/mg of proteins. The assay of all glycohydrolases was performed at a final assay mixture concentration of 0.08% (w/v) Triton X-100 or 0.35% (w/v) octylglucoside.

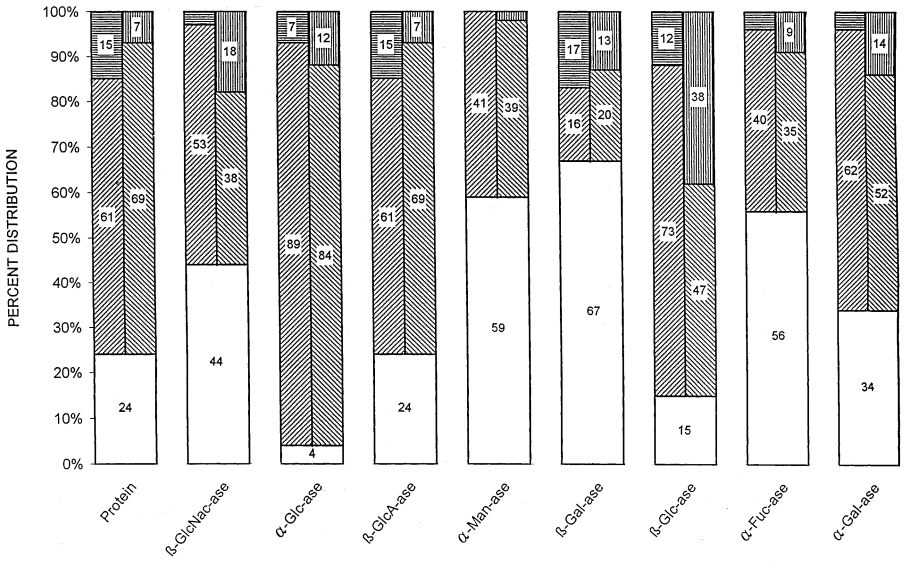


Fig. 2. Percent distribution of protein and different glycohydrolases of human erythrocyte plasma membrane in: (a) the non-integral, peripheral membrane-associated portion, released by 1.0 mol/l NaCl treatment (open bar); (b) the integral membrane-bound portion solubilized by Triton X-100 (hatched top right to bottom left) or octylglucoside (hatched top left to bottom right) treatment; and (c) the integral membrane = bound fraction resistant to detergent treatment (horizontally hatched, Triton X-100; vertically hatched, octylglucoside). Percent values were calculated on total protein and enzyme activities recovered in the supernatants and pellets obtained after each treatment assuming an equal distribution of losses in the different portions. The experimental data reported in Tables 2 and 3 were used. The figures reported in the columns correspond to percent values.

## 4. Discussion

The aim of the present investigation was to establish how several glycohydrolases, namely α- and β-Glc-ase, α- and β-Gal-ase, β-GlcNAc-ase, β-GlcA-ase, α-Fuc-ase and α-Man-ase, are associated or bound to the plasma membrane of human erythrocytes. First, optimal assay conditions were worked out for all tested enzymes. The data generated confirm previous reports [11] and, thanks to the improved assays, extend the knowledge on some properties of these enzymes. In fact, the specific activity values we observed were substantially higher than those reported previously [23,24], and the coefficients of analytical variation (both intra-run and day-to-day run) were in the range 1.4–7.3% (a performance quite satisfactory for membrane-bound enzymes). Moreover, we observed that high salt (NaCl) concentrations, and even more, detergent treatment (Triton X-100, octylglucoside) strongly affect (either inhibiting or activating) most of the enzymes investigated. The results obtained (summarized in Fig. 2) regarding the mode of membrane association/binding of each enzyme are consistent with the following pattern: (a) part of the enzyme can be released by treatment with 1.0 mol/l NaCl, indicating a membrane association mainly based on a network of ionic bonds. Presumably, this portion consists of a secreted form of the enzyme, or blood plasma-derived enzyme, adhering to the erythrocyte surface [29]; (b) a second part can be solubilized by treatment with detergents (Triton X-100, octylglucoside) known to differently affect specific membrane microdomains [13,16–18,30,34]. Therefore, this portion of the enzyme should belong to the affected microdomain; (c) a third minor part is constituted by an integral protein pertaining to membrane domains resistant to either one or the other detergent [13]; (d) none of the investigated enzymes appears to be sensitive to PIPLC treatment, excluding a membrane anchoring by a GPI bridge or, at least, a PIPLC-sensitive bridge. Curiously, acidic sialidase (pH 4.7) markedly differs from the other glycohydrolases in that it is mainly linked via a GPI anchor, does not exhibit any loose association to the membrane, and is completely resistant to Triton X-100 solubilization [6]. Of course, owing to the well-known existence in erythrocytes of a network of molecular connections between membrane- and cytoskeleton-linked proteins, it cannot be excluded that the observed pattern of membrane-associated erythrocyte glycohydrolases may be due, at least in part, to a connection of these enzymes with the cytoskeleton. It should be emphasized that, although displaying a similar general pattern of mode of association/anchoring to the membrane, differences in the distribution of the studied glycohydrolases among the different modes of association anchoring are remarkable. In the case of β-Gal-ase and α-Man-ase the major part (70% and 61%, respectively) is loosely associated, whereas for α-Glc-ase the major part is released by detergent treatment (89% and 85% with Triton X-100 and octylglucoside, respectively), the other glycohydrolases having an intermediate behavior. These observations are consistent with the notion that glycohydrolases have a different 'geographical' distribution in the erythrocyte membrane, where some domains are richer in some enzymes (for instance α-Glc-ase, β-GlcA-ase, α-Gal-ase in domains more sensitive to the used detergents) and other domains richer in other enzymes, like β-Glc-ase, which is present mostly in the octylglucoside-resistant membrane fraction, or like β-Gal-ase, α-Man-ase and α-Fuc-ase, most of which is associated in a loose way.

Two important questions arise from the presented results: do the different modes of membrane anchoring by the individual glycohydrolases reflect post-translational modifications of a common polypeptide, or the occurrence of isozymes of different primary or quaternary structures? What could be predicted about the different attachment modes of these enzymes from the knowledge of their polypeptide sequence de-

rived from the corresponding cDNAs? To the latter purpose it should be kept in mind that the tested glycohydrolases are membrane-bound or associated enzymes, for none of which the cDNA is known at present. Glycohydrolases with available cDNA are the lysosomal α-Man-ase [35,36], β-GlcNAc-ase [37], β-Glc-ase [38,39], α-Fuc-ase [40], α-Gal-ase [41], α-Glc-ase [42], β-GlcA-ase [43], and the endoplasmic reticulum [44] and Golgi apparatus [45] α-Man-ase. Except for the brush border glycosidases [46], the only plasma membrane-bound glycohydrolase having coded cDNA is sialidase [47]. Anyhow, the cDNA of the plasma membrane sialidase is different from that of the lysosomal and cytosolic sialidases [48,49], and the cDNAs of the lysosomal, endoplasmic reticulum and Golgi apparatus α-Man-ases are also different, suggesting that the relationship between structural features and modes of plasma membrane anchoring relationship can be predicted only relying on the cDNA of the individual plasma membrane-bound enzymes.

In conclusion, the present investigation, based on the development of reliable assays for ghost-bound glycohydrolases, provides a tentative view of how these enzymes are linked to the membrane: loose association to the surface, integral insertion, possible occurrence in different membrane microdomains. Experiments are presently in progress aiming at (a) ascertaining the possible involvement of individual isozymes of each glycohydrolase having a different mode of membrane anchoring or association, (b) establishing the presence of glycohydrolases in isolated membrane microdomains, (c) searching for the cDNA of these enzymes in order to study structure/membrane anchoring relationships and (d) exploring the behavior of these enzymes in the plasma membrane of erythrocytes during physiological and pathological ageing.

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