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Identification of a Sialoglycopeptide Released by Self-Digestion from Human Erythrocyte Membranes

By AUGUSTA BROVELLI, GIOSUE PALLAVICINI, FABIOLA SINIGAGLIA, CARLO L. BALDUINI and CESARE BALDUINI
Institute of Biological Chemistry, Faculty of Sciences, University of Pavia, 27100 Pavia, Italy

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Membranes from human O Rhesus-positive erythrocyte 'ghosts' were tested *in vitro* for their ability to digest their own glycoproteins. 'Ghost' membranes incubated in Tris/HCl buffer, pH 7.4, release a sialoglycopeptide, which contains glucosamine, galactosamine, galactose and mainly polar amino acids. Chemical composition, molecular size and aggregation properties suggest that this glycopeptide may be a fragment of glycophorin.

The presence of glycoproteins on the external surface of human erythrocyte membranes is described in several reviews (Guidotti, 1972; Hughes, 1973) and reports (Winzler, 1969; Marchesi *et al.*, 1972, 1973). Their carbohydrate moieties are antigenic determinants (Moston, 1962; Cook & Eylar, 1965) and receptors for viruses and plant agglutinins (Springer *et al.*, 1966). Sialic acid residues are responsible for the negative charge at the cell surface (Eylar *et al.*, 1962) and are involved in M- and N-group specificity (Ebert & Jurgen, 1972). The sialic acid content decreases as erythrocytes age (Balduini *et al.*, 1974; Baxter & Beeley, 1975), and its removal from erythrocyte membranes by neuraminidase digestion significantly decreases the half-life of these cells (Jancik & Schauer, 1974; Durocher *et al.*, 1975; Gattegno *et al.*, 1975). To investigate the mechanism determining hydrolysis of sialic acid from membranes *in vivo*, we have suggested that an enzyme system exists providing for sialic acid removal in suitable conditions. To check this hypothesis we have tested *in vitro* for the conditions in which 'ghost' membranes can release their sialic acid. The product of this self-digestion was isolated by chromatography on Dowex 2 (X8) and gel filtration on Bio-Gel P-2, P-6 and P-30 columns, and its chemical composition was characterized.

Experimental

Preparation of erythrocyte 'ghosts'

Fresh human blood (70–100ml) of the O Rh^+ (Rhesus positive) group was collected for each experiment from the veins of donors by using 3.8% (w/v) sodium citrate as anti-coagulant. Plasma and buffy coat (leucocytes) were carefully removed after centrifugation at 4°C at 1000*g* for 15min, and the erythrocytes were then suspended in 5mM-Tris/HCl buffer, pH 7.4, containing 1mM-EDTA, lysed and washed as described by Marchesi & Palade (1967). These 'ghost' membranes were then used for the self-digestion experiments.

Self-digestion experiments

'Ghost' membranes were suspended in 0.5vol. of 0.05M-Tris/HCl buffer, pH 7.4, and incubated for progressively longer periods at 37°C, with shaking. Control experiments were performed by heating the 'ghosts' for 10min at 100°C or incubating intact 'ghost' membranes at 4°C. After incubation, the samples were centrifuged at 25000*g* for 15min; the supernatant was tested for free and linked sialic acid. pH-dependence of sialic acid release was tested with different buffers in the pH range 3.6–9.0, by using 0.1M-sodium acetate buffer, phosphate buffer (0.06M-KH_2PO_4 and 0.06M-Na_2HPO_4 appropriately mixed) and 0.05M-Tris/HCl buffer respectively for pH ranges 3.6–5.6, 5.6–6.8 and 7.0–9.0.

Isolation of 'ghost' self-digestion products

The supernatant from self-digestion experiments was applied to a column (1.5cm×4.0cm) of Dowex 2 (X8; acetate form), equilibrated with 0.1M-acetic acid. After washing with water, elution was carried out with 7ml of 1M-sodium acetate, pH 4.6. A portion of the eluate from Dowex 2, containing 100–150μg of sialic acid, was filtered on a Bio-Gel P-2 column (1.5cm×85cm); elution was with double-distilled water, flow rate was 7ml/h, and 4.5ml fractions were collected. Protein elution was monitored at 280nm with an ISCO UA4 absorbance monitor. The presence of sialic acid was assayed in each fraction by the Svennerholm (1958) test. Fractions corresponding to each peak were pooled, concentrated to a known volume and analysed. Gel filtrations on Bio-Gel P-6 and Bio-Gel P-30 columns (1.5cm×85cm) were performed in the same conditions, with an effluent flow of 13ml/h.

Analytical methods

Hexosamines and amino acids were determined with the Hitachi–Perkin–Elmer liquid chromatograph by the method of Moore & Stein (1951). Sialic acid was measured by the Svennerholm (1958) and Warren (1959) methods. Total sialic acid content of 'ghost' membranes was determined after hydrolysis of dried 'ghosts' for 1 h at 80°C in 0.05 M-H_2SO_4 as previously reported (Balduini *et al.*, 1974).

Gas–liquid chromatography

Trimethylsilyl derivatives of neutral sugars were analysed as reported by Cetta *et al.* (1972).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

This was carried out on sodium dodecyl sulphate-solubilized 'ghost' membranes as described by Fairbanks *et al.* (1971), except that the current used was 3–3.5 mA/tube. Acrylamide concentration was 5.6% (w/v).

The gels were stained with Coomassie Blue and periodic acid–Schiff reagent (Fairbanks *et al.*, 1971). The electrophoretograms were recorded with a Saitron 803 densitometer (Florence, Italy), by using the yellow–green filter.

Results

Isolation of sialic acid-containing material

The sialic acid release reaches its highest value (50 nmol/ml of packed 'ghosts') after 4–5 h of incubation, and is pH-dependent, with two optima at pH 4.6 and 7.4–7.6. This release does not take place when 'ghosts' are preincubated for 10 min at 100°C or when the incubation is carried out at 4°C.

The sialic acid detected in the incubation medium is not in a free form, but it is linked by a glycosidic bond, as indicated by the Svennerholm (1958) and Warren (1959) tests. The products of membrane self-digestion at pH 7.4 were chromatographed on a column (1.5 cm × 4 cm) of Dowex 2 (X8); the eluate from the column contained sialic acid, glucosamine, galactosamine and mainly polar amino acids.

Gel filtration on Bio-Gel P-2, P-6 and P-30

To purify the eluate from Dowex 2 (X8) and to investigate its molecular size, gel filtration of the sialic acid-containing material on Bio-Gel P-2, P-6 and P-30 was performed. The elution patterns from Bio-Gel P-2 and P-6, recorded at 280 nm, resulted in three peaks; the first, corresponding to the void volume, contains 95–98% of the sialic acid of the sample, and also glucosamine, galactosamine, galactose and mainly polar amino acids. The second peak, retarded by the column, is mainly composed of polar amino acids and does not contain carbohydrate residues. The third peak corresponds to the sodium acetate present in the Dowex effluent buffer.

The E_{280} elution pattern from Bio-Gel P-30 also shows three peaks, all retarded by the gel. The first peak contains 90–95% of the sialic acid of the sample and the same carbohydrate residues as in the first peak from Bio-Gel P-2 and P-6. The second peak contains only amino acids and the third contains sodium acetate.

A low absorbance in the void-volume region was also observed. The chemical composition of the first peak eluted from Bio-Gel P-30 is reported in Table 1.

It is worth mentioning that in some experiments the glycopeptide peak was freeze-dried, dissolved in water and then rechromatographed on the same Bio-Gel P-30 column; a different distribution of the sample results in these conditions. A portion is eluted with the same volume as in the first chromatography, but the remaining material appears in the void-volume region; its chemical composition overlaps with that of the retarded glycopeptide. This behaviour may result from an aggregation phenomenon reminiscent of the reversible polymerization of glycophorin monomers in solution (Janado *et al.*, 1973; Janado, 1974).

Table 1. *Chemical composition of the sialic acid-containing material isolated by gel filtration on Bio-Gel P-30*

Gel filtration was carried out after incubation of 'ghost' membranes at pH 7.4 and separation of sialic acid-containing material on Dowex 2 (X8). Values are the average ± S.D. of three experiments. Basic amino acids, being present in very small amounts, were determined only in some experiments and are not reported in the Table.

	Content (μmol/100 μmol)
Sialic acid	29.14 ± 2.23
Glucosamine	1.91 ± 0.57
Galactosamine	6.76 ± 1.78
Galactose*	Present
Aspartic acid	7.75 ± 2.05
Threonine	7.65 ± 1.30
Serine	11.84 ± 1.38
Glutamic acid	12.06 ± 1.50
Proline	Traces
Glycine	7.88 ± 1.74
Alanine	5.64 ± 0.58
Valine	3.17 ± 1.05
Isoleucine	2.12 ± 0.94
Leucine	4.08 ± 0.56

* Qualitatively determined by g.l.c.

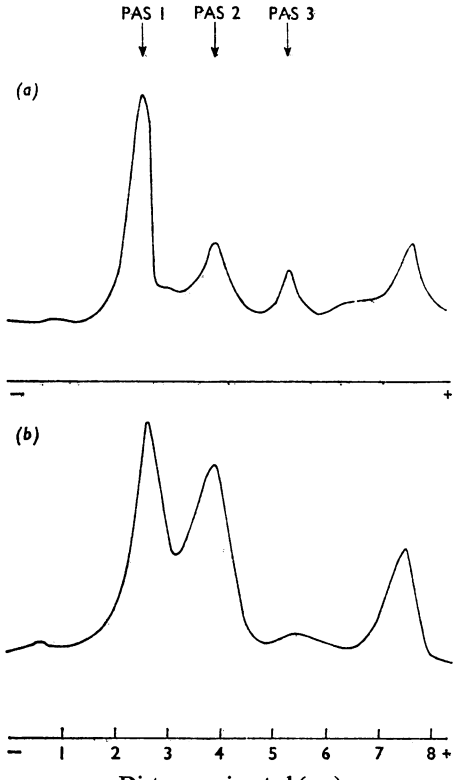

Fig. 1. *Sodium dodecyl sulphate / polyacrylamide-gel electrophoresis of sodium dodecyl sulphate-solubilized 'ghost' membranes before (a) and after (b) 4h incubation at pH7.4*

The electrophoresis was carried out as described by Fairbanks *et al.* (1971): the current used was 3–3.5mA/tube. Acrylamide concentration was 5.6% (w/v). Gels were stained with periodic acid–Schiff reagent (PAS); electrophoretograms were recorded with a Saitron densitometer by using the yellow–green filter.

Sodium dodecyl sulphate / polyacrylamide-gel electrophoresis

The electrophoretograms, after staining of incubated and intact 'ghost' membranes with periodic acid–Schiff reagent, are reported in Fig. 1. An evident modification of the normal pattern appears after the self-digestion process, consisting of an increase in the ratio of peaks PAS 2/PAS 1 and of a decrease in the PAS 3 band.

Discussion

Our results agree well, and partially explain the above-mentioned evidence on the role of sialic acid in the determination of erythrocyte life-span (Jancik & Schauer, 1974; Durocher *et al.*, 1975; Gattegno *et al.*, 1975). It has been proved that O Rh^+ erythrocyte 'ghost' membranes can release sialic acid-containing glycopeptides from the membrane itself. This phenomenon shows the characteristics of an enzyme action: it is pH-dependent, with two optima at pH4.6 and pH7.4–7.6; heat-treated membranes, like membranes incubated at 4°C, do not give rise to surface glycoprotein breakdown; the sialic acid release reaches its highest value after 4–5h of incubation.

The glycopeptide recovered in the medium was characterized after isolation on a Dowex 2 (X8) column filtration on Bio-Gel columns of different porosity; the sialic acid-containing material, excluded by Bio-Gel P-2 and P-6, was retarded by Bio-Gel P-30 and eluted as a single peak. The carbohydrate core was composed of sialic acid, glucosamine, galactosamine and galactose; in the protein moiety, mainly polar amino acids were present.

As to the origin of this glycopeptide, it must be remarked that its mol.wt. is presumably lower than 30000; moreover, it was calculated that its sialic acid content accounts for about 35–60% of the total membrane sialic acid. This evidence, together with carbohydrate and protein composition, supports the hypothesis that the glycopeptide released in the self-digestion experiments is a portion of the glycophorin molecule, which contains about 80% of the total membrane sialic acid (Winzler, 1969). The aggregation phenomenon shown by the released glycopeptide in solution is further evidence that it comes from glycophorin, whose aggregating properties have been elucidated (Janado *et al.*, 1973; Janado, 1974). In any case, the glycoprotein assembly, after membrane self-digestion, is sharply modified, as proved by the periodic acid–Schiff reagent pattern after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of incubated 'ghosts'.

As for the mechanism by which a sialic acid-containing glycopeptide is split from the 'ghosts', this may be attributed to a proteinase activity linked to the membrane. The presence of proteinases able to digest 'ghost' glycoproteins on erythrocyte membranes has been reported by several authors (Morrison & Neurath, 1953; Moore *et al.*, 1970; Bernacki & Bosmann, 1972; Tokes & Chambers, 1975); their optimum pH values were similar to those at which we observe maximum sialic acid release. However, the product of glycoprotein hydrolysis and the biological meaning of this mechanism have never been investigated.

On the basis of the above-mentioned evidence on the decrease of sialic acid in old erythrocyte membrane (Balduini *et al.*, 1974; Baxter & Beeley, 1975) and the role of sialic acid in determining erythrocyte survival in the circulation (Jancik & Schauer, 1974; Durocher *et al.*, 1975; Gattegno *et al.*, 1975), the possible role of membrane proteinases could be to determine the

membrane modifications typical of the old erythrocyte; as a consequence of these modifications, the erythrocyte could be recognized by the spleen and destroyed. It is evident that, if this hypothesis is true, a mechanism must exist that is responsbile for the inhibition of the proteinase activities in the young cell and for their activation during the aging process.

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