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Are Polyphosphoinositides Associated with Glycophorin in Human Erythrocyte Membranes?

By SHIVENDRA D. SHUKLA, ROGER COLEMAN, J. BRYAN FINEAN and ROBERT H. MICHELL
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

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Glycophorin prepared by a lithium di-iodosalicylate-extraction/phenol-partition method was rich in polyphosphoinositides (phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-bisphosphate), but glycophorin extracted by Triton X-100 showed no such enrichment. The enrichment observed in the former preparations appeared not to be caused by pre-existing association between glycophorin and polyphosphoinositides in the human erythrocyte membrane, but to be largely a consequence of the preparative procedures.

Glycophorin is the major sialoglycoprotein of human erythrocyte membrane (Furthmayr, 1977). Marchesi and his colleagues, by using ^{32}P labelling (Shapiro & Marchesi, 1977) and ^{31}P n.m.r. (Armitage *et al.*, 1977), have presented evidence that glycophorin preparations isolated from erythrocytes by extraction with lithium di-iodosalicylate contain appreciable quantities of polyphosphoinositides (phosphatidyl-*myo*-inositol 4-phosphate or diphosphoinositide, and phosphatidylinositol 4,5-bisphosphate or triphosphoinositide) and this has been confirmed by Buckley (1978). Indeed, the studies of Armitage *et al.* (1977) have gone so far as to suggest a specific association between the hydrophobic membrane-spanning domain of glycophorin and diphosphoinositide. By contrast, van Zoelen *et al.* (1977) found that the lipids associated with glycophorin isolated by this method were particularly rich in phosphatidylserine; they made no mention of polyphosphoinositides, despite the fact that they used acidic extraction procedures that would have extracted these lipids.

Interactions between polyphosphoinositides and particular membrane proteins could be of substantial importance, especially because Ca^{2+} -stimulated polyphosphoinositide breakdown may be implicated in the control of erythrocyte shape (Allan & Michell, 1978) and the monoester phosphate groups of polyphosphoinositides show rapid turnover (Hawthorne & White, 1975). We have therefore investigated in greater detail the distribution of polyphosphoinositide in various fractions obtained during the isolation of glycophorin by two different procedures. We conclude that the reported association between polyphosphoinositide and isolated glycophorin (Armitage *et al.*, 1977) is largely circumstantial. In general, our results indicate that the majority of the polyphosphoinositides of the membrane are not associated with glycophorin.

Materials and Methods

Materials

Human blood was obtained from the regional blood-transfusion centre and used within 4–5 days of donation. Lithium 3,5-di-iodosalicylate was purchased from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K., or prepared by neutralization of 3,5-di-iodosalicylate with LiOH (Marchesi & Andrews, 1971). Triton X-100 was purchased from Rohm & Hass, Croydon, Surrey, U.K. All other chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K., or Sigma, and were of analytical-reagent grade.

Methods

Preparation of 'ghosts.' Human erythrocytes were rejuvenated to restore their polyphosphoinositide content (Shukla *et al.*, 1978). Haemoglobin-free 'ghosts' were prepared from these cells by repeated lysis and washing in 20 mM-Tris/HCl, pH 7.4, containing 1 mM-EDTA.

Procedures for glycophorin isolation. (1) The procedure of Marchesi & Andrews (1971). Packed 'ghosts' (1 vol.) were suspended in 1 vol. of 0.6 M-lithium 3,5-di-iodosalicylate in 0.1 M-Tris/HCl, pH 7.5, and were stirred for 15 min at room temperature (25°C). Chilled water (2 vol.) was then added and the suspension was stirred for a further 10 min at 4°C. It was then centrifuged at 45000g for 90 min at 4°C: the lithium 3,5-di-iodosalicylate-insoluble pellet was kept for analysis. The lithium 3,5-di-iodosalicylate-soluble supernatant was mixed with an equal volume of freshly prepared aqueous 50% (w/v) phenol. The mixture was stirred at 4°C for 15 min and centrifuged at 4000g for 1 h at 4°C to give a lower (phenol) phase and an upper (aqueous) phase. The upper phase was separated and dialysed against several changes of water at 4°C over a period of 30–

36h. The dialysed material was concentrated by freeze-drying and contained all the glycophorin (lithium 3,5-di-iodosalicylate-soluble glycophorin fraction).

(2) The procedure of Gerritsen *et al.* (1978). Packed 'ghosts' (1 vol.) were treated with 3 vol. of 0.1% (v/v) Triton X-100 in 20mM-Tris/HCl, pH 7.0, at 4°C for 20 min. The suspension was then centrifuged at 35000g at 4°C for 15 min. The Triton X-100-soluble supernatant, which was freeze-dried, contained all the glycophorin (Triton-soluble glycophorin fraction). The insoluble residual pellet (Triton-insoluble pellet) was also retained for analysis.

Analytical methods. The polypeptides of the various fractions were resolved by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969) and stained by the 'Stains All' reagent (King & Morrison, 1976). This gave, on the same gel, a red colour with polypeptides and a blue colour with glycopolypeptides.

For the extraction of lipids, 1 ml of aqueous sample was mixed with 4 ml of chloroform/methanol (2:1, v/v) containing HCl (1 ml of 12M-HCl in 150 ml of chloroform/methanol) and stirred intermittently for 15 min at room temperature. The suspension was briefly centrifuged to separate two phases. The upper phase was discarded and the lower phase plus interfacial material was washed successively with 1.0 ml of 1M-HCl and 0.5 vol. of chloroform/methanol/1M-HCl (3:48:47, by vol.). The two phases were again separated and the upper phase and interfacial material were discarded. The lower phase was washed 3 times with 0.4 vol. of 0.05M-CaCl₂/methanol/chloroform (47:48:3, by vol.) and then dried under vacuum. Re-extraction of the interfacial material by acidified chloroform/methanol did not extract any additional lipids.

Results

The distribution of total phospholipids and of polyphosphoinositides amongst the various fractions are presented in Table 1, and these results are summarized in Scheme 1.

Gel electrophoresis showed that the lithium 3,5-di-iodosalicylate-soluble glycophorin fraction was very rich in glycoprotein material with the behaviour expected of glycophorin, whereas this was absent from the lithium 3,5-di-iodosalicylate-insoluble pellet. By contrast, lithium 3,5-di-iodosalicylate extracted only about half of the total phospholipid and just over one-third of the total polyphosphoinositides from the 'ghosts.' During subsequent phenol partition almost all of the phospholipids, with the exception of polyphosphoinositides, went into the phenol-rich lower phase, with the result that about 10% of the phospholipid molecules present in the lithium 3,5-di-iodosalicylate-soluble glyco-

Table 1. *Distribution of erythrocyte phospholipids and polyphosphoinositides during the isolation of glycophorin by two different procedures*
Individual fractions were isolated as described under 'Methods' and in Scheme 1. Lipids were extracted and analysed for phosphate as described under 'Methods.'
Results are presented as means $\pm$ s.e.m. for the numbers of experiments indicated in parentheses. Abbreviation: n.d., none detected.

Material analysed	Phospholipid of (P μ mol/10 ml of packed 'ghosts')				Molar percentage of total phospholipids			
	Total phospholipid		Diphosphoinositide		Diphosphoinositide		Triphosphoinositide	
'Ghosts'	24.5 $\pm$ 1.1 (10)	0.19 $\pm$ 0.03 (10)	0.35 $\pm$ 0.02 (10)	0.48	0.39	0.48		
Lithium 3,5-di-iodosalicylate-insoluble pellet	11.6 $\pm$ 1.1 (5)	0.1 $\pm$ 0.01 (3)	0.33 $\pm$ 0.03 (3)	0.97	0.44	0.97		
Lithium 3,5-di-iodosalicylate/phenol lower phase	12.6 $\pm$ 1.9 (5)	n.d.	n.d.	n.d.	n.d.	n.d.		
Lithium 3,5-di-iodosalicylate-soluble glycophorin fraction	0.94 $\pm$ 0.1 (5)	0.07 $\pm$ 0.02 (3)	0.18 $\pm$ 0.03 (3)	7.6	4.4	7.6		
Triton-insoluble pellet	18.6 $\pm$ 0.9 (5)	0.13 $\pm$ 0.01 (5)	0.24 $\pm$ 0.01 (5)	0.44	0.35	0.44		
Triton-soluble glycophorin fraction	3.0 $\pm$ 0.3 (5)	0.03 $\pm$ 0.01 (5)	0.07 $\pm$ 0.01 (5)	0.78	0.51	0.78

Scheme 1. Fractions obtained during isolation of glycoporphin by two different procedures. The details of the procedures were as described in the Materials and Methods section. Values represent the percentage of total phospholipid or total polyphosphoinositides of the 'ghosts'.

phorin fraction were polyphosphoinositides, compared with only about 0.8% in the initial 'ghost' preparations.

It seemed possible that a major factor contributing to this relative enrichment of polyphosphoinositides in the lithium 3,5-di-iodosalicylate-soluble glycoporphin fraction was a tendency of the highly charged polyphosphoinositides to distribute into the aqueous phase of the phenol-partition step, whereas other less-polar phospholipids went into the phenol-rich phase. Support for this view came from experiments in which polyphosphoinositides extracted from 'ghosts' by using acidified chloroform/methanol were found to distribute quantitatively ($\geq 95\%$) into the upper phase of the phenol/water system even in the absence of glycoporphin, provided that they were partitioned in their free-acid forms or as univalent-cation salts (as they are likely to be after lithium 3,5-di-iodosalicylate extraction).

When 'ghosts' were extracted with Triton X-100 less than 20% of the total protein of the 'ghosts' was 'solubilized,' but this fraction contained all of the glycoporphin. In this case the 'solubilized' glycoporphin was accompanied by about 10–20% of the total phospholipid and of the polyphosphoinositides (Table 1 and Scheme 1), despite the fact that any relatively strong ionic association between glycoporphin and polyphosphoinositides might have been expected to survive this rather mild 'solubilization' procedure.

Discussion

The present studies confirm that the lipids that are isolated with glycoporphin when the lithium 3,5-di-iodosalicylate/phenol procedure is used are substantially enriched in polyphosphoinositides, compared with the phospholipid of the starting erythrocyte 'ghosts.' In the original report by Armitage *et al.* (1977) no quantitative estimate of this enrichment was offered, and subsequent reports have either emphasized the high proportion of acidic lipids associated with glycoporphin, but apparently without detection of polyphosphoinositides (van Zoelen *et al.*, 1977), or have indicated that rather more than one-third of the lipid molecules isolated with the lithium 3,5-di-iodosalicylate-soluble glycoporphin fraction are either diphosphoinositide or triphosphoinositide (Buckley, 1978). Our estimate falls between these extremes, at about one-tenth of the total lipid in such preparations. The reason for these differences, particularly for the difference between Buckley's data and ours, are unknown, but it may be relevant that the white 'ghosts' that were isolated by Buckley from outdated blood-bank blood, and then incubated with ATP, appeared to contain far more polyphosphoinositides than were observed in the present

studies, where membranes were prepared from 'rejuvenated' erythrocytes.

It is clear from Table 1 and Scheme 1 that this relative enrichment of the glycophorin fraction with respect to polyphosphoinositides occurred at the phenol-partition step of the isolation procedure, rather than during the initial lithium 3,5-di-iodosalicylate extraction. Furthermore, we have demonstrated that when extracted erythrocyte lipids, in the form of univalent cation salts or free acids, are distributed between phenol and water, then polyphosphoinositides go selectively into the aqueous phase. Although this process may be enhanced by the presence of proteins in the upper phase, these observations suggest that the selective enrichment of polyphosphoinositides in the glycophorin-containing fraction may well depend mainly on the solubility characteristics of polyphosphoinositides rather than on any specific tendency to associate with glycophorin. This behaviour of polyphosphoinositides in their more polar forms is essentially similar to that reported previously by Dawson (1965), except that in those experiments the apolar phase of the phase-partition system was chloroform rather than phenol.

The suggestion that the association between the lithium 3,5-di-iodosalicylate-soluble glycophorin fraction and polyphosphoinositides is coincidental receives further support from the observation that when glycophorin was extracted with Triton X-100 then the glycophorin-rich fraction contained less than half as much polyphosphoinositides as the lithium 3,5-di-iodosalicylate-soluble glycophorin fraction, with no selective concentration of polyphosphoinositides into the mixture of lipids that was extracted with glycophorin.

Although these results indicate that the published information that has pointed towards some specificity of association between polyphosphoinositides and glycophorin does not constitute good evidence for a pre-existing association in the erythrocyte membrane, they do not eliminate the possibility of some such association with a small proportion of the membrane's polyphosphoinositides.

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