^{31}P Nuclear Magnetic Resonance Evidence for Polyphosphoinositide Associated with the Hydrophobic Segment of Glycophorin A†

Ian M. Armitage,* Donald L. Shapiro, Heinz Furthmayr,‡ and Vincent T. Marchesi

ABSTRACT: Glycophorin A, the major human erythrocyte sialoglycoprotein, contains a significant amount of phosphorus when isolated by the lithium diiodosalicylate–phenol procedure. Only a small percentage (approximately 1%) of this phosphorus is phosphoprotein. ^{31}P nuclear magnetic resonance (NMR) analysis of glycophorin A has identified the remaining phosphorus content as phospholipid in origin. From the ^{31}P chemical shifts, the phospholipid has been identified as diphosphoinositide. ^{31}P NMR spectra of the peptides produced by trypsin hydrolysis of glycophorin A reveal that all the diphosphoinositide is closely associated with the hydrophobic region of the protein, suggesting that there is a specific affinity between this phospholipid and the intramembranous portion of glycophorin A.

Glycophorin A is the major sialoglycoprotein of the human red blood cell membrane (Furthmayr et al., 1975). Recent studies of its primary structure (Tomita and Marchesi, 1975) and the results of attempts to radiolabel it in situ (Silverberg et al., 1976) indicate that a short segment of its polypeptide chain spans the lipid bilayer. The amino acid sequence of this portion of the polypeptide chain is also extremely rich in nonpolar amino acids (Tomita and Marchesi, 1975), consistent with its presumed location within the interior of the membrane.

Studies on the phosphorylation of glycophorin A in intact red blood cells indicated that a relatively small amount of ^{32}P inorganic phosphate (approximately 1 mol per 100 mol) was incorporated into serine and threonine residues of the glycophorin A molecule (Shapiro and Marchesi, 1977). However, approximately 100 times more phosphorus was found to be associated with the glycoprotein (Shapiro and Marchesi, 1977) when direct chemical determinations were carried out. ^{31}P NMR[1] was considered the most direct and nonperturbing method to determine the nature and the position of the phosphorus associated with the glycophorin A molecules.

The glycophorin A preparations used in these studies were obtained by a purification procedure using lithium diiodosalicylate (LIS) to dissociate red cell ghost membranes followed by partitioning of the solubilized proteins in the aqueous phase of a 25% phenol–water mixture (Marchesi and Andrews, 1974). Solubilized sialoglycoproteins were subsequently delipidated with ethanol, chloroform–methanol (2:1), and chloroform–methanol–12 N HCl (200:100:1) and the final purified product was obtained by gel filtration in *N,N*-dimethyllaurylamine *N*-oxide (Ammonyx-LO detergent). A comparative ^{31}P NMR analysis was carried out on the glycoprotein preparations at each stage of the purification and on the peptides produced by trypsin hydrolysis of purified glycophorin A.

Materials and Methods

Preparation of erythrocyte membranes (Dodge et al., 1963), isolation of glycophorin by the LIS–phenol procedure (Marchesi and Andrews, 1974), purification of glycophorin A (Furthmayr et al., 1975), and the preparation of the peptides by digestion with trypsin (Jackson et al., 1973) were as previously described.

Treatment of glycophorin with phospholipase C was as follows: Phospholipase C, chromatographically pure (Worthington), was reacted with glycophorin at a ratio of 20 units of enzyme to 60 mg of glycophorin. Reaction conditions were: 0.1 M Tris-HCl (pH 7.0), 0.1 mM $ZnCl_2$, 10 mM $CaCl_2$, 37 °C for 2 h. The reaction was stopped by lowering the temperature to 4 °C and the reaction components were extensively dialyzed against water at 4 °C. The dialyzate was recovered by lyophilization and solubilized in Ammonyx buffer (0.5% Ammonyx-LO, 0.1 M NaCl, 20 mM Tric-HCl, pH 7.2) for study by NMR. The phospholipase C contained no phosphatase activity against histone.

Gel filtration of glycophorin in sodium dodecyl sulfate was as follows: Glycophorin A was solubilized in a solution containing 0.2% sodium dodecyl sulfate, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA and applied to a 95 × 2.5 cm column of Sepharose 6B equilibrated with the same buffer and maintained at a temperature of 45 °C. Under these conditions glycophorin A eluted as a single peak of approximate molecular weight 55 000 (Furthmayr and Marchesi, 1976). The glycophorin-containing fractions were pooled and extensively dialyzed against water at 22 °C. The dialyzate was lyophilized and the glycophorin solubilized in Ammonyx buffer.

^{31}P NMR spectra were recorded on a FT-Bruker HFX-90 MHz spectrometer operating at 36.4 MHz for ^{31}P at 28 ± 2 °C. Deuterium oxide (D_2O), internal, or in a 3-mm coaxial capillary insert, was used as a field-frequency lock. All spectra were obtained under conditions of proton noise decoupling. A spectral width of 5000 Hz was used to maximize the S/N improvement from a 5000-Hz band width crystal filter with an acquisition time of 0.2 s. For all spectra shown an interpolation expansion routine was employed providing a resolution of 1.22 Hz/point. Solutions of glycophorin and peptide frag-

† From the Section of Physical Sciences and the Departments of Pathology and Pediatrics, Yale University School of Medicine, New Haven, Connecticut 06510. *Received July 23, 1976.* This work was supported by Grants 5RO1GM-19,929, Membrane Center Grant P17-GM21714, Research Corporation, and AM18778-01 from the National Institutes of Health and acknowledgment is made to the donors of The Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research.

‡ Supported by Public Health Service Training Grant GM-02140-03.

[1] Abbreviations used: NMR, nuclear magnetic resonance; P_i, inorganic phosphate; LIS, lithium diiodosalicylate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

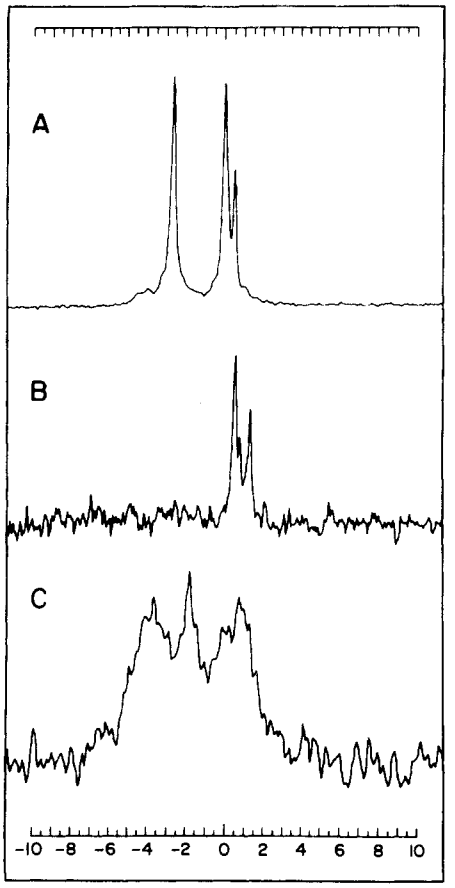

FIGURE 1. ^{31}P NMR spectra of soluble red cell membranes, phospholipid extracts, and glycophorin. (A) soluble red cell membranes to saturation in 3% sodium dodecyl sulfate, pH 8.3; (B) combined neutral and acid chloroform-methanol extract in 0.5% Ammonyx-LO in H_2O, pH 8.1; (C) 1.4×10^{-3} M glycophorin in H_2O-NaOH was added to raise the pH to 7.4.

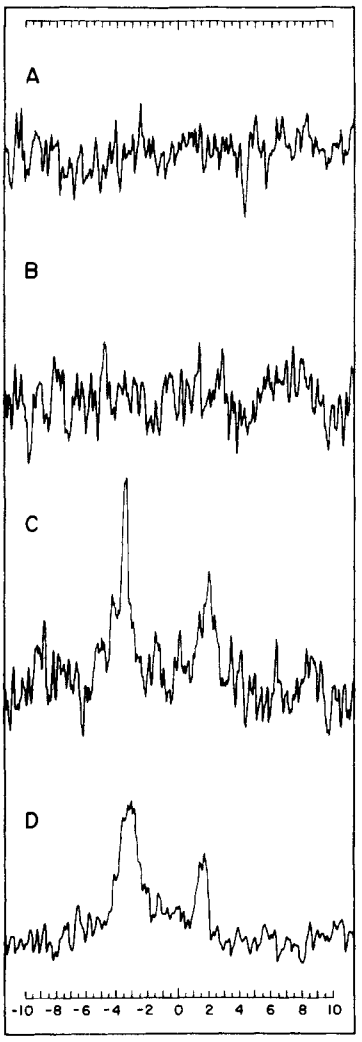

FIGURE 2: ^{31}P NMR spectra of glycophorin A and the peptides obtained from trypsin hydrolysis of glycophorin A. (A) Glycopeptides (1.1×10^{-3} M) from the N-terminal portion of glycophorin A in H_2O, pH 4.8; (B) 1.2×10^{-3} M C-terminal peptide from glycophorin A in H_2O, pH 5.3; (C) 0.9×10^{-3} M hydrophobic peptide in 0.5% Ammonyx-LO in H_2O, pH 11.9; (D) 1.2×10^{-3} M pure glycophorin A in 0.5% Ammonyx buffer (see Materials and Methods), pH 7.2.

ments ranged from 0.9 to 1.4×10^{-3} M in concentration. Analysis of some samples was possible at their native pH in water. However, for reasons of solubility and for optimal high-resolution ^{31}P spectra and chemical-shift separation, a variety of pH and detergent conditions were found necessary. ^{31}P chemical shifts were determined relative to external 85% H_3PO_4.

Pure L-α-glycerylphosphorylinositol phosphate (diphosphoinositide) was a gift of Dr. Bernard Agranoff, Department of Biological Chemistry, University of Michigan.

Results

^{31}P NMR of Whole Erythrocyte Membranes, Phospholipid Extract, and Glycophorin A. The ^{31}P NMR spectrum of soluble red cell membranes in 3% sodium dodecyl sulfate (pH 8.3) is shown in Figure 1A. The resonance to lowest field, −2.63 ppm, is assigned to free phosphate residual from the membrane isolation procedure. The resonances at 0 and +0.53 ppm are due to the major phospholipids of the human erythrocyte membrane. On the basis of previous ^{31}P chemical shift data for lipids (Assmann et al., 1974), the resonance at 0 ppm is assigned to L-α-glycerylphosphorylethanolamine and the resonance at +0.53 ppm to L-α-glycerylphosphorylcholine. However, because of the similarity in ^{31}P chemical shift for these lipids, the ^{31}P resonances from L-α-glycerylphosphorylserine and sphingomyelin are also undoubtedly represented in these resonances. The extract from the delipidation procedures (neutral and acid chloroform-methanol) used in the purification of glycophorin contains the ^{31}P lipid resonances shown in Figure 1B.

Initial attempts to study glycophorin A purified by the LIS-phenol procedure were carried out in aqueous solution. Adjusting the pH of this solution to pH 7.4 with NaOH gave rise to the ^{31}P spectrum shown in Figure 1C. At this pH there is a clear distinction between phosphate monoesters and diesters on the basis of the ionization state of phosphorus and this is represented in this spectrum. The resonance at −3.73 ppm corresponds to a normal phosphate monoester and that at +0.63 ppm to a diester phosphate. The resonance at −1.92 ppm is assigned to free phosphate. The unusually broad ^{31}P resonances from a protein of 31 000 molecular weight may be due to an aggregated state of the protein under these solvent conditions and/or the immobilization of bound lipid association with this aggregated state. Alternatively, the line width may be governed by the rate of chemical exchange between free and bound lipid. It is significant to mention that for several different samples of glycophorin A the pattern shown in Figure 1C was totally reproducible.

^{31}P NMR Spectra of Glycophorin A Peptides. Glycophorin

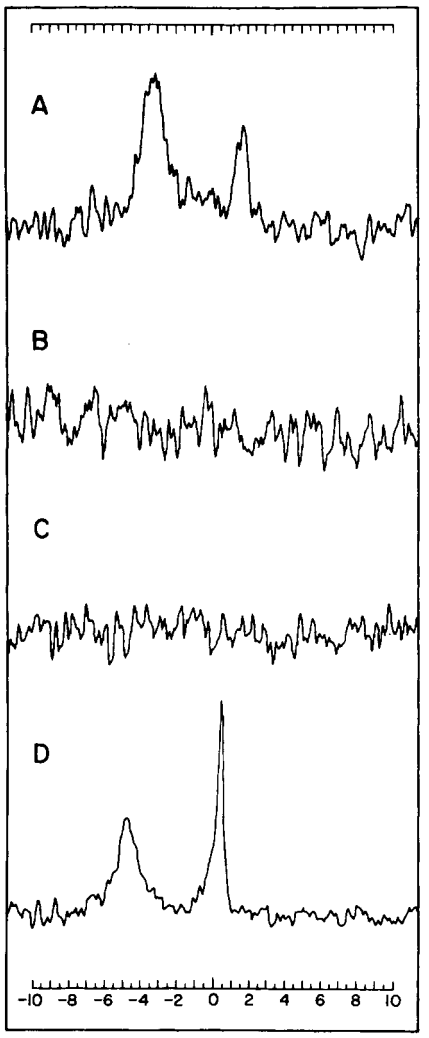

FIGURE 3: ^{31}P NMR spectra of glycophorin A and diphosphoinositide. (A) Pure glycophorin A (1.2×10^{-3} M) in 0.5% Ammonyx buffer (see Materials and Methods), pH 7.2; (B) 1.1×10^{-3} M glycophorin A, treated with phospholipase C, in 0.5% Ammonyx buffer, pH 7.2; (C) 1.0×10^{-3} M glycophorin A isolated by gel filtration in sodium dodecyl sulfate, in 0.5% Ammonyx buffer, pH 7.2; (D) 1×10^{-3} M L-α-glycerylphosphorylinositol phosphate in 0.5% Ammonyx-LO in H_2O, pH 7.4.

A was subjected to trypsin hydrolysis which yielded three distinct peptide classes: (1) glycopeptides from the N-terminal portion of the molecule, (2) the C-terminal peptide containing 30 amino acids, and (3) a hydrophobic peptide of 35 amino acid residues (Tomita and Marchesi, 1975). ^{31}P NMR of 1.0×10^{-3} M solutions of glycopeptides and the C-terminal peptide run under exactly the same conditions as whole glycophorin A gave rise to the spectra shown in Figures 2A and 2B. As is evident, neither of the samples contained any of the phosphate present in the whole protein. ^{31}P NMR analysis of the hydrophobic peptide fragment gave rise to a spectrum (Figure 2C) which accounts for the total phosphate concentration of pure glycophorin A (Figure 2D). The slightly narrower resonances from the peptide fragment probably reflect its lower molecular weight.

The Chemical Nature of Phosphate Resonances. In an effort to determine the chemical nature of the compound responsible for these phosphate resonances, two approaches were utilized which could distinguish between phosphoprotein and phospholipid phosphorus.

The first technique was to treat a sample of glycophorin A with phospholipase C which hydrolyzes the α-carbon esters of glycerides. The ^{31}P NMR spectrum of a sample so treated is shown in Figure 3B and shows an absence of resonances, indicating that both were of phospholipid origin. The second technique utilized was to isolate glycophorin A by gel filtration in sodium dodecyl sulfate. This procedure, which will not dissociate covalently bound protein phosphate, was also successful in eliminating the phosphate resonances as shown in Figure 3C.

Figure 3D is the ^{31}P NMR spectrum of diphosphoinositide. The resonances associated with the hydrophobic segment of glycophorin are similar to those of diphosphoinositide and are considered to arise from this phospholipid. Except for its more phosphorylated analogue triphosphoinositide, this is the only phospholipid in the erythrocyte membrane which contains both monoester and diester phosphates.

Precise ^{31}P chemical shifts, relative to that of H_3PO_4, for the various compounds discussed above and for some model reference compounds are collected in Table I.

TABLE I: ^{31}P Chemical Shifts of Phospholipids and Glycophorin.

Compound	pH	Model[b]	Chemical Shift[a] ppm from 85% H_3PO_4
Soluble red cell membranes	8.3	−2.70[c]	−2.63
			0
			+0.53
Phospholipid extract	8.1		+0.45
			+1.27
Glycophorin A	7.4	−4.15[d]	ca. −3.73
			−1.92
		+0.95[e]	ca. +0.63
Glycophorin A[f]	7.2	−4.05[d]	−3.2
		+0.95[e]	+1.37
Hydrophobic peptide	11.9	−4.50[d]	−3.5
		ca. +0.75[e]	+1.15
L-α-Glycerylphosphorylinositol	7.4		−4.50
			+0.85

[a] Precision of the measurements is ±0.04 ppm for sharp resonances and ca. ±0.1 ppm for broad resonances. [b] Model compounds are as designated in footnotes *c–f*. [c] P_i. [d] Myoinositol 2-monophosphate. [e] L-α-Glycerylphosphorylcholine. [f] Purified by gel filtration in Ammonyx-LO.

Discussion

These findings suggest that the intramembranous portion of glycophorin A has a specific affinity for polyphosphoinositide. The biological implications of this association are interesting since polyphosphoinositides have such unusual solubility properties and there is a considerable amount of evidence which suggests that they play an important role in the function of excitable membranes (Hokin, 1969).

The assignment of the ^{31}P resonances from glycophorin to bound diphosphoinositide is based on the following considerations. Elimination of the resonances with sodium dodecyl sulfate and phospholipase C treatment indicates that both resonances arise from a phospholipid. In addition, the spectrum indicates the presence of mono- and diester phosphate in approximately equimolar amounts and polyphosphoinositides

are the only erythrocyte membrane phospholipids which contain both mono- and diester phosphates. It is likely that small amounts of triphosphoinositide are also present, as studies (Shapiro and Marchesi, 1977) indicate that diphosphoinositide accounts for 80% and triphosphoinositide for 20% of the polyphosphoinositide extractable from erythrocyte membranes by the procedures employed. Equimolar quantities of phosphatidic acid, which contains a single monoester phosphate, and a phospholipid such as phosphatidylethanolamine which contains a single diester phosphate could also account for the spectrum observed. However, these phospholipids are extractable with neutral chloroform–methanol while the polyphosphoinositides are the major class of phospholipids which are not readily extractable from tissues with neutral organic solvents. The use of acidified organic solvent mixtures significantly enhances their removal (Hauser and Eichberg, 1973), but removal may still be incomplete as these studies verify. Finally, the chemical shifts and relative peak areas for the ^{31}P resonances of pure diphosphoinositide, Figure 3D, are in accord with the spectrum obtained from the hydrophobic segment of glycophorin. The resonance to lowest field, −4.5 ppm, is assigned to the monoester phosphate and the resonance at +0.85 ppm to the diester phosphate. The much broader resonance from the monoester resonance at −4.5 ppm is due to the presence of small amounts of paramagnetic impurities which preferentially complex this dianionic phosphate in the absence of other chelating agents. Addition of 16 mM EDTA (not shown) produced a sharpening of this resonance with a resultant line width similar to that of the diester phosphate. The chemical shift remains unchanged (Cohn and Hughes, 1962). The low-field position of this resonance, −4.5 ppm, compared with that of phosphatidic acid at the same pH, −0.35 ppm, may simply be a result of a difference in the phosphate p*K* or, if one applies the recent empirical correlation proposed by Gorenstein (Gorenstein, 1975), may reflect a smaller (<104°) O-P-O bond angle for the inositol phosphate. From the crystal structure of myoinositol 2-phosphate monohydrate (Yoo et al., 1974), the smallest O-P-O bond angle is 104° between two protonated oxygens which in the dianionic form may be reduced to ca. 102° and would account for a chemical shift similar to the observed chemical shift. The ^{31}P resonances from pure glycophorin A appear at −3.2 and +1.37 ppm and are assigned to phosphate mono- and diester, respectively (Figure 3A). The chemical-shift difference of 1.3 and 0.52 ppm to high field for mono- and diester, respectively, further indicates that there exists a strong association between the phospholipid and protein. Whether this shift is the result of a shift in the phospholipid p*K* upon association or is the result of an expansion of both O-P-O bond angles in the lipid induced by the surrounding protein structure is not known. Nevertheless, the latter possibility offers a reasonable explanation since interaction through one oxygen of both phosphates would be consistent with a strong steric repulsion for the other oxygen at the same point of attachment to the protein.

The physiological significance of this association between polyphosphoinositide and glycophorin A cannot yet be assessed since we cannot rule out the possibility that polyphosphoinositide is bound nonspecifically to the hydrophobic segment of glycophorin A during the isolation or purification procedure employed in this study. While the amphipathic nature of polyphosphoinositides suggests a physiological interaction with certain proteins, the same characteristics could predispose such molecules to artifactual interactions when in situ relationships are disrupted during solubilization. Salts of polyphosphoinositides may be highly water soluble, and, in aqueous solutions hydrophobic and ionic interactions may act cooperatively to produce a strong binding affinity particularly to proteins which contain hydrophobic segments. Interactions between polyphosphoinositides and proteins such as albumin and cytochrome *c* can occur and have been shown to be dependent on pH, ionic strength, and the nature of ionic species present (Palmer, 1971; Dawson, 1965).

In favor of the idea that polyphosphoinositide and glycophorin A represent a meaningful association is the fact that only 1 mol of lipid is bound per mol of peptide and this association resists disruption by commonly used organic solvent systems. Since the polyphosphoinositide remains bound to the glycoprotein peptide in organic media, this association may also be favored within the hydrophobic milieu of the lipid bilayer. In this regard, studies on the interaction of lipids and proteins in myelin suggest an important role for highly anionic phospholipids in the structure of myelin membranes (Braun and Radin, 1969). Similarly, the concept of a boundary of lipid fixed to the intramembranous portion of a protein has been suggested by studies on mitochondrial cytochromes (Jost et al., 1973; Dehlinger et al., 1974).

References

Assmann, G., Sokolaski, E. A., and Brewer, H. B., Jr. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 549–553.

Braun, P. E., and Radin, N. S. (1969), *Biochemistry 8,* 4310.

Cohn, M., and Hughes, T. R., Jr. (1962), *J. Biol. Chem. 237,* 176–181.

Dawson, R. M. C. (1965), *Biochem. J. 97,* 134–138.

Dehlinger, P. J., Jost, P. C., and Griffith, O. H. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2280–2284.

Dodge, J. T., Mitchell, C., and Hanahan, D. J. (1963), *Arch. Biochem. Biophys. 100,* 119–130.

Furthmayr, H., and Marchesi, V. T. (1976), *Biochemistry 15,* 1137–1144.

Furthmayr, H., Tomita, M., and Marchesi, V. T. (1975), *Biochem. Biophys. Res. Commun. 65,* 113–121.

Gorenstein, D. G. (1975), *J. Am. Chem. Soc. 97,* 898–900.

Hauser, G., and Eichberg, J. (1973), *Biochim. Biophys. Acta 326,* 201–209.

Hokin, L. E. (1969), *Ann. N.Y. Acad. Sci. 165,* 695–709.

Jackson, R. L., Segrest, J. P., Kahane, I., and Marchesi, V. T. (1973), *Biochemistry 12,* 3131.

Jost, P. C., Griffith, O. H., Capaldi, R. A., and Vanderkooi, G. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 480–484.

Marchesi, V. T., and Andrews, E. P. (1974), *Science 174,* 1247.

Palmer, F. B. St. C. (1971), *Biochim. Biophys. Acta 231,* 134–144.

Shapiro, D. L., and Marchesi, V. T. (1977), *J. Biol. Chem. 252,* 508–517.

Silverberg, M., Furthmayr, H., and Marchesi, V. T. (1976), *Biochemistry 15,* 1448–1454.

Tomita, M., and Marchesi, V. T. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2964–2968.

Yoo, C. S., Blank, G., Pletcher, J., and Sax, M. (1974), *Acta Crystallogr., Sect. B 30,* 1983–1987.