Purification and characterization of a major glycoprotein in rat hepatoma plasma membranes

One of the membrane proteins released by phosphatidylinositol-specific phospholipase C

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A major glycoprotein of rat hepatoma plasma membranes was selectively released as a soluble form by incubating the membrane with phosphatidylinositol-specific phospholipase C. The soluble form corresponding to the glycoprotein was also prepared by butan-1-ol extraction of microsomal membranes at pH 5.5, whereas extraction at pH 8.5 yielded an electrophoretically different form with a hydrophobic nature. The soluble glycoprotein extracted at pH 5.5 was purified by sequential chromatography on concanavalin A-Sepharose, Sephacryl S-300 and anti-(alkaline phosphatase) IgG-Sepharose, the last step being used to remove a contaminating alkaline phosphatase. The glycoprotein thus purified was a single protein with M_r 130000 in SDS/polyacrylamide-gel electrophoresis, although it behaved as a dimer in gel filtration on Sephacryl S-300. The glycoprotein was analysed for amino acid and carbohydrate composition. The composition of the carbohydrate moiety, which amounted to 64% by weight, suggested that the glycoprotein contained much larger numbers of *N*-linked oligosaccharide chains than those with *O*-linkage. It was confirmed that the purified glycoprotein was immunologically identical not only with that released by the phospholipase C but also with the hydrophobic form extracted with butan-1-ol at pH 8.5. The results indicate that the glycoprotein of rat hepatoma plasma membranes, which has an unusually high content of carbohydrate, is another membrane protein released by phosphatidylinositol-specific phospholipase C, as documented for alkaline phosphatase, acetylcholinesterase and Thy-1 antigen.

INTRODUCTION

Growing evidence suggests that cell-surface glycoproteins play an important role in essential cellular functions such as recognition, adhesion, regulation of cell growth and differentiation (Wylie *et al.*, 1979; Rauvala *et al.*, 1981; Ocklind & Öbrink, 1982). These functions are known to be altered during malignant transformation of cells (Nicolson, 1976a). The molecular basis of these alterations is in part due to modifications in the oligosaccharide and polypeptide composition of the plasma membrane (Warren *et al.*, 1973; Rapin & Burger, 1974; Nicolson, 1976b). Despite current interest in cell-surface glycoproteins, little has been reported with regard to molecular details of glycoproteins present in the plasma membranes of malignant cells. This may be in part due to the difficulties in extracting glycoproteins in water-soluble form from the membranes. Membrane proteins with hydrophobic domains are in general not easily purified. Thus most of the studies have dealt with glycopeptide fractions released by treatment of intact cells or membranes with proteolytic enzymes (Smith *et al.*, 1973; Codrington *et al.*, 1978; Nato & Bourrillon, 1982).

We previously reported that alkaline phosphatase, a membrane-bound glycoprotein, was released as a soluble form from plasma membranes by treatment with phosphatidylinositol-specific phospholipase C (Kominami *et al.*, 1985). The same soluble form of alkaline phosphatase was also obtained by butan-1-ol extraction of plasma membranes at low pH (pH 5.5), although the

same treatment at pH 8.5 yielded a hydrophobic form that was easily aggregated without a detergent (Miki *et al.*, 1985, 1986). During the purification of alkaline phosphatase and its comparison between the normal liver and ascites hepatoma AH-130 cells, we found that the hepatoma plasma membranes contained a major glycoprotein that was released as a soluble form by treatment with either phosphatidylinositol-specific phospholipase C or butan-1-ol at pH 5.5. In the present paper we describe the purification and characterization of the major glycoprotein. The glycoprotein was found to contain an unusually high content (64% by weight) of carbohydrate and to be apparently different from those glycoproteins so far characterized in other hepatoma cells (Funakoshi & Yamashina, 1976; Nakajo *et al.*, 1979; Nato & Bourrillon, 1982).

MATERIALS AND METHODS

Materials

Concanavalin A-Sepharose (8 mg of concanavalin A/ml of gel) and Sephacryl S-300 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Thyroglobulin, ferritin and catalase were from Sigma Chemical Co. (St Louis, MO, U.S.A.). Acrylamide gel reagents, SDS and *p*-nitrophenyl phosphate were from Nakarai Chemicals (Kyoto, Japan); RNA polymerase B was from Seikagaku Kogyo (Tokyo, Japan); 5-bromoindol-3-yl phosphate was from Wako Pure Chemicals (Tokyo, Japan). Other chemicals used were of reagent grade.

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Phosphatidylinositol-specific phospholipase C was purified from *Bacillus cereus* as described previously (Kominami *et al.*, 1985). Anti-(alkaline phosphatase) IgG-Sepharose was prepared as described previously (Miki *et al.*, 1986).

Hepatoma cells and preparation of plasma-membrane and microsomal fractions

Ascites hepatoma AH-130 cells (Odashima, 1964) were transplanted into and harvested from the intraperitoneal cavity of male Donryu rats (Ikehara *et al.*, 1977, 1978). Plasma membranes of the hepatoma cells were isolated by the method developed by Emmelot & Bos (1969) after contaminating blood cells had been removed. Microsomal fraction (referred to below simply as 'microsomes') was prepared by centrifugation of a post-mitochondrial fraction at 105000 *g* for 1 h.

Treatments for release of membrane glycoproteins

Microsomes (8–10 mg of protein/ml) were treated with butan-1-ol at 25 °C for 60 min in 50 mM-sodium acetate buffer, pH 5.5, for preparation of soluble forms of the glycoprotein and alkaline phosphatase, and in 50 mM-Tris/HCl buffer, pH 8.5 for membrane-bound forms of these proteins (Miki *et al.*, 1985, 1986). Aqueous phases obtained after centrifugation at 30000 *g* for 30 min were dialysed against 50 mM-NaCl/1 mM-MgCl₂/0.02% NaN₃/20 mM-Tris/HCl buffer, pH 7.5. When phospholipase C was used for release of these proteins, the plasma-membrane suspension (2–3 mg of protein/ml) was incubated with the purified phosphatidylinositol-specific phospholipase C (final concn. 0.01 unit/ml) in 20 mM-Tris/HCl buffer, pH 7.5, at 37 °C for 2 h (Kominami *et al.*, 1985). The treated sample was centrifuged at 105000 *g* for 60 min to separate the released proteins from the membranes.

Purification of the major glycoprotein

Microsomes of hepatoma cells were suspended in 50 mM-sodium acetate buffer, pH 5.5, and treated with butan-1-ol (final concn. 25%, v/v) at 25 °C for 60 min. After the mixture had been centrifuged at 30000 *g* for 30 min, the resulting aqueous phase was taken and adjusted to pH 7.5 with 1 M-Tris/HCl, followed by centrifugation at 105000 *g* for 60 min. The supernatants were adjusted to contain 1 mM-MgCl₂, 1 mM-CaCl₂ and 1 mM-MnCl₂, and applied on to a concanavalin A-Sepharose column (2.5 cm × 20 cm) previously equilibrated with 1 mM-MgCl₂/1 mM-CaCl₂/1 mM-MnCl₂/20 mM-Tris/HCl buffer, pH 7.5. The column was washed with 500 ml of the same buffer, followed by elution with 0.4 M- α -methyl D-glucoside in 0.5 M-NaCl/20 mM-Tris/HCl buffer, pH 7.5. Fractions (15 ml) were collected and the protein concentrations were determined by the method of Lowry *et al.* (1951), with albumin as standard. Fractions comprising the protein peak were pooled and concentrated to about 5 ml by ultrafiltration in an Amicon Diaflow cell with an XM-50 membrane. The concentrated sample was applied to a Sephacryl S-300 column (5 cm × 90 cm) previously equilibrated with 50 mM-NaCl/1 mM-MgCl₂/0.02% NaN₃/20 mM-Tris/HCl buffer, pH 7.5. The column was eluted with the same buffer at a flow rate of 23 ml/h, and 7.4 ml fractions were collected. After determination of protein distribution, fractions comprising each protein peak were subjected to SDS/polyacrylamide-gel electrophoresis,

followed by staining for protein and carbohydrate. Fractions containing the major glycoprotein were pooled. Since the sample thus prepared was found to be contaminated with alkaline phosphatase, the major glycoprotein was finally purified by passing the preparation through an anti-(alkaline phosphatase) IgG-Sepharose column (Miki *et al.*, 1986).

Polyacrylamide-gel electrophoresis

Standard disc gel electrophoresis was carried out in 7.5% (w/v) polyacrylamide gels by the procedure of Davis (1964) with a slight modification as described previously (Ikehara *et al.*, 1977). SDS/polyacrylamide-gel electrophoresis was performed with two different systems. For determination of the M_r of the purified major glycoprotein, the same system as that of Laemmli (1970) was employed [7.5% (w/v) polyacrylamide gels]. The M_r was determined by co-electrophoresis of the marker protein RNA polymerase B, the subunits of which are β' (M_r 180000), β (M_r 140000), α (M_r 100000), α (M_r 42000) and α (M_r 38000). For separation of soluble and membrane-bound forms of the glycoprotein and alkaline phosphatase, samples in 0.1 M-sodium phosphate buffer, pH 7.5, were incubated at 37 °C for 60 min in the presence of 1% (w/v) SDS but in the absence of a reducing agent, and subjected to electrophoresis in SDS/5.6% (w/v) polyacrylamide gels (Weber & Osborn, 1969). Gels were stained for protein with Coomassie Brilliant Blue R-250 (Laemmli, 1970), and for carbohydrate by the periodate/Schiff procedure (Zacharius *et al.*, 1969). Gels were also stained for alkaline phosphatase activity with 5-bromoindol-3-yl phosphate as substrate (Ikehara *et al.*, 1977).

Analysis of amino acid composition

Acid hydrolysates were prepared by digesting samples in 6 M-HCl at 110 °C *in vacuo* for 24 h as described by Moore & Stein (1963). Amino acid analysis was performed on a Hitachi model 835 amino acid analyser by the procedure of Spackman *et al.* (1958).

Analysis of carbohydrate composition

Sialic acid was determined by the thiobarbituric acid method of Warren (1959), with *N*-acetylneuraminic acid as standard, after samples had been hydrolysed in 0.05 M-H₂SO₄ at 80 °C for 60 min. For determination of neutral and amino sugars, samples were hydrolysed in 2.5 M-trifluoroacetic acid for 8 h at 100 °C. Hydrolysates were separated and analysed as described previously (Ikehara *et al.*, 1981).

Immunological analysis

Antibodies against the major glycoprotein were raised in rabbits. Double gel immunodiffusion was carried out in a 1% (w/v) agarose gel containing 50 mM-sodium Veronal buffer, pH 8.6 and 0.02% NaN₃ as described previously (Kawahara *et al.*, 1982).

Enzyme assays

Alkaline phosphatase activity was determined with *p*-nitrophenyl phosphate as substrate (Ikehara *et al.*, 1977). Phosphatidylinositol-specific phospholipase C activity was determined by the method of Low (1981), with some modifications as described previously (Kominami *et al.*, 1985). One unit of each enzyme activity was

Table 1. Distribution of protein and alkaline phosphatase after treatment of plasma membranes with phosphatidylinositol-specific phospholipase C

Plasma-membrane suspension (212 mg/100 ml) in 20 mM-Tris/HCl buffer, pH 7.5, was incubated with phosphatidylinositol-specific phospholipase C (0.01 unit/ml) at 37 °C for 2 h. The membrane suspension was then centrifuged at 105000 *g* for 60 min. The resulting soluble and sedimentable fractions were assayed for protein and alkaline phosphatase activity.

Plasma-membrane sample	Protein		Alkaline phosphatase	
	(mg)	(%)	(units)	(%)
Before treatment	212	100	450	100
After treatment and centrifugation				
Precipitated	202	95.8	7	1.5
Supernatant	10.8	5.1	441	98.0

defined as the amount of enzyme catalysing the hydrolysis of 1 μ mol of substrate/min at 37 °C.

RESULTS

Release of the major glycoprotein from plasma membranes by phosphatidylinositol-specific phospholipase C

The plasma-membrane preparation from hepatoma cells was enriched in alkaline phosphatase, an ectoenzyme, by 23-fold as compared with the specific activity of cell homogenates. When the membrane suspension was incubated with purified phosphatidylinositol-specific phospholipase C, 5.1% of protein and 98% of alkaline phosphatase were released from the plasma membranes (Table 1). Incubation without the phospholipase C yielded 2.5% of protein and 1.5% of alkaline phosphatase in the soluble fraction. The phospholipase C used here exerted a phospholipase C activity specific for phosphatidylinositol but not for other phospholipids and had no proteolytic activity (Kominami *et al.*, 1985). Thus the results indicate that 2.6% of protein and 96.5% of alkaline phosphatase were specifically released from the membrane by the phosphatidylinositol-specific phospholipase C action.

The samples before and after treatment with the phospholipase C were analysed by SDS/polyacrylamide-gel electrophoresis under non-reducing conditions (Fig. 1). When gels were stained for carbohydrate, it was found that the plasma membranes before treatment with the phospholipase C contained at least five periodate/Schiff-positive components, PAS-1 to PAS-5, although they were very faintly stained under these conditions (Fig. 1a, gel 1). The soluble fraction obtained after phospholipase C treatment contained an intensely stainable band (PAS-1' in gel 3), which was found neither in the original membranes nor in the residual membrane fraction after treatment and centrifugation (gels 1 and 2). Components PAS-3, PAS-4 and PAS-5 remained in the membrane fraction, although component PAS-2 was recovered in both fractions. On the other hand, alkaline phosphatase,

Fig. 1. SDS/polyacrylamide-gel electrophoresis of major glycoproteins and alkaline phosphatase of hepatoma plasma membrane before and after treatment with phosphatidylinositol-specific phospholipase C

Hepatoma plasma membranes were treated with phosphatidylinositol-specific phospholipase C followed by centrifugation, as shown in Table 1. Samples obtained were analysed by electrophoresis on SDS/polyacrylamide gels (5.6%, w/v) under non-reducing conditions. Gels were stained for carbohydrate (a) and for alkaline phosphatase activity (b). Gel 1, plasma membranes before the phospholipase C treatment; gels 2 and 3, precipitate and supernatant respectively obtained after centrifugation of the phospholipase-C-treated sample. PAS-1 to PAS-5 and PAS-1' indicate periodate/Schiff-reagent-positive bands. In (a) gels 1 and 2, 100 μ g of protein/gel; gel 3, 30 μ g of protein/gel. In (b) 0.1 unit of alkaline phosphatase/gel.

which was identified by activity staining, showed different mobilities in electrophoresis before and after its release from the membranes; the solubilized enzyme migrated more slowly than the membrane-bound form (Fig. 1b). The relationship between the two forms of alkaline phosphatase and their electrophoretic mobilities may also be considered for components PAS-1 and PAS-1', suggesting that the soluble major glycoprotein, PAS-1', was derived from the membrane-bound form PAS-1. This may be supported by the fact that PAS-1 could not be detected in the membrane fraction after phospholipase C treatment (Fig. 1a, gel 2). Taken together, these results indicate that the major glycoprotein was selectively released from the membranes by phosphatidylinositol-specific phospholipase C, as observed for alkaline phosphatase (Kominami *et al.*, 1985).

Major glycoprotein prepared by butan-1-ol extraction

We previously demonstrated that soluble and membrane-bound forms of alkaline phosphatase were selectively prepared by butan-1-ol extraction of the plasma membranes at pH 5.5 and at pH 8.5 respectively (Miki *et al.*, 1985, 1986). The soluble form prepared at pH 5.5

Fig. 2. Polyacrylamide-gel electrophoresis of major glycoproteins prepared by butan-1-ol extraction of microsomes

Hepatoma microsomes were treated with butan-1-ol at pH 5.5 or at pH 8.5 as described in the Materials and methods section. Each aqueous phase was dialysed and subjected to electrophoresis on SDS/polyacrylamide gels (a) under the same conditions as in Fig. 1, or on standard polyacrylamide gels (7.5%, w/v) at pH 8.6 (b). Gels were stained for carbohydrate. Gels 1 and 2, samples obtained at pH 8.5 and at pH 5.5 respectively; gel 3, sample released by phosphatidylinositol-specific phospholipase C. Each gel received 30 μ g of protein.

was found to have the same molecular properties as those of the form obtained by phospholipase C treatment (Miki *et al.*, 1985).

The same procedure of butan-1-ol extraction was applied for preparation of the glycoprotein. As shown in Fig. 2(a), the glycoprotein prepared at pH 5.5 (gel 2) migrated in SDS/polyacrylamide-gel electrophoresis to the same position as that obtained by treatment with phosphatidylinositol-specific phospholipase C (gel 3) and more slowly than the glycoprotein prepared at pH 8.5 (gel 1). In addition, when the same samples were subjected to electrophoresis in the absence of SDS, the glycoprotein prepared at pH 5.5 and that obtained by treatment with phospholipase C had the same mobility (Fig. 2b, gels 2 and 3), whereas the glycoprotein prepared at pH 8.5 remained at top of the gel, possibly because of aggregation (gel 1). These results demonstrate that the glycoprotein extracted with butan-1-ol at pH 5.5 is the same soluble form as that released by phospholipase C, whereas the glycoprotein extracted at pH 8.5 still retains a hydrophobic nature of the membrane protein.

Purification of the major glycoprotein

The butan-1-ol extract of microsomal membranes prepared at pH 5.5 was used for purification of the glycoprotein. When the extract was subjected to concanavalin A-Sepharose chromatography, the protein was adsorbed on the column and eluted with α -methyl D-glucoside. The eluate was concentrated and applied to a Sephacryl S-300 column. The elution profile is shown

Fig. 3. Gel filtration of the major glycoprotein on a Sephacryl S-300 column

The sample obtained by concanavalin A-Sepharose chromatography was applied to a Sephacryl S-300 column (5 cm $\times$ 90 cm) and eluted with 50 mM-NaCl/1 mM-MgCl₂/0.02% NaN₃/20 mM-Tris/HCl buffer, pH 7.5 (a). Fractions (7.4 ml) were collected and assayed for protein (●) and alkaline phosphatase activity (○). The void volume of the column (V_0) is indicated by a large arrow. Fractions indicated by small arrows were subjected to electrophoresis in SDS/polyacrylamide gels (8.0%, w/v) under reducing conditions, followed by staining for carbohydrate (b) and for protein (c). An equal volume (70 μ l) of sample was applied to each lane. Lanes 1–8, fraction nos. 114, 115, 117, 119, 121, 123, 125 and 126 respectively. An arrow in (c) indicates the position of alkaline phosphatase.

Fig. 4. SDS/polyacrylamide-gel electrophoresis of the purified major glycoprotein

The purified major glycoprotein was subjected to electrophoresis in SDS/polyacrylamide gels (7.5%, w/v) under reducing conditions. Gel 1, stained for protein (40 μ g/gel); gel 2, stained for carbohydrate followed by staining for protein (5 μ g/gel); gel 3, stained for carbohydrate (5 μ g/gel). Marker proteins used are, from top to bottom, β' , β , α , α and α subunits of RNA polymerase B.

in Fig. 3(a). Fractions of each protein peak were analysed by SDS/polyacrylamide-gel electrophoresis, followed by staining for carbohydrate and protein. It was found that a major peak eluted before alkaline phosphatase contained the major glycoprotein (Fig. 3b). The apparent M_r of the major glycoprotein under the conditions of the gel filtration was estimated to be about 270000, by chromatography on the same column of the standards thyroglobulin (M_r 670000), ferritin (M_r 460000), catalase (M_r 232000) and IgG (M_r 160000). Since some fractions were contaminated with alkaline phosphatase (Fig. 3c), the pooled fraction was further subjected to anti-(alkaline phosphatase) IgG-Sepharose chromatography to remove the contaminating alkaline phosphatase. Flow-through fractions were collected and concentrated. The final preparation was found to contain a single protein with M_r 130000, when analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions (Fig. 4). The major glycoprotein thus purified was strongly stained with periodate/Schiff reagents (5 μ g applied to gel 3), but not easily stained with Coomassie Brilliant Blue (40 μ g to gel 1), as observed for glycophorin in human erythrocyte ghosts (Fairbanks *et al.*, 1971). The difference in its M_r values determined by gel filtration (Fig. 3a) and by SDS/polyacrylamide-gel electrophoresis (Fig. 4) suggests that the

Table 2. Amino acid composition of the major glycoprotein

The purified major glycoprotein of hepatoma AH-130 was hydrolysed in 6 M-HCl at 110 °C *in vacuo* for 24 h, and analysed for amino acid composition. Data are compared with those for major glycoproteins of other rat hepatoma cells.

Amino acid	Source of major glycoprotein ...	Amino acid composition (residues/1000 residues)		
		Hepatoma AH-130*	Hepatoma AH-66†	Hepatoma AH-66‡
Lysine		54	54	25
Histidine		5	19	23
Arginine		12	36	26
Aspartic acid		125	102	106
Threonine		177	100	131
Serine		133	99	118
Glutamic acid		64	113	123
Proline		39	67	91
Glycine		28	76	98
Alanine		103	66	61
Half-cystine		11	17	—
Valine		110	56	53
Methionine		16	18	16
Isoleucine		26	41	25
Leucine		51	75	63
Tyrosine		25	27	14
Phenylalanine		21	34	27

* Present study.

† Funakoshi & Yamashina (1976).

‡ Nakajo *et al.* (1979).

native form of the major glycoprotein may exist as a dimer.

Chemical composition of the major glycoprotein

The purified major glycoprotein was analysed for amino acid composition (Table 2). When compared with the amino acid compositions of major glycoproteins isolated from rat hepatoma AH-66 cells (Funakoshi & Yamashina, 1976; Nakajo *et al.*, 1979), the present major glycoprotein was found to have higher contents of aspartic acid/asparagine, threonine, serine, alanine and valine residues, but lower contents of histidine, arginine, glutamic acid/glutamine, proline and glycine residues. The results suggest that the present major glycoprotein is a molecule different in primary structure from those of AH-66 cells.

The major glycoprotein was also analysed for carbohydrate composition. The carbohydrate moiety amounted to 64% on a weight basis. As shown in Table 3, mannose, galactose, glucosamine and sialic acid were major components, and galactosamine and fucose were also present in relatively small quantities. Since mannose and galactosamine are characteristic components of *N*- and *O*-linked oligosaccharide chains respectively of glycoproteins, these results indicate that the major glycoprotein contains both *N*- and *O*-linked oligosaccharide chains. The data were compared with carbohydrate compositions of major glycoproteins from hepatoma AH-66 cells. The present major glycoprotein was found to have much higher contents of mannose, glucosamine, galactose and fucose residues than those in

Table 3. Carbohydrate composition of the major glycoprotein

The purified major glycoprotein of hepatoma AH-130 was analysed for carbohydrate composition as described in the Materials and methods section. The M_r value of 130000 was used for calculation of residues per protein molecule (mol/mol). Data are compared with those reported for major glycoproteins of other rat hepatoma cells.

Carbohydrate	Source of major glycoprotein...	Carbohydrate composition					
		Hepatoma AH-130*		Hepatoma AH-66†		Hepatoma AH-66‡	
		(%, w/w)	(mol/mol)	(%, w/w)	(mol/mol)	(%, w/w)	(mol/mol)
Mannose		12.2	88	2.8	25	2.9	26
Galactose		15.7	113	12.4	110	9.0	80
Fucose		2.0	16	0.5	5	0.2	2
Glucosamine		12.9	94	9.9	72	10.3	75
Galactosamine		4.4	32	7.4	54	15.1	110
Sialic acid		16.8	76	10.9	57	21.3	111
Total weight (%)		64.0		43.9		58.8	

* Present study.

† Funakoshi & Yamashina (1976).

‡ Nakajo *et al.* (1979).

AH-66 glycoproteins, although the content of galactosamine residues was much lower (Table 3). These results suggest that the present major glycoprotein contains predominantly *N*-linked oligosaccharide chains, whereas the carbohydrate moieties of AH-66 glycoproteins consist mainly of *O*-linked oligosaccharide chains.

Immunological comparison of the major glycoprotein with other preparations

The major glycoprotein purified as above was used as the antigen for the preparation of specific antibodies in rabbits. The anti-(major glycoprotein) serum obtained was used for immunological characterization of different

preparations of the glycoprotein. As shown in Fig. 5, with the antiserum both preparations of the soluble form, butan-1-ol-extracted at pH 5.5 and phosphatidylinositol-specific-phospholipase-C-solubilized, formed single precipitin lines that completely fused with each other, demonstrating that the glycoprotein obtained by the phospholipase C treatment is immunologically identical with the major glycoprotein prepared by butan-1-ol extraction at pH 5.5. In addition, the major glycoprotein extracted with butan-1-ol at pH 8.5 was also immunologically identical with the major glycoprotein prepared at pH 5.5 (Fig. 5), in spite of their different behaviour in polyacrylamide-gel electrophoresis (Fig. 2). Taken together, these results demonstrate that the purified major glycoprotein is the same molecule as those prepared from the plasma membranes, by phospholipase C treatment and by butan-1-ol extraction at pH 8.5.

DISCUSSION

The major glycoprotein characterized in the present study is a glycoprotein tightly bound to the plasma membrane. The glycoprotein was not released from membranes by treatment with either high salt concentration (0.5 M-NaCl), EDTA or even 0.1% Triton X-100. When extracted at pH 8.5 with butan-1-ol and extensively dialysed against an appropriate buffer containing no detergent, the glycoprotein was easily aggregated, did not migrate on standard polyacrylamide-gel electrophoresis (Fig. 2b, gel 1), and was eluted at the void volume of a Sephacryl S-300 column in the absence of detergents (results not shown). Such characteristics are commonly observed for membrane proteins with hydrophobic domains. Membrane proteins are usually solubilized by treatment with appropriate detergents or chaotropic agents (Funakoshi & Yamashina, 1976; Nakajo *et al.*, 1979), or alternatively by proteolytic removal of hydrophobic domains (Cordington *et al.*, 1978; Nato & Bourrillon, 1982).

The hepatoma glycoprotein, however, was easily solubilized from membranes by treatment with phosphat-

Fig. 5. Immunological identification of different preparations of the major glycoprotein

Double gel immunodiffusion was carried out in 1% (w/v) agarose gel containing 50 mM-Veronal buffer, pH 8.6. Centre well, antiserum against the purified major glycoprotein; well 1, purified major glycoprotein; well 2, butan-1-ol extract (pH 5.5); well 3, sample prepared by treatment with phosphatidylinositol-specific phospholipase C; well 4, butan-1-ol extract (pH 8.5).

idylinositol-specific phospholipase C or with butan-1-ol at pH 5.5, under the same conditions as those under which alkaline phosphatase was released as a soluble form from membranes (Kominami *et al.*, 1985; Miki *et al.*, 1985, 1986). Under non-reducing conditions, the soluble form of the glycoprotein migrated on SDS/polyacrylamide-gel electrophoresis more slowly than the membrane-bound form (Figs. 1a and 2a), as observed between the two forms of alkaline phosphatase (Fig. 1b). Under reducing conditions, however, there was no significant difference in electrophoretic mobility between the two forms (Kominami *et al.*, 1984). These observations may be interpreted in terms of the previous findings that proteins that are incompletely denatured, because of intramolecular disulphide bonds or other structural features, exhibit anomalous SDS binding and thus anomalous mobilities on SDS/polyacrylamide-gel electrophoresis (Fish *et al.*, 1970; Phillips & Agin, 1977).

The finding that treatment of microsomal membranes with butan-1-ol at pH 5.5 yielded the same soluble form of the glycoprotein as that obtained by treatment with phosphatidylinositol-specific phospholipase C suggests the presence of an endogenous factor with activity similar to that of the bacterial phosphatidylinositol-specific phospholipase C. In fact, Low & Zilversmit (1980) have suggested the existence of phosphatidylinositol-specific phospholipase C in kidney microsomes that is activated by butan-1-ol and involved in the release of alkaline phosphatase from membranes. Our results indicate that the phosphatidylinositol-specific-phospholipase-C-like enzyme has optimum pH at about 5.5, as demonstrated for phosphoinositide-specific phospholipase C with optimum pH at 5.0 in rat liver plasma membranes (Melin *et al.*, 1986). We previously reported that hepatoma alkaline phosphatase, as opposed to the liver enzyme, was not effectively prepared as the soluble form from the purified plasma membranes by butan-1-ol extraction even at pH 5.5 (Miki *et al.*, 1985). The soluble hepatoma alkaline phosphatase, however, was obtained in good yield from a crude microsomal fraction containing lysosomes as well as plasma membranes under the same conditions of treatment (Miki *et al.*, 1986). This is the same for the glycoprotein, which was extracted as the soluble form when the crude microsomal fraction was used for butan-1-ol extraction at pH 5.5 (Fig. 2b). These results suggest the possibility that the enzyme involved in release of the glycoprotein and alkaline phosphatase has different subcellular localizations in the liver and the hepatoma.

On the basis of the fact that butan-1-ol extraction at pH 5.5 yielded the same soluble major glycoprotein as effectively as the bacterial phosphatidylinositol-specific phospholipase C, we utilized the former procedure for the preparation of the major glycoprotein, because the purified phosphatidylinositol-specific phospholipase C was not so easily obtained in large quantity. Once the major glycoprotein was released from membranes by this method, it no longer required detergents for its handling and was purified by simple steps as described above. The purified major glycoprotein was found to have M_r 130000 on SDS/polyacrylamide-gel electrophoresis. However, the major glycoprotein was eluted at a position corresponding to M_r 270000, possibly as a dimer, on Sephacryl S-300 chromatography, for which we used a buffer with relatively low ionic strength (50 mM-NaCl/1 mM-MgCl₂, 20 mM-Tris/HCl buffer, pH

7.5) for its better separation from alkaline phosphatase (Fig. 3a). Thus the native major glycoprotein may exist as a dimer, but this is not due to a disulphide bond, because the purified major glycoprotein had a similar M_r value on SDS/polyacrylamide-gel electrophoresis under reducing and non-reducing conditions (results not shown).

The major glycoprotein thus purified had unusually high content of carbohydrate (64% by weight), and was hardly stained with Coomassie Brilliant Blue after polyacrylamide-gel electrophoresis, as observed for glycophorin in human erythrocytes (Fairbanks *et al.*, 1971). When compared with major glycoproteins from other rat hepatomas so far characterized (Funakoshi & Yamashina, 1976; Nakajo *et al.*, 1979; Nato & Bourrillon, 1982), the present major glycoprotein was found to be distinctly different from these glycoproteins in molecular mass and in amino acid and carbohydrate composition. In particular, carbohydrate composition analysis suggests that the present major glycoprotein mainly contains *N*-linked oligosaccharides, whereas glycoproteins from other sources have *O*-linked oligosaccharides as major components.

Most membrane proteins have hydrophobic peptide domains that interact with the membrane lipid bilayer. Recent investigations, however, are revealing that some membrane proteins are anchored in the membrane by covalent attachment to phosphatidylinositol (for review see Low *et al.*, 1986). Detailed information on such attachment is available for variant surface glycoproteins of *Trypanosoma brucei* (Ferguson *et al.*, 1985), Thy-1 antigen (Low & Kincade, 1985), acetylcholinesterase (Futerman *et al.*, 1985) and alkaline phosphatase (Ikezawa *et al.*, 1976; Low & Zilversmit, 1980; Kominami *et al.*, 1985). Each of the proteins is easily released as a soluble form from the membrane by phosphatidylinositol-specific phospholipase C. Thus the major glycoprotein described in the present paper is strongly suggested to be one of these proteins with unique attachment to the membrane.

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