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Isolation and characterization of two individual glycoprotein components from human milk-fat-globule membranes

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Two individual glycoprotein components from human milk-fat-globule membranes (MFGM) has been purified by selectively extracting the membrane glycoproteins followed by lectin affinity chromatography and gel filtration on Sephadex G-200 in the presence of protein-disaggregating agents. The purified glycoprotein components, termed 'epithelial-membrane glycoprotein' (EMGP-155 and EMGP-39) have estimated molecular weights of 155000 and 39000 respectively, and yield a single band under reducing conditions on sodium dodecyl sulphate/polyacrylamide gel. EMGP-155 and EMGP-39 contain 21.0% and 7.0% carbohydrate by weight, with fucose (13.5%, 12.4%), mannose (3.7%, 6.2%), galactose (28.5%, 22.6%), *N*-acetylglucosamine (17.8%, 7.4%) and sialic acid (36.4%, 51.4%) of the carbohydrate moiety respectively. For both the glycoprotein components, aspartic and glutamic acid and serine are the major amino acid residues.

Structural glycoproteins as components of mammalian membrane play an important role in several functions of the cell, including cell recognition (Woodruff & Gesner, 1969), contact phenomena (Hughes, 1976; Roseman, 1970), growth control (Hughes, 1976) and immunological recognition of tumour cells (Hughes, 1976; Hynes, 1976). However, a few attempts have been made to isolate and characterize membrane glycoproteins from sources other than erythrocyte membrane (Glick, 1974). A detailed study of the major human erythrocyte sialoglycoprotein has permitted presentation of a rather detailed model for glycoprotein and membrane association (Marchesi & Furthmayer, 1976). For other tissues, most effort has been directed towards the studying of fragments released from the cell surface by proteolysis (Farrar & Harrison, 1978; Steck *et al.*, 1971; Tomich *et al.*, 1976). Although proteolytically cleaved fragments of cell-surface molecules are valuable in terms of studying the cell-surface topology, such fragments cannot yield much information about the nature and assembly of transmembrane protein in the lipid bilayer and the portion that extends into the cytoplasm through the membrane. In solid tissues, such as mammary gland, purification and characterization of unfragmented membrane glycoproteins may facilitate our understanding of several functions of cells, since these glycoproteins are said to play important roles in the functioning of the cell.

A source, alternative to breast tissues, of plasma membrane from mamary epithelial cells is found in milk (Keenan *et al.*, 1970). The membranes from such a source not only provide a higher yield of the membrane components but are also virtually free from contamination with cytoplasmic constituents. The cream fraction of milk consists of fat droplets stabilized in milk by an external membrane. This membrane layer known as the milk-fat-globule membrane (MFGM) is derived from the apical plasma membrane of mammary secretory epithelial cells (Bargmann *et al.*, 1961; Wooding, 1971*a*,*b*). Therefore MFGM represents an excellent source of material for the investigation of glycoproteins from the surface of plasma membrane of mammary epithelial cells.

SDS/polyacrylamide-gel-electrophoretic analysis of bovine MFGM reveals about six major Coomassie Blue-staining protein and about six periodate/Schiff-staining glycoprotein components (Anderson *et al.*, 1972; Kitchen, 1974; Kobylka & Carraway, 1972; Mangino & Brunner, 1975; Mather & Keenan, 1975). In order to purify individual

Abbreviations used: MFGM, milk-fat-globule membrane(s); SDS, sodium dodecyl sulphate; EMGP, epithelial-membrane glycoprotein.

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components, a variety of reagents have been used to solubilize MFGM proteins, and selective extraction of individual proteins has been achieved in some cases (Kobylka & Carraway, 1972; Mangino & Brunner, 1975; Mather & Keenan, 1975). Partial purification of four protein components by gel-filtration chromatography in the presence of ionic detergent has been reported (Cawston *et al.*, 1976). Two major glycoprotein components from bovine MFGM have been purified and chemically characterized (Freudenstein *et al.*, 1979; Snow *et al.*, 1977). Other researchers concentrated on isolating fragments released from MFGM by proteolysis (Harrison *et al.*, 1975; Steck *et al.*, 1971; Tomich *et al.*, 1976).

Initial study of human MFGM indicated that the MFGM from both human and bovine sources appear to have a similar protein pattern on SDS/polyacrylamide gels (Imam *et al.*, 1981; Martel *et al.*, 1973). Apart from one major glycoprotein component (Imam *et al.*, 1981), purification and chemical characterization of major glycoprotein components from human MFGM has not been achieved.

The present paper reports the continuation of these studies, which resulted in the purification of two more individual glycoprotein components from human MFGM. Amino acid and monosaccharide compositions, and some of their physical properties, are included in the present paper. The purified glycoprotein components were termed 'epithelial-membrane glycoproteins (EMGP-155 and EMGP-39)' to indicate their origin and estimated molecular weights.

Materials and methods

Materials

All reagents were obtained as described previously (Imam *et al.*, 1981).

Methods

Source of milk. Collection and transportation of human breast milk was carried out as described previously (Imam *et al.*, 1981).

Preparation of cream and MFGM. Both cream and MFGM were prepared as described previously (Imam *et al.*, 1981).

Lectin affinity chromatography of milk-fat-globule membrane glycoproteins. The $MgCl_2$ extract of glycoproteins from human MFGM were fractionated by lectin affinity chromatography as described previously (Imam *et al.*, 1981).

Gel filtration of glycoproteins from MFGM. The fraction unbound to the concanavalin A–Sepharose 4B (see Imam *et al.*, 1981) was solubilized in 0.05 M-Tris/HCl buffer, pH 9.0, containing 5 mM-EDTA, 5 mM-2-mercaptoethanol and 20% (v/v) dimethylformamide. The solubilized sample was chromatographed on a column (2.5 cm × 30 cm), which was packed with Sephadex G-200 and equilibrated with the above buffer solution. The rest of the chromatography was performed as described previously (Imam *et al.*, 1981). The fractions eluted at each peak were pooled, dialysed exhaustively against distilled water at 4°C and freeze-dried.

SDS/polyacrylamide-gel electrophoresis. Initial extract of MFGM and column fractions resulting from the chromatographies of MFGM extracts were examined by electrophoresis on polyacrylamide gels as described previously (Imam *et al.*, 1981).

Protein determination. Protein contents were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Monosaccharide and amino acid analyses. Both monosaccharides and amino acid residues were analysed as described previously (Imam *et al.*, 1981).

Production of antibodies. New Zealand White rabbits were immunized with the purified glycoprotein components termed EMGP-155 and EMGP-39. The scheme for immunization was the same as that described previously (Imam *et al.*, 1981).

Ouchterlony double diffusion. Immunoprecipitation reactions were carried out by double diffusion in agarose by Imam *et al.* 1981).

Results

Lectin affinity chromatography of glycoproteins from MFGM

This approach was made with a view to purifying non-concanavalin A-binding glycoprotein(s). The $MgCl_2$ extract of washed cream was separated into fractions by using concanavalin A–Sepharose 4B [see Fig. 2 of Imam *et al.* (1981)]. The unbound fractions contained mostly components 3 and 19, termed EMGP-155 and EMGP-39 respectively, as revealed by SDS/polyacrylamide-gel electrophoresis (results not shown).

Chromatographic isolation of EMGP-155 and EMGP-39

The unbound protein fraction from the concanavalin A–Sepharose 4B column was solubilized and chromatographed on a Sephadex G-200 column in the presence of dimethylformamide. An elution profile is illustrated in Fig. 1. Approx. 80% of total protein applied was eluted in two major peaks. The protein eluted at each peak yielded a single band on SDS/polyacrylamide gels (Fig. 2). The material eluted at peaks 1 and 2 corresponds to components 3 and 19 respectively and stained with both Coo-

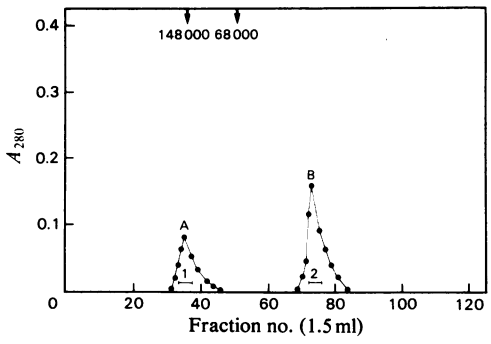

Fig. 1. *Sephadex G-200 chromatography of the unadsorbed fraction from lectin affinity column in 20% (v/v) dimethylformamide*

The $MgCl_2$ extract of washed cream that did not bind to the concanavalin A–Sepharose 4B column was subsequently chromatographed on a Sephadex G-200 column (2.5 cm × 29 cm) in 0.05 M-Tris/HCl buffer, pH 9.0, containing 5 mM-EDTA, 5 mM-2-mercaptoethanol and 20% (v/v) dimethylformamide. The column flow rate was 6.0 ml/h. Fractions (1.5 ml) were collected and monitored for A_{280}. Fractions taken for further analyses are designated 1 and 2. Marker proteins of known molecular weights (148000 and 68000) were used to calibrate the column.

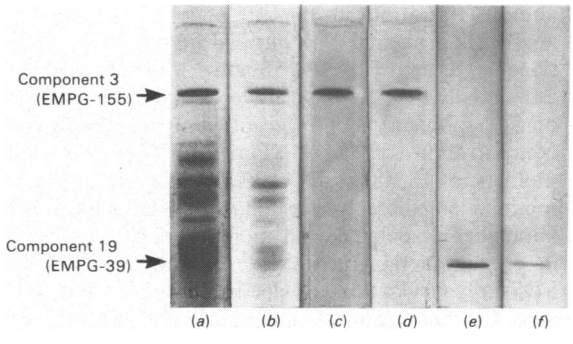

Fig. 2. *SDS/polyacrylamide-gel electrophoresis of unfractionated protein, $MgCl_2$ extract and fractions 1 and 2 from the Sephadex G-200 column chromatography of human milk-fat-globule-membrane proteins*

Unfractionated protein, $MgCl_2$ extract and Fractions 1 and 2 from the Sephadex G-200 column chromatography (see Fig. 1) of human milk-fat-globule-membrane protein were analysed by SDS/polyacrylamide-gel electrophoresis as described in the text. Gels (*a*), (*b*), (*c*) and (*e*) are stained with Coomassie Blue and gels (*d*) and (*f*) with periodate/Schiff reagents. Gel (*a*) contained unfractionated protein from milk-fat-globule membranes; (*b*) the $MgCl_2$ extract of the milk-fat-globule membranes; (*c*) and (*d*) the Fraction 1 from the Sephadex G-200 column chromatography; (*e*) and (*f*) the Fraction 2 from the Sephadex G-200 column chromatography.

massie Blue and periodate/Schiff reagent. On 7.5% acrylamide gels these glycoprotein components have an estimated molecular weight of 155000 and 39000.

Chemical characterization

To compare the purified EMGP-155 and EMGP-39 from human MFGM and with glycoprotein from other sources, quantitative amino acid and carbohydrate analyses were performed.

Amino acid analysis. The amino acid compositions of EMGP-155 and EMGP-39 are shown in Table 1. Tryptophan was not determined, as it is totally destroyed under the hydrolysis conditions. Aspartic acid, glutamic acid and leucine were the major amino acid residues for both EMGP-155 and EMGP-39 (Table 1).

Carbohydrate analysis. The carbohydrate analyses of isolated EMGP-155 and EMGP-39 are shown in Table 1. For EMGP-155 and EMGP-39, approx. 21 and 7% respectively of the molecule by

Table 1. *Amino acid and monosaccharide compositions of purified glycoprotein components from human milk-fat-globule membrane*

Abbreviations used: n.d., not detectable; n.r., not recorded.

	Composition (mol %)	
	Component 3	Component 19
Cysteic acid	0.5	0.3
Methionine sulphoxide	0.9	0.4
Aspartic acid	10.2	12.7
Threonine	6.6	7.6
Serine	6.9	10.9
Glutamic acid	14.6	8.7
Proline	9.8	5.9
Glycine	6.6	6.6
Alanine	6.9	7.6
Valine	5.9	5.9
Methionine	0.3	n.d.
Isoleucine	5.5	5.9
Leucine	8.6	9.0
Tyrosine	3.1	2.4
Phenylalanine	3.3	5.0
Lysine	6.8	6.1
Histidine	1.1	2.1
Arginine	2.3	2.1
Fucose	13.5	12.4
Mannose	3.7	6.2
Galactose	28.5	22.6
Glucose	n.r.	n.r.
N-Acetylgalactosamine	n.d.	n.d.
N-Acetylglucosamine	17.8	7.4
Sialic acid	36.4	51.4

weight was carbohydrate (Table 1). The percentage carbohydrate compositions of EMGP-155 and EMGP-39 were different from those of the bovine proteins (Basch *et al.*, 1976; Freudenstein *et al.*, 1979). The percentage of fucose, galactose and sialic acid was higher, and the percentage of mannose and *N*-acetylglucosamine was lower, than was observed with the bovine product (Basch *et al.*, 1976; Freudenstein *et al.*, 1979). No *N*-acetylgalactosamine could be detected in either of these glycoprotein components.

Certain amounts of glucose were found in the column eluates of EMGP-155 and EMGP-39 preparations. This could be reduced by extensive dialysis, indicating that the glucose was being 'bled' from the Sephadex column during chromatography. Hence glucose values have not been included in the calculations.

Immunological characterization

Purified glycoprotein components, including EMGP-70 (Imam *et al.*, 1981), were analysed for any immunological similarity. Immunoprecipitin lines were observed only between antigens and their corresponding antisera, suggesting that all three glycoprotein components were immunologically distinct (Fig. 3). No immunological cross-reactivity was observed between antisera to purified components and bovine milk-fat-globule membrane and also bovine skimmed milk. Neither EMGP-155 nor EMGP-39 have shown an immunoprecipitin line with rabbit antibodies to human whole plasma proteins, serum albumin, α_1-acid glycoprotein, α_1-antitrypsin, lactoferrin, α-lactalbumin, casein, secretory piece of immunoglobulin A, carcinoembryonic antigen, glycophorin and mouse mammary-tumour virus.

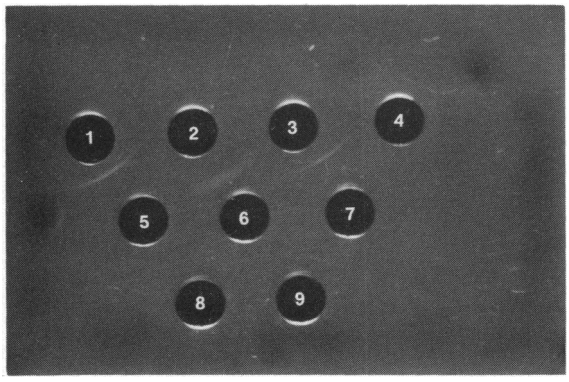

Fig. 3. *Comparison of antigenic properties of purified glycoprotein components from human-milk-fat-globule membrane*

The immunodiffusion plate shows the immunoprecipitin line obtained between the individual antigen and its corresponding antiserum. No immunological cross-reactivity between any of the components was observed. The experiment was performed by adding antigens and rabbit antisera to the wells as described below: Wells 5, 6 and 7 contain antisera to EMGP-155, EMGP-70 and EMGP-39 respectively; wells 1 and 9 contain EMGP-155; wells 2 and 4, EMGP-70; wells 3 and 8, EMGP-39.

Discussion

A selective solubilization of individual proteins from biological membranes could be achieved by including a selective initial-extraction step. The successful isolation and purification of glycophorin from human erythrocyte membranes owes much to a similar approach, which included an initial selective extraction of the membranes with lithium 3,5-di-iodosalicylate, followed by chromatography (Marchesi & Andrews, 1971). Letarte-Muirhead *et al.* (1975) made use of such an approach to solubilize and purify Thy-1 glycoprotein molecules from deoxycholate extracts of rat thymocyte membranes. Snow *et al.* (1977) have also included an initial and selective extraction of bovine MFGM to purify a glycoprotein component.

The purification procedures consisted of extraction of human milk-fat-globule membrane with $MgCl_2$, followed by lectin affinity chromatography and gel filtration in the presence of protein-disaggregating agents. The purified glycoprotein components, termed EMGP-155 and EMGP-39, yielded individually a single band under reducing conditions on SDS/polyacrylamide gels at a polyacrylamide-gel concentration of 7.5% (Fig. 2). The estimated mol.wts. of 155000 and 39000 are only an approximation, as these values may be influenced by anomalies associated with mobility of glycoproteins in gels (Anderson *et al.*, 1974). The technique of SDS/polyacrylamide-gel electrophoresis is not reliable for estimates of the molecular weights of glycoproteins from biological membranes. All these values may be overestimates of the true molecular weight, since they are derived by comparison with marker proteins, which probably bind much less SDS than do the membrane molecules (Helenius & Simons, 1975).

It is possible that the MFGM carry glycoproteins that may be unique to mammary epithelial cells. As a first step in this direction, comparative analyses of amino acid and carbohydrate compositions of the purified glycoproteins from human MFGM and erythrocyte membranes reveal major differences [see Table 1 of Imam *et al.* (1981)]. However, the amino acid compositions observed for both EMGP-155 and EMGP-39 were similar to those reported for glycoproteins purified from bovine MFGM (Basch *et al.*, 1976; Freudenstein *et al.*, 1979).

Immunological tests also reveal differences. Extracts of bovine milk-fat-globule membrane failed to give an immunoprecipitin line in agar against antisera to human milk-fat-globule membrane ($MgCl_2$ extract of human washed cream), EMGP-155 and EMGP-39. Furthermore, EMGP-155 and EMGP-39 appear to be immunologically unrelated to the human and bovine milk proteins (e.g. casein, lactoferrin and α-lactalbumin), secretory piece of immunoglobulin A, glycophorin, plasma proteins, carcinoembryonic antigen and mouse mammary-tumour virus.

EMGP-70 (Imam *et al.*, 1981), EMGP-155 and EMGP-39 appear to be immunologically distinct molecules, since no immunological cross-reactivity was observed between these components (Fig. 3).

The purification of cell-surface glycoprotein components from human milk-fat-globule membranes, which appear to derive from mammary epithelial cells (Wooding, 1971*a*,*b*), may permit studies of their structural and functional role in secretion and/or in transport across the membrane of epithelial cells. Antisera to such components, if found to be specific for human mammary epithelial cells, could be an important tool in the identification of a cell type in mixed population of breast cells in culture.

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