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Isolation and characterization of a major glycoprotein from milk-fat-globule membrane of human breast milk

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A major periodate–Schiff-positive component from milk-fat-globule membrane of human breast milk 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-dissociating agents. The purified glycoprotein, termed epithelial membrane glycoprotein (EMGP-70), has an estimated mol.wt. of 70000 and yields a single band under reducing conditions on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The glycoprotein contains 13.5% carbohydrate by weight, with fucose, mannose, galactose, *N*-acetylglucosamine and sialic acid 17.2, 17.0, 21.1, 7.9 and 36.6% respectively of the carbohydrate moiety. Aspartic and glutamic acid and serine are the major amino acid residues.

Glycoproteins as components of mammalian membranes are important because of their possible role in cell recognition (Woodruff & Gesner, 1969) and adhesion (Roseman, 1970), immunological recognition (Hughes, 1976) and provisions of receptor sites for viruses (Gottschalk *et al.*, 1972) and hormones (Cuatrecasas, 1973). Detailed structural studies in the carbohydrate portion of membrane glycoproteins have been largely confined to the erythrocyte membrane (Glick, 1974), and a detailed model of glycoprotein–membrane association (Marchesi & Furthmayer, 1976) is available. For other tissues, most researchers have studied fragments released from cells by proteolysis (Steck *et al.*, 1971; Harrison *et al.*, 1975; Farrar & Harrison, 1978).

A source of membranes from mammary epithelial cells can be found in the milk-fat fraction (Keenan *et al.*, 1970). The cream of milk consists of fat globules stabilized by an external membrane. This membrane layer, known as the milk-fat-globule membrane is mainly derived from the apical plasma membrane of mammary secretory cells (Patton & Keenan, 1975).

Bovine milk-fat-globule membrane reveals at least six major Coomassie Blue-staining proteins and about six major periodate–Schiff-staining glycoprotein components when analysed on sodium dodecyl sulphate/polyacrylamide gels (Kobylka & Carraway, 1972; Anderson *et al.*, 1972, 1974; Mather & Keenan, 1975). A variety of reagents have been used to solubilize milk-fat-globule-membrane proteins, and selective extraction of individual proteins has been achieved in some cases (Kobylka & Carraway, 1972; Mangino & Brunner, 1975; Mather & Keenan, 1975). Swope *et al.* (1968) and Anderson & Cawston (1975) obtained glycoprotein components from the bovine milk-fat-globule membrane by differential centrifugation and extraction. Some researchers have prepared fragments released from milk-fat-globule membrane by proteolysis (Harrison *et al.*, 1975; Farrar & Harrison, 1978; Tomich *et al.*, 1976).

Two major glycoprotein components from bovine milk-fat-globule membrane have been purified and characterized (Snow *et al.*, 1977; Freudenstein *et al.*, 1979), but no isolation of glycoproteins from human breast-milk-fat-globule membrane has yet been reported. We now report the purification of a major glycoprotein component from human breast-milk-fat-globule membrane, its amino acid and monosaccharide compositions and some of its physical and immunological properties. The purified glycoprotein was termed epithelial membrane glycoprotein (EMGP-70) to indicate its origin and estimated molecular weight.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Materials and methods

Materials

Sephadex G-200, Sephadex G-25 and Sepharose 4B were obtained from Pharmacia, Hounslow, Middx., U.K. Acrylamide, *NN'*-methylenebisacrylamide, *NNN'N'*-tetramethylethylenediamine, sodium dodecyl sulphate and ammonia-free glycine were obtained from Eastman–Kodak, Liverpool, U.K. Calibration proteins were obtained from Boehringer, Lewes, Sussex, U.K. Lactoperoxidase (EC 1.11.1.7) and Tris were obtained from Sigma, Poole, Dorset, U.K. Coomassie Blue G-250 and Schiff reagent were obtained from Raymond A. Lamb, Alperton, Middx., U.K. All remaining reagents used were of the highest purity available from BDH, Poole, Dorset, U.K.

Rabbit anti-(human albumin), α_1-acid glycoprotein and α_1-antitrypsin were obtained from Miles Laboratories, Slough, Berks., U.K. Rabbit anti-(human lactoferrin) and secretory piece of immunoglobulin A were obtained from Dakopatts, Copenhagen, Denmark. Other antisera were generously given: anti-(α-lactalbumin) from Mrs. U. Stevens, anti-(mouse mammary tumour virus) from Dr. A. Leathem, anti-glycophorin from Dr. P. A. W. Edwards and anti-(carcinoembryonic antigen) from Dr. D. A. Darcy.

Methods

Source of milk. Freshly secreted human pooled breast milk was obtained from the milk bank of Sorrento Maternity Hospital, Birmingham, U.K., and the Sutton Area Health Authorities, Sutton, Surrey, U.K.

The samples from Birmingham were frozen before transportation. No detectable release of milk fat or loss of membrane material was observed when the results obtained with the frozen milk were compared with the results from the unfrozen milk obtained locally.

Preparation of cream. Cream was separated from the milk by centrifugation at $3000\,g_{av.}$ and 30°C for 15min. The cream was washed four times in 5vol. of 0.01M-Tris/HCl buffer, pH7.5, containing 0.25M-sucrose and 1mM-$MgCl_2$ at 30°C, the percentage of cream being adjusted to 33% (v/v) between washes (Anderson & Cheeseman, 1971). The effectiveness of the washing technique in removing major skim-milk proteins (e.g., casein and α-lactalbumin) was checked by testing the dialysed and freeze-dried proteins from the washings for the presence of casein and α-lactalbumin by SDS/polyacrylamide-gel electrophoresis. The freeze-dried materials were also analysed on Ouchterlony agar diffusion plates by using rabbit antisera to caseins and α-lactalbumin by the method of Anderson & Cheeseman (1971).

Preparation of milk-fat-globule membranes. Washed and well-packed cream (33g wet wt.) was suspended in 0.05M-Tris/HCl buffer, pH7.5, to a final volume of 100ml in a stoppered glass flask. The suspension was first kept in the cold-room (4°C) for 3–4h and then allowed to reach room temperature. When the temperature of the suspension reached 12–14°C, the flask was shaken on a laboratory shaker until butter formed. In this process membranes are released from the fat globules, which then adhere to each other to form butter. After the formation of butter was complete, the mixture was warmed to 37°C to release membrane trapped by butter granules. Milk-fat-globule membrane was obtained as a light-brown pellet after centrifugation at $100000\,g_{av.}$ and 37°C for 1h. The membrane was washed twice with water by centrifugation under the same conditions. The washed milk-fat-globule membrane was finally freeze-dried and stored at −20°C until further use.

Extraction of milk-fat-globule-membrane protein with $MgCl_2$. The extraction of the major glycoproteins of milk-fat-globule membrane with $MgCl_2$ was carried out by the method of Mather & Keenan (1975). Washed and well-packed cream (3.3g wet wt.) was suspended in 0.05M-Tris/HCl buffer, pH7.5, containing 1.5M-$MgCl_2$, to give a final volume of 10ml. The suspension was mixed gently at room temperature for 15min and the cream separated by centrifugation at $12000\,g_{av.}$ and 22°C for 15min. The rest of the experimental procedure was carried out at 4°C. The supernatant was re-centrifuged at $100000\,g_{av.}$ for 1h and the residual cream removed by aspiration. A small pellet was also obtained. The clear supernatant was dialysed exhaustively against 0.05M-Tris/HCl buffer, pH7.5. A slight precipitate that formed during dialysis was removed by centrifugation at $100000\,g_{av.}$ for 1h. The supernatant (extract) was concentrated in an ultrafiltration cell (model 52, Amicon, High Wycombe, Bucks., U.K.) fitted with a PM10 Diaflo ultrafilter by using 136kPa pressure from a cylinder of N_2. The concentrated extract was analysed by SDS / polyacrylamide-gel electrophoresis and assayed for protein and amino acids. Extracts were stored frozen at −20°C until further use.

Lectin affinity chromatography of milk-fat-globule-membrane glycoproteins. Extracts of glycoprotein(s) from human milk-fat-globule membrane were fractionated by lectin affinity chromatography based on the methods of Allan & Crumpton (1972) and Snow *et al.* (1977). All manipulations during this experiment were carried out at 4°C unless otherwise stated. A column (10.0cm × 0.6cm) was packed with concanavalin A covalently coupled to the CNBr-activated Sepharose 4B and was equilibrated with 0.05M-Tris/HCl buffer, pH7.9, containing 0.15M-NaCl and 0.25% (w/v) sodium deoxycholate, which also served as the running buffer.

The $MgCl_2$ extracts of washed cream protein (4.0mg) were mixed with 0.05M-Tris/HCl buffer (2.5ml), pH7.9, containing 0.5% (w/v) sodium deoxycholate at room temperature for 15min. The mixture was centrifuged at $100000\,g_{av.}$ and 22°C for 1h to remove any insoluble material. The clear supernatant was applied to the concanavalin A–Sepharose column at a flow rate of 1.0ml/h. After sample application, the column was washed with the running buffer at a flow rate of 8.0ml/h until the A_{280} of the effluent returned to the baseline. Fractions (1.5ml) were collected. Bound glycoprotein was eluted with the same buffer solution containing 0.1M-α-methyl mannoside. The column was further washed with the buffer solution containing 0.1M-α-methyl mannoside. The column was further washed with the buffer solution containing 0.01M-α-methyl mannoside and with a buffer containing 1.0M-NaCl. The protein–sodium deoxycholate complex was dialysed against 40% (v/v) methanol with frequent changes at 22°C for 12h, as described by Kobylka & Carraway (1972). The protein solution was then dialysed for 12h at room temperature against frequent changes of distilled water to remove the methanol.

Gel filtration of milk-fat-globule-membrane glycoprotein. All manipulations during this experiment were carried out at 4°C. A column (2.5cm × 29cm) was packed with Sephadex G-200 and equilibrated with 0.05M-Tris/HCl buffer, pH9.0, containing 5mM-EDTA, 5mM-2-mercaptoethanol and 20% (v/v) dimethylformamide, which also served as the running buffer. Dimethylformamide, a protein-dissociating agent, previously used for the dissociation of lipase from κ-casein by Fox *et al.* (1966), was investigated for its ability to dissociate and fractionate milk-fat-globule-membrane proteins. The bound material to the concanavalin A-Sepharose 4B column was solubilized in the running buffer and chromatographed on the Sephadex G-200 column. A flow rate of 6ml/h was maintained. Fractions (1.5ml) were collected and A_{280} of each measured on a spectrophotometer. The fractions eluted at the peak were pooled, dialysed exhaustively against water and freeze-dried.

SDS/polyacrylamide-gel electrophoresis. Initial extract of milk-fat-globule membrane and column fractions resulting from the chromatography of milk-fat-globule-membrane extracts were examined by electrophoresis at room temperature on polyacrylamide gels containing methylenebisacrylamide, SDS, EDTA and 2-mercaptoethanol by the method of Weber & Osborn (1969), with 0.05M-Tris/HCl, pH7.0, as the buffer system. Gels were prepared to give a 7.5% (w/v) acrylamide concentration. After a 30min pre-run, 50–200μg of protein was applied to the gels and the electrophoresis continued for 3–4h at 3mA/gel. Gels were stained for protein and carbohydrate by the method of Anderson *et al.* (1974) and Fairbanks *et al.* (1971) respectively. Molecular weights of proteins were estimated by comparison of the milk-fat-globule-membrane-protein bands with bovine serum albumin and α-, β- and β'-polypeptide subunits of RNA polymerase used as molecular-weight standards (Boehringer).

Enzymic iodination of purified glycoprotein. The purified glycoprotein, termed 'epithelial membrane glycoprotein' (hereafter referred to as EMGP-70) was enzymically iodinated by the method of Morrison & Bayse (1970) and Miyachi & Chrambach (1972). Lactoperoxidase (50μg/ml) and EMGP-70 (500μg/ml) solutions were freshly made in 0.05M-sodium phosphate buffer, pH7.4. Solutions of sodium metabisulphite (0.5mg/ml) and KI (31.1mg/ml) containing bovine serum albumin (0.1%, w/v) were prepared in the same buffer. Iodinations were carried out at room temperature in small glass vials with stirring. The reaction mixture consisted of $Na^{125}I$ solution (10μl; containing 0.97mCi and 0.56nmol of ^{125}I), EMGP-70 (20μl) and lactoperoxidase solution (10μl). The reaction was started by addition of 0.86mM-H_2O_2 (10μl) and the reaction mixture was stirred for 15min at room temperature. The reaction was terminated by the addition of sodium metabisulphite (100μl) and KI (200μl) solutions.

Separation of ^{125}I bound to EMGP-70 from free ^{125}I was carried out on a Sephadex G-25 column (25cm × 0.7cm) equilibrated with 0.05M-sodium phosphate buffer, pH7.4, containing bovine serum albumin (0.1%, w/v). The reaction mixture was applied to the column and eluted with the equilibrating buffer at a flow rate of 10ml/h under gravity at room temperature. Fractions (1.0ml) were collected. Samples (10μl) from each fraction were pipetted out in polystyrene tubes and the radioactivity counted on an automatic gamma-scintillation counter (LKB Wallac). The ^{125}I-labelled EMGP-70 solution was treated with SDS (3%, w/v) / 5mM-2-mercaptoethanol / 5mM-EDTA in 0.05M-Tris/HCl buffer, pH7.0, for 1h at 37°C on a water bath. The aqueous fractions were dialysed overnight against 0.05M-Tris/HCl buffer, pH7.0, containing SDS (0.1%, w/v), 5mM-2-mercaptoethanol and 5mM-EDTA. The dialysed material containing solubilized ^{125}I-labelled EMGP-70 was subjected to SDS / polyacrylamide-gel electrophoresis. The gels were frozen and sliced into 1.0mm sections with a gel slicer (The Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.). The radioactivity in each gel section was counted in an automatic gamma-scintillation counter (LKB Wallac).

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

Monosaccharide analysis. Monosaccharides were analysed by Dr. J. Westwood of this Institute by the method of Clamp *et al.* (1971), by using a Perkin–Elmer F-30 gas chromatograph containing two matching 1.83 m × 0.64 cm (6 ft × 0.25 in) glass columns packed with 2.5% silicone gum rubber (E301) on AW-DMCS Chromosorb G (80–100 mesh). After injection of the samples, the temperature of the column was raised from 120°C at the rate of 1°C/min to 210°C. Mannitol and perseitol were used as internal standards.

Amino acid analysis. Amino acid analyses were performed by Dr. J. Walker of this Institute. The analyses were carried out by using an automatic amino acid analyser (JLC 6AH) after hydrolysis of samples using 6 M-HCl under an atmosphere of N_2 at 110°C for 24 h.

Production of antibodies. New Zealand White rabbits were immunized with an $MgCl_2$ extract of washed cream and with the purified glycoprotein (EMGP-70).

Immunization was initiated subcutaneously in the nuchal area with 1.0 ml mixture of $MgCl_2$ extract of washed cream (0.5 mg of protein/ml) in 50% (v/v) complete Freund's adjuvant. Secondary and subsequent (booster) immunizations were given fortnightly. On 15 days after the second booster injections, the rabbits were bled from their ear veins. Antisera were stored at −20°C in small portions.

The immunization scheme with EMGP-70 was the same as above, with the exception of the protein concentration. Each rabbit was immunized with 1.0 ml mixture of EMGP-70 (0.2 mg of protein/ml) in 50% (v/v) complete Freund's adjuvant.

Ouchterlony double diffusion. Immunoprecipitation reactions were carried out by double diffusion in agarose. 0.05 M-Tris/HCl buffer, pH 7.5, containing agarose (1.0%, w/v), Triton X-100 (0.1%, v/v) and NaN_3 (0.02%, w/v) was incubated on a water bath until the solution became clear. When the gel solution cooled to a temperature of about 50–60°C, it was pipetted out on to immunodiffusion plates (9.5 cm × 4.5 cm) and left to gel at room temperature. Wells of various diameter were made in the gel using a cutter and template kit (Miles Laboratories, Slough, Bucks., U.K.). The presence of antibodies was determined by setting up antiserum against appropriate antigen preparation on immunodiffusion gel plates as described below.

The glycoprotein extracted from washed cream with $MgCl_2$ and EMGP-70 (1–5 mg of protein/ml) were solubilized in 0.05 M-Tris/HCl buffer, pH 7.5, containing Triton X-100 (0.1%, v/v) and NaN_3 (0.02%, w/v). Solutions were centrifuged on a microcentrifuge (Beckman microfuge B) at 4°C to remove any insoluble materials. Samples (10 μl) were applied to outer wells formed in the agarose layer, whereas the centre wells contained antisera (10 μl) to either $MgCl_2$ extract of washed cream or EMGP-70. EMGP-70 was also tested against antisera to human lactoferrin, α-lactalbumin, caseins, whole plasma, serum albumin, α_1-acid glycoprotein, α_1-antitrypsin, secretory piece of immunoglobulin A, carcinoembryonic antigen, glycophorin and mouse mammary-tumour-virus glycoprotein.

Results

SDS/polyacrylamide-gel-electrophoretic analysis

The membrane preparations obtained both directly from washed cream and from isolated milk-fat-globule membrane consistently showed 19 polypeptide components (Fig. 1). Of these 19 components, six Coomassie Blue-positive major size classes of polypeptide of 155000, 70000, 58000, 52000, 42000 and 39000 mol.wt. (components 3, 12, 14, 16, 18 and 19 respectively) were present. Five of these major polypeptides (components 3, 12,

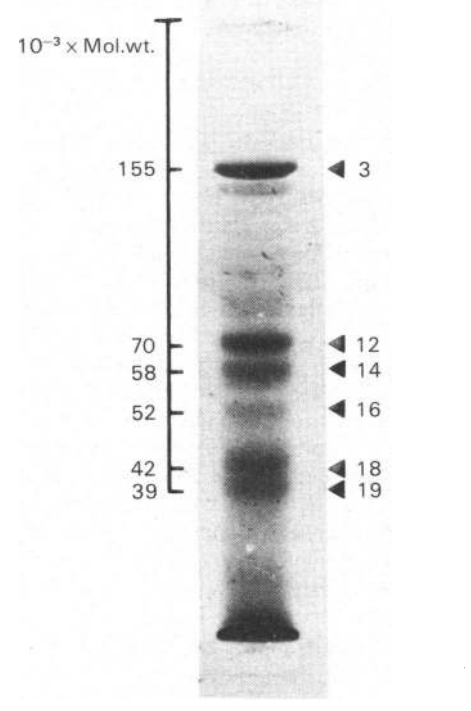

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of proteins from washed cream*

The separation of proteins from human milk-fat globule membrane is shown. The sample was extracted from thrice-washed cream and prepared for electrophoresis as described in the Materials and methods section. The gel was stained with Coomassie Blue. The major protein bands routinely seen in gels after electrophoresis are numbered (on the right) from the top of the gel. The numbers on the left indicate molecular weight.

16, 18 and 19) were also periodate–Schiff-reagent-positive.

Glycoprotein extraction. The extraction of well-packed and washed human cream (3.3 wet wt.) with $MgCl_2$ yielded 11–15 mg dry wt. of the extract, of which about 60% was protein. The same yield, namely 7–9 mg of milk-fat globule-membrane protein from 100 ml of whole milk was obtained from either washed cream or isolated milk-fat-globule membrane. The protein extracts were analysed by SDS/polyacrylamide-gel electrophoresis. Some selectivity in the extraction of the proteins was observed. Glycoprotein components corresponding to components 3, 12, 14 and 19 and also relatively minor amounts of components 16 and 18 were present in the extract. Components 3, 14, 18 and 19 were partially extracted, whereas component 12 (EMGP-70) was mostly extracted by this procedure.

Lectin affinity chromatography of milk-fat-globule-membrane glycoproteins

Since milk-fat-globule membrane is known to bind through its glycoprotein(s) to concanavalin A (Horisberger *et al.*, 1977), we decided to determine which components were involved in binding to this lectin. It was found possible to purify the glycoproteins further by lectin affinity chromatography. Washed cream extracted with $MgCl_2$ was separated into two fractions using concanavalin A–Sepharose 4B, as shown in Fig. 2. Approx. 85% of the added protein was recovered from the column. The bound fractions contained mostly component 12 (EMGP-70), with small traces of component 18, as revealed

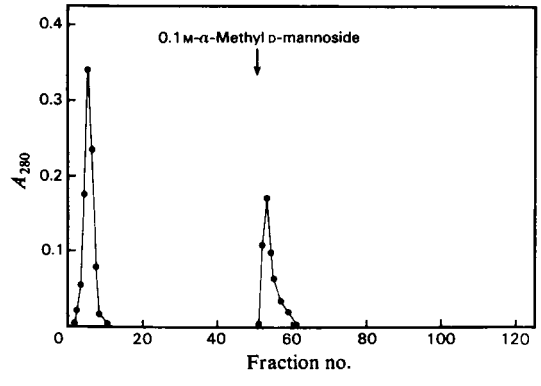

Fig. 2. *Lectin affinity chromatography of milk-fat-globule-membrane glycoproteins on concanavalin A–Sepharose 4B in 0.25% (w/v) sodium deoxycholate*

Samples (5.0 mg of protein) were applied to a column (10.0 cm × 0.6 cm) of concanavalin A–Sepharose 4B conjugate in 0.05 M-Tris/HCl buffer, pH 7.9, containing 0.25% (w/v) sodium deoxycholate and 0.15 M-NaCl. Specifically bound glycoproteins were eluted with the above buffer solution containing 0.1 M-α-methyl D-mannoside. The column flow rate was 1.0 ml/h. Fractions (1.5 ml) were collected and monitored for A_{280}.

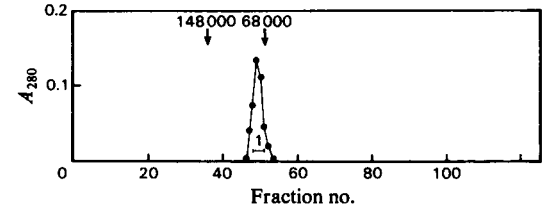

Fig. 3. *Sephadex G-200 chromatography of the adsorbed fraction from lectin affinity column (Fig. 2) in 20% (v/v) dimethylformamide*

Samples (bound material from concanavalin A–Sepharose 4B column) were applied to a column (2.5 cm × 29 cm) of Sephadex G-200 in 0.05 M-Tris/HCl buffer, pH 9.0, containing 5 mM-EDTA and 20% (v/v) dimethylformamide. The column flow rate was 6.0 ml/h. Fractions (1.5 ml) were collected and monitored. Fractions taken for further analyses are designated 'I'.

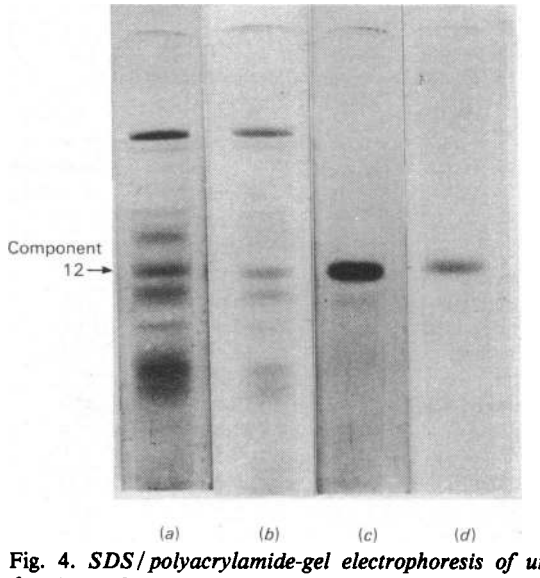

Fig. 4. *SDS/polyacrylamide-gel electrophoresis of unfractionated proteins, $MgCl_2$ extract and Fraction 1 from the Sephadex G-200 column chromatography of human milk-fat-globule-membrane protein*

Unfractionated protein, $MgCl_2$ extract and Fraction 1 from the Sephadex G-200 column chromatography (see Fig. 3) of human milk-fat-globule-membrane protein were analysed by SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section. Gels (*a*)–(*c*) are stained with Coomassie Blue and gel (*d*) with Periodate–Schiff reagent. Gel (*a*) contains unfractionated protein from milk-fat-globule membrane; (*b*) the $MgCl_2$ extract of the milk-fat globule membrane; (*c*) the Fraction 1 from the Sephadex G-200 column chromatography; (*d*) same as in gel (*c*).

by SDS/polyacrylamide gel electrophoresis (result not shown).

Chromatographic isolation of EMGP-70. The adsorbed glycoproteins from the concanavalin A–Sepharose column were solubilized and chromatographed on a Sephadex G-200 column in the presence of dimethylformamide. An elution profile is illustrated in Fig. 3. Approx. 80% of total protein applied was eluted in a single major peak. The protein eluted at the peak yielded a single band on SDS/polyacrylamide gels (Fig. 4). This band corresponded to electrophoretic component 12 and stained with both Coomassie Blue and periodate–Schiff reagent. On 7.5% acrylamide gels, this glycoprotein has an estimated mol.wt. of 70000.

Enzymic iodination of EMGP-70

EMGP-70 was enzymically iodinated with ^{125}I. Approx. 45% of the total radioactivity applied was incorporated. The ^{125}I-labelled EMGP-70 migrated at the same position as unlabelled EMGP-70 (component 12) on SDS/polyacrylamide gels, confirming that the major band (Fig. 4) was derived from component 12.

^{125}I-labelled EMGP-70 was also subjected to polyacrylamide-gel electrophoresis in the absence of SDS. Most of the labelled EMGP-70 applied remained at the upper portion of the gel (result not shown). This result suggests that the EMGP-70 in the absence of protein-dissociating agent forms large aggregates. Such an observation is consistent with its strong hydrophobic domains, which are associated with integral membrane proteins (Williams, 1978). Preliminary studies of a radioimmunoassay system for EMGP-70 show that the antigenic properties are retained after iodination.

Chemical characterization

To compare the purified EMGP-70 from human milk-fat-globule membrane with glycoprotein from

Table 1. *Comparison of amino-acid and monosaccharide compositions of isolated glycoprotein from bovine erythrocyte membranes, bovine milk-fat-globule membrane (MFGM), human erythrocyte membranes and human milk-fat-globule membrane*

References: [a] Hudson *et al.* (1975); [b] Basch *et al.* (1976); [c] Freudenstein *et al.* (1979); [d] Furthmayr *et al.* (1975); n.r., not recorded; n.d., not detected.

	Composition (mol%)					
		Bovine MFGM isolated glycoprotein			Human MFGM	
	Bovine erythrocyte glycoprotein[a]	Glycoprotein B[b]	Band 12[c]	Human erythrocyte glycophorin A[d]	$MgCl_2$ extract	EMGP-70
Cysteic acid	n.r.	2.6	1.0	0.1	2.4	0.8
Methionine sulphoxide	n.r.	n.r.	n.r.	n.r.	0.5	0.4
Aspartic acid	5.4	10.8	9.9	9.0	10.0	11.0
Threonine	12.1	7.3	7.0	14.3	5.1	6.1
Serine	10.2	6.4	6.6	15.4	7.6	9.9
Glutamic acid	13.5	10.0	8.2	15.3	10.4	9.4
Proline	11.8	4.6	5.1	9.7	2.3	5.0
Glycine	7.5	9.1	8.8	6.1	7.8	8.1
Alanine	6.5	6.0	6.5	7.3	8.9	9.4
Valine	6.4	5.3	8.0	11.1	5.2	6.4
Methionine	n.d.	1.0	2.6	1.8	0.5	n.d.
Isoleucine	4.6	5.2	6.3	9.8	4.7	3.3
Leucine	5.4	7.9	9.8	8.0	8.1	8.6
Tyrosine	1.3	3.3	2.4	3.8	2.9	2.5
Phenylalanine	1.7	4.6	5.1	2.5	4.4	5.4
Lysine	3.1	4.4	4.7	5.2	6.3	6.0
Histidine	1.3	2.6	2.7	5.2	2.4	2.5
Arginine	6.9	4.2	5.2	6.1	5.1	4.7
				(g/100 g)*		
Fucose	0.4	n.r.	3.2	1.0	13.8	17.2
Mannose	1.2	7.6	14.8	2.9	9.5	17.0
Galactose	42.0	7.3	8.2	10.7	35.0	21.1
Glucose		4.9	4.9	0.6	n.r.	n.r.
N-Acetylgalactosamine	12.0	6.3	17.3	10.8	n.d.	n.d.
N-Acetylglucosamine	29.0	22.1	44.4	6.7	20.5	7.9
Sialic acid	16.0	51.6	7.2	20.7	21.1	36.6

* Applies only to values in this column.

other sources, quantitative amino-acid-residue and carbohydrate analyses were performed.

Amino-acid analysis. The amino-acid compositions of EMGP-70 and the $MgCl_2$ extracts of milk-fat-globule membrane resembled that of bovine fractions (Kobylka & Carraway, 1972; Cawston *et al.*, 1976; Freudenstein *et al.*, 1979). These bovine proteins are characterized by a relative abundance of glutamic acid and aspartic acid, alanine, leucine, glycine and serine and deficiency of methionine and cysteine (Basch *et al.*, 1976; Freudenstein *et al.*, 1979) and are shown for comparison in Table 1.

Carbohydrate analysis. The carbohydrate analyses of $MgCl_2$ extracts of milk-fat-globule membrane and isolated EMGP-70 are shown in Table 1. For EMGP-70, approx. 13.5% of the molecule by weight was carbohydrate and 86% was protein. The molar percentage of fucose (17.2), mannose (17.0), galactose (21.1), *N*-acetylglucosamine (7.9) and sialic acid (36.6) corresponded to a molar ratio of 2:2:3:1:5. The percentage carbohydrate compositions of EMGP-70 were different from those of the bovine proteins (Freudenstein *et al.*, 1979) (Table 1). 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 proteins from bovine milk-fat-globule membranes. No *N*-acetylgalactosamine could be detected in EMGP-70.

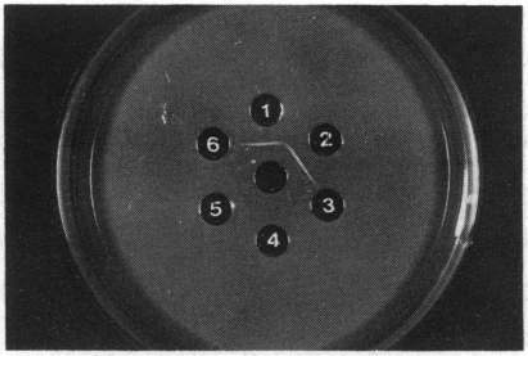

Fig. 5. *Comparison of antigenic property of extract of glycoproteins from human milk-fat-globule-membrane with $MgCl_2$ and EMGP-70*

The immunodiffusion plate shows the immunoprecipitin line obtained when rabbit antiserum to a $MgCl_2$ extract of glycoproteins from human milk-fat-globule membrane was made to react against a similar extract and EMGP-70 by the methods described in the text. The centre well contains antiserum to the $MgCl_2$ extract. Peripheral wells contain: (1) EMGP-70; (2) the $MgCl_2$ extract; (3) lactoferrin; (4) α-lactalbumin; (5) caseins; (6) pooled plasma.

A certain amount of glucose was found in the column effluents of EMGP-70 preparations. This could be decreased 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.

Ouchterlony double diffusion. Rabbit antisera raised against the $MgCl_2$ extract of glycoproteins from washed cream showed two parallel immunoprecipitin lines with the $MgCl_2$ extract and a single immunoprecipitin line with EMGP-70 (Fig. 5). Reactions of identity were observed between Triton X-100-solubilized $MgCl_2$ extract of washed cream and EMGP-70 (Fig. 5). This confirms that EMGP-70 has retained its antigenic properties during the purification procedure. Neither the $MgCl_2$ extract of milk-fat-globule membrane nor EMGP-70 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, caseins, secretory piece of IgA, carcinoembryonic antigen, glycophorin, and glycoproteins from mouse mammary-tumour virus.

Discussion

The present study shows that the pattern of major proteins from human milk-fat-globule membrane on SDS/polyacrylamide gels is similar to that of bovine milk-fat-globule membrane (Freudenstein *et al.*, 1979; Mather, 1978; Mather & Keenan, 1975). The presence of two major glycoprotein components of human milk-fat-globule membrane with estimated mol.wts. of 160000 and 84000, which correspond closely to components 3 and 12 in the present study, has been reported by Martel *et al.* (1973).

Kobylka & Carraway (1972) showed that milk-fat-globule-membrane proteins could be separated by gel filtration in detergent solutions. The glycoproteins of human milk-fat-globule membrane can be selectively obtained by extraction with high concentration of $MgCl_2$. This procedure can be applied directly to samples of cream without intermediate isolation of the membrane fraction. Attempts to fractionate these glycoprotein components by gel chromatography in aqueous buffers were frustrated by the formation of aggregates containing multiple species. This difficulty was overcome by including protein-dissociating agents such as sodium deoxycholate and dimethylformamide in the chromatographic procedures. In the present study, lectin affinity chromatography and gel filtration in the presence of sodium deoxycholate and dimethylformamide respectively were combined to isolate EMGP-70. The purified EMGP-70 gave a single band on SDS/polyacrylamide-gel electrophoresis at a polyacrylamide-gel concentration of 7.5%. The possibility of microheterogeneity of the

carbohydrate portions of the glycoproteins is not excluded. The estimated mol.wt. of 70000 may be affected by the anomalies associated with transport of glycoproteins on gels (Anderson *et al.*, 1974).

There are differences between the amino-acid compositions of glycoproteins of human milk-fat-globule membrane and of human erythrocyte membranes (Table 1). Similar differences have also been reported between the glycoproteins of bovine milk-fat-globule membrane and bovine erythrocyte membranes (Hudson *et al.*, 1975; Snow *et al.*, 1977). In human milk-fat-globule-membrane glycoproteins the diacidic amino acids and the hydroxy amino acids accounted for 22 and 16% of the total respectively. In glycoproteins from human erythrocyte membranes the proportions are reversed (Furthmayr *et al.*, 1975). The composition of EMGP-70, with the absence of *N*-acetylgalactosamine, excludes the possibility of an *N*-acetylgalactosamine–hydroxy amino acid linkage that is found in the erythrocyte membrane glycoproteins (Furthmayr *et al.*, 1975; Kornfeld & Kornfeld, 1976).

The purification of a glycoprotein from human milk-fat-globule membrane derived from the mammary secretory epithelium will permit studies of its structural and functional role in secretion. The substance may be a valuable aid in the study of the function of mammary epithelial cells in tissue culture.

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