High- M_r glycoprotein profiles in human milk serum and fat-globule membrane

Makoto SHIMIZU,*† Kunio YAMAUCHI,* Yosuke MIYAUCHI,* Toshio SAKURAI,* Kazuhisa TOKUGAWA* and Robert A. J. McILHINNEY†

*Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan, and †Ludwig Institute for Cancer Research, Royal Marsden Hospital, Sutton, Surrey, U.K.

Gradient-polyacrylamide-gel electrophoresis of human milk serum separated three high- M_r glycoprotein bands. The properties of the components corresponding to the three bands (tentatively termed 'Components C, B and A' in their order of migration) were compared by staining with four monoclonal antibodies and lectins. Components B and C both reacted with the four antibodies, but Component A did not. Components B and C were stained with peanut (*Arachis hypogaea*) agglutinin (PNA) and wheat (*Triticum*)-germ agglutinin (WGA), Component A being stained with soya-bean (*Glycine max*) agglutinin as well as PNA and WGA. These results suggest that Components B and C were related molecules, whereas Component A was markedly different from them. The reactivities of Components B and C were the same as those of PAS-0, a high- M_r periodate/Schiff (PAS)-positive glycoprotein previously isolated from human milk fat-globule membrane (MFGM). Component C, whose electrophoretic mobility was the same as PAS-0, could have been a soluble form of PAS-0. A high- M_r glycoprotein having the same properties as Component A was also observed in MFGM. The amino acid composition of the isolated Component A was significantly different from that of Component C and PAS-0, high threonine and serine contents being characteristic of Component A. The carbohydrate content of Component A was 65–80%, and was much higher than that of Component C and PAS-0. Fucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine and sialic acid were each detected as constituent sugars of Component A. Component A represents, therefore, a new high- M_r glycoprotein species in human milk serum and MFGM. Since these glycoproteins were high- M_r mucin-like glycoproteins, the names 'HM glycoprotein-A' and 'HM glycoprotein-C' were proposed for Component A and Component C (PAS-0) respectively.

INTRODUCTION

The milk fat-globule membrane (MFGM) that encloses the fat droplets secreted in milk is known to be derived from the apical-cell membranes of mammary secretory cells (Patton & Keenan, 1975). Studies by many researchers in the past two decades have revealed that bovine MFGM contains a variety of proteins and glycoproteins (Keenan *et al.*, 1983). However, the information available for human MFGM proteins and glycoproteins is still limited.

By SDS/polyacrylamide-gel electrophoresis using 7.5–10% (w/v)-acrylamide gels, the existence of several PAS-positive components has been demonstrated in human MFGM (Murray *et al.*, 1979; Freudenstein *et al.*, 1979; Imam *et al.*, 1981; Shimizu & Yamauchi, 1982; Heid *et al.*, 1983). A unique hydrophobic glycoprotein having an M_r of 67000 was isolated by Heid *et al.* (1983) and named 'butyrophilin'. Three glycoproteins with M_r 155000, 70000 and 39000 (named EMGP-155, 70 and 39 respectively) have been isolated and characterized by Imam *et al.* (1981, 1982).

Shimizu & Yamauchi (1982) isolated another type of glycoprotein in human MFGM, which had a high M_r and did not migrate in 10% acrylamide gel on SDS/poly-

acrylamide-gel electrophoresis. Chemical and biochemical analyses of this glycoprotein, termed 'PAS-0', showed that this was a mucin-like glycoprotein containing approx. 50% (w/w) carbohydrate. The existence of such a high- M_r mucin-like glycoprotein in human MFGM has been confirmed by other investigators in the field of cancer research. By using antisera or monoclonal antibodies raised against human defatted cream or MFGM, they found in MFGM an antigenic glycoprotein having a large M_r (Ceriani *et al.*, 1983; Burchell *et al.*, 1983; Hilken *et al.*, 1984b). The properties of the glycoproteins reported suggest that they are the same molecule as PAS-0.

Heyderman *et al.* (1979) showed that a high- M_r glycoprotein that reacted with the antisera against human MFGM was also present in skim milk. This glycoprotein molecule, termed 'epithelial-membrane antigen' (EMA), has recently been isolated from skim milk by Ormerod *et al.* (1983), and its similarity with PAS-0 has been elucidated by immunological means (Ormerod *et al.*, 1985). However, the identity of EMA is still obscure, because the chemical composition of EMA varied from one preparation to another (Ormerod *et al.*, 1983).

In the present study we have analysed the high- M_r fraction of the milk serum from some samples of

Abbreviations used: MFGM, milk fat-globule membrane; SDS, sodium dodecyl sulphate; PAS, periodate/Schiff; EMA, epithelial-membrane antigen; PNA, peanut agglutinin; SBA, soya-bean agglutinin; Con A, concanavalin A; WGA, wheat-germ agglutinin; e.l.i.s.a., enzyme-linked immunosorbent assay.

† To whom correspondence and reprint requests should be addressed.

colostrum and mature milk. A new high- M_r glycoprotein having a larger molecular size than that of PAS-0 was found in most of the milk samples. This glycoprotein was also found to be present in MFGM.

MATERIALS AND METHODS

Materials

The rabbit polyclonal anti-EMA (Ormerod *et al.*, 1983) antibody was a gift from Dr. M. G. Ormerod, and the monoclonal antibody 115D8 (Hilkens *et al.*, 1984a) was generously given by Dr. J. Hilkens of The Netherlands Cancer Institute, Amsterdam, The Netherlands. The binding of 115D8 to PAS-0 has been confirmed by Hilkens *et al.* (1984b). The monoclonal antibodies M8 (Foster *et al.*, 1982) and TW 19.5 (Gore, 1984) were provided by Dr. P. A. W. Edwards and Dr. M. E. Gore respectively of the Ludwig Institute for Cancer Research. The monoclonal antibody 77.1, which was raised against a human bladder carcinoma, was a gift from Dr. I. Summerhayes of the Institute of Cancer Research, London, U.K. The latter three antibodies reacted with both EMA and PAS-0 as demonstrated previously (Ormerod *et al.*, 1985). From the reaction pattern of each of these antibodies, as well as from their tissue distributions, it is clear that each reacts with a different epitopes carried on sugar side chains (McIlhinney *et al.*, 1985; K. Steele & M. G. Ormerod, personal communication). The affinity-purified and peroxidase-conjugated sheep anti-mouse IgG and phosphatase-conjugated goat anti-rabbit IgG were purchased from Cappel Laboratories and Kirkegaard and Perry Laboratories respectively. The peroxidase-conjugated PNA, WGA, SBA and ConA were purchased from Seikagaku Kogyo Co. Ltd., Tokyo, Japan.

Preparation of milk serum and fat-globule membrane

Samples of mature milk (more than 1 month after parturition) and colostrum (within 1 week of parturition) were obtained from healthy mothers. The milk serum prepared from acidified skim milk as described by Azuma *et al.* (1985) was dialysed against water and freeze-dried. MFGM was prepared as described previously (Shimizu & Yamauchi, 1982).

Column chromatography

Gel filtration was carried out in 15 mM-Tris/HCl buffer, pH 8.0, containing 100 mM-NaCl and 10 mM-sodium deoxycholate, with a column (1.5 cm $\times$ 60 cm) of Sepharose CL-4B (Pharmacia) at 4 °C.

Anion-exchange chromatography was carried out on a column (1.8 cm $\times$ 10 cm) of DEAE-Sephacel (Pharmacia) that had been equilibrated with 50 mM-Tris/HCl buffer, pH 8.0 containing 0.1% α -dodecyl- ω -hydroxy-poly(oxyethane-1,2-diyl) ('polyoxyethylene lauryl ether') with an average of nine polyoxyethylene chains per molecule ($C_{12}E_9$; Nakarai Chemicals). $C_{12}E_9$ in the recovered fractions was removed by the use of Bio-Beads SM2 (Bio-Rad Laboratories) (Moriyama *et al.*, 1984).

SDS/polyacrylamide-gel electrophoresis and Western blotting

SDS/polyacrylamide-gel electrophoresis was carried out either by the method of Laemmli (1970) or by that of Weber & Osborn (1969), a gradient acrylamide gel being used.

The electrophoresed gels were directly stained or transferred electrophoretically to nitrocellulose membranes (Bio-Rad). The transferred nitrocellulose strips were stained by using lectin-conjugated peroxidase as described by Moroi & Jung (1984). For immunological staining, the strips were first treated with phosphate buffered saline (10 mM-sodium phosphate buffer, pH 7.4) containing 1% (w/v) bovine serum albumin and 5% (v/v) pig serum, and then incubated with diluted hybridoma supernatants containing the monoclonal antibodies. After incubating for 90 min at 40 °C and then overnight at 4 °C, the strips were washed with 1% bovine serum albumin/phosphate-buffered saline. The second antibody (horseradish peroxidase-conjugated sheep anti-mouse IgG) was added and the mixture was incubated at 40 °C for 90 min. The strips were washed with 1% bovine serum albumin/phosphate-buffered saline, and then stained by immersing in phosphate-buffered saline containing 0.02% 3,3'-diaminobenzidine and 0.005% H_2O_2 .

E.l.i.s.a

The antigen solution (10 μ g/100 μ l in phosphate-buffered saline) was applied to the wells of 96-well polystyrene microtitre plates. After being left overnight at room temperature, the wells were washed with phosphate-buffered saline containing 0.05% Tween 20 and then incubated with phosphate-buffered saline containing 1% ovalbumin for 2 h at room temperature. After three washes with Tween/phosphate-buffered saline, diluted rabbit anti-EMA serum (100 μ l) was added before incubating for 2 h. After washing the wells with Tween/phosphate-buffered saline, goat anti-rabbit IgG-phosphatase conjugate (30 ng/100 μ l) was added and incubated at room temperature for 2 h. The unbound second antibody was removed and the wells were washed with Tween/phosphate-buffered saline. A 100 μ l portion of *p*-nitrophenyl phosphate disodium salt solution (1 mg/ml) was then added to each well and the A_{405} in the wells after 30 min was measured.

Chemical analysis

Amino acid and hexosamine analyses were performed on a Hitachi 835 amino acid analyser as described previously (Shimizu & Yamauchi, 1982). Individual neutral sugars were determined as trimethylsilyl derivatives (Clamp *et al.*, 1972). G.l.c. was performed on a Shimadzu GC-7A gas chromatograph as described previously (Shimizu & Yamauchi, 1982). Sialic acid was determined with a Shimadzu LC-4A h.p.l.c. instrument equipped with a Zorbax NH_2 column (4.6 mm $\times$ 150 mm). Chromatography was performed at 38 °C in 20 mM- $NH_4H_2PO_4$ /methanol (1:1, v/v) at pH 2.66. The eluate was monitored at 210 nm and the sialic acid content was determined by using authentic *N*-acetylneuraminic acid as the standard.

RESULTS

Profiles of the high- M_r glycoprotein fraction in human milk serum

The high- M_r protein fraction obtained from different human milk serum samples by gel filtration (Fig. 1) was analysed by SDS/polyacrylamide-gel electrophoresis. Fig. 2 shows polyacrylamide-gel-electrophoretic patterns of the high- M_r glycoproteins from one mother at various

Fig. 1. Typical gel-filtration pattern of human milk serum protein on Sepharose CL-4B

The serum protein from mature milk was dissolved in 15 mM-Tris/HCl buffer, pH 8.0, containing 100 mM-NaCl/10 mM-sodium deoxycholate/130 mM-2-mercaptoethanol, and applied to a column. Similar patterns were observed for the colostrum samples. The F1 and F2 fractions (fractions nos. 9–17) were combined and used as a 'high- M_r protein fraction'.

stages of lactation. In the mature milk sample, three bands of high- M_r (A–C) were detected by PAS. The components corresponding to these three bands were tentatively termed 'Components A, B and C' in the present study. In contrast, the fractions from the transitional milk and colostrum of this mother did not show these bands. In colostrum, a broad band having a similar electrophoretic mobility to band A was predominant. SDS/polyacrylamide-gel electrophoresis of the high- M_r fractions of several colostrum and mature milk samples from different mothers indicated that the pattern varied according to the individual mother, although Component A was clearly observed in all colostrum samples (results not shown). Band C had almost the same electrophoretic mobility as that of PAS-0, a high- M_r glycoprotein previously purified from human MFGM (Shimizu & Yamauchi, 1982).

From gel filtration using Sepharose CL-4B (Fig. 1), Component A was eluted in the void-volume fraction (F-1), whereas Component B and C were eluted in the F-2 fraction (Fig. 1). In order to examine the homology or similarity between these high- M_r glycoproteins, their binding activities towards lectins and certain monoclonal antibodies were investigated. After SDS/polyacrylamide-gel electrophoresis of F1 and F2 the separated high- M_r glycoproteins were transferred to nitrocellulose strips, which were then stained immunologically with the antibodies. The results are summarized in Table 1. Components B and C, together with PAS-0, reacted with these monoclonal antibodies, whereas Component A did not (no staining). The strips were also stained with four lectins, the results being shown in Table 1. PNA and WGA reacted with each of the three components (A–C) together with PAS-0, whereas SBA reacted with

Fig. 2. SDS/polyacrylamide-gel electrophoresis patterns of the high- M_r glycoprotein fractions from one mother at various stages of lactation

(a) PAS-0 (as a control); (b) from colostrum (within 5 days after parturition); (c) from transitional milk (1–2 weeks); and (d) from mature milk (more than 1 month). SDS/polyacrylamide-gel electrophoresis was carried out by the procedure of Weber & Osborn (1969), with a 2–10% (w/v)-acrylamide gradient gel. The sample load was 50 μ g for (a) and 100 μ g for (b–d). The gels were stained with PAS-reagent.

Table 1. Reactivity of the high- M_r glycoproteins in human milk with monoclonal antibodies and lectins

Key to symbols: +, reacted; $\pm$, reacted, but weakly; –, did not react.

Glycoproteins...	Reaction			
	Component A	B	C	PAS-0
Monoclonal antibodies				
115D8	–	+	+	+
M8	–	+	+	+
77.1	–	+	+	+
TW19.5	–	$\pm$	$\pm$	$\pm$
Lectins				
PNA	+	+	+	+
WGA	+	+	+	+
SBA	+	–	–	–
Con A	–	–	–	–

Component A alone. Con A did not react with any of these glycoproteins. These results suggest that Components B and C were molecules related to PAS-0, but Component A was quite distinct.

Fig. 3. Isolation of Component C by Sepharose CL-4B chromatography

The human mature milk serum protein was applied to a column as described in Fig. 1. F-III (fractions nos. 14–15) was recovered and used for the further purification of Component C. SDS/polyacrylamide-gel electrophoresis patterns of F-II–F-IV stained by PAS reagent are shown in the inset Figure.

Fig. 4. Purification of Component C by DEAE-Sephacel chromatography

F-III (defined in Fig. 3) was applied to a column. The first peak (F-IIIA) eluted at 0.05 M-NaCl was recovered for further study.

Isolation of components A and C

The serum protein samples, in which band C was predominant, were chosen and chromatographed on Sepharose CL-4B as described in the Materials and methods section. The gel-filtration pattern (Fig. 3) showed that Component C was concentrated in the F-III fraction, and this fraction was rechromatographed on the same column. The F-III fraction so obtained was passed through a column of Bio-Beads SM-2 and then dialysed against 50 mM-Tris/HCl buffer, pH 8.0, containing 0.1% $C_{12}E_8$. The dialysed fraction was applied to a DEAE-Sephacel column, and elution was carried out by increasing the NaCl concentration. Component C was

Fig. 5. SDS/polyacrylamide-gel-electrophoretic patterns of the purified Component C

(a) Purified Component C; (b) whole milk serum protein (as a control). SDS/polyacrylamide-gel electrophoresis was carried out by the procedure of Laemmli (1970), with a 3–14% gradient gel. Gels were stained with Coomassie Blue R-250 (CB) for protein and PAS reagent (PAS) for carbohydrate.

eluted at < 0.05 M-NaCl, as shown in Fig. 4. This fraction was passed through a Bio-Beads SM-2 column and then extracted with cold methanol/chloroform (1:2, v/v). The aqueous phase was recovered and the residual solvent was removed by evaporation followed by dialysis against water. After dialysis, the solution was centrifuged to remove insoluble materials. The soluble fraction gave a single PAS-positive band on SDS/polyacrylamide-gel electrophoresis (Fig. 5a).

Component A was isolated from one colostral serum sample which contained the glycoprotein in a relatively high concentration. The serum sample was chromatographed on Sepharose CL-4B, the void-volume fraction being recovered and rechromatographed on the same column. The second void-volume fraction was passed through a Bio-Beads SM-2 column, dialysed against water and freeze-dried. A small amount of lipid contained in the fraction was extracted with cold ethanol/diethyl ether (1:1, v/v). This fraction also gave a single PAS-positive band on SDS polyacrylamide-gel electrophoresis (Fig. 6a).

A high- M_r glycoprotein similar to Component A was also detected in human MFGM (Fig. 6e), and was isolated from MFGM of a mature milk sample by repeated Sepharose CL-4B chromatography. The void-volume fraction gave a single band on SDS/polyacrylamide-gel electrophoresis (Fig. 6c). The reactivity of Component A from MFGM with the monoclonal antibodies and lectins was the same as that of Component A from milk serum (results not shown).

Chemical composition of the high- M_r glycoproteins

The amino acid composition of the high- M_r glycoproteins can be compared in Fig. 7. The composition of the purified Component C was very similar to that of PAS-0

Fig. 6. SDS/polyacrylamide-gel-electrophoretic patterns of Component A prepared from human milk serum and MFGM

(a) Component A from milk serum (colostrum); (b) total milk serum glycoproteins; (c) Component A from MFGM; (d) PAS-0 from MFGM; and (e) total MFGM glycoproteins. SDS/polyacrylamide-gel electrophoresis was carried out by the procedure of Laemmli (1970) with a 3–14% -acrylamide gradient gel. Gels were stained by PAS reagent for carbohydrate. Δ , $\blacktriangle$, Glycoproteins with similar M_r to those identified by: $\blacktriangle$, Imam *et al.* (1982); Δ , Heid *et al.* (1983).

Fig. 7. Profiles of amino acid composition for high- M_r glycoproteins isolated from human milk

(a) —, Component C; ----, PAS-0; (b) —, Component A from serum; ----, that from MFGM. Values are expressed as mol/100 mol of the total amino acids. The one-letter code for amino acids is used.

Table 2. Carbohydrate composition of the high- M_r glycoproteins in human milk

Values are expressed in mg/100 mg of the protein moiety (calculated from the sum of the amounts of individual amino acids).

	Content (mg/100 mg)					
	Fuc	Gal	GlcNAc	GalNAc	NAcNeu	Total
Component A						
From colostrum	22.4	196	104	54.2	27.8	404.4
From MFGM	12.4	83.6	52.2	30.7	20.7	199.4
of mature milk						
Component C*	11.1	52.4	19.9	14.7	19.7	117.8
PAS-0†	7.8	49.6	21.0	15.9	11.9	106.2

* Average for two preparations (from mature milk).
† Average for three preparations (from mature milk).

(Fig. 7a). Component A from the milk serum showed an amino acid composition similar to that from MFGM (Fig. 7b); however, the compositions of Component C and A were distinguishably different.

The carbohydrate composition of the glycoproteins is given in Table 2. All of the glycoproteins contained, as carbohydrate, fucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine and sialic acid. Although the carbohydrate content varied according to the sample (probably because of the microheterogeneity on the carbohydrate chains), the composition of Component C was similar to that of PAS-0. However, Component A seemed to carry a much higher amount of carbohydrate than Component C or PAS-0.

Reactivity of Components A and C with anti-EMA

In order to reveal the relationship between EMA and Components A–C, the reactivity of Components A and C with anti-EMA was examined by e.l.i.s.a. Both Components A and C were observed to react with anti-EMA.

DISCUSSION

The present study reveals that the human milk serum contained a high- M_r glycoprotein (Component C) that is very similar to the glycoprotein PAS-0 that had previously been isolated from human MFGM (Shimizu & Yamauchi, 1982). The pattern of reactivity of these two glycoproteins with some monoclonal antibodies and lectins, as well as the similarities in their amino acid and sugar compositions, suggest that they may be the same molecule. However, PAS-0 is a membranous glycoprotein present in MFGM, whereas Component C exists in milk serum in a soluble form. This difference in their distribution in milk might be accounted for by a subtle difference between the two molecules. Detailed analyses of the both glycoproteins, including the analysis of their carbohydrate structure, are required to resolve this matter.

The relationship between Components B and C could not be elucidated in the present study. However, the reactivity pattern of Components B and C with the

antibodies and lectins (Table 1) strongly suggested that Component B was a dimeric form of Component C.

The existence of another high- M_r glycoprotein species, termed 'Component A', was also observed in milk serum and MFGM. Since the electrophoretic mobility of Component A was very low, it was difficult to determine the homogeneity of this component. Component A could be a heterogeneous set of glycoproteins whose composition varied depending on the individual mother and lactation period. In the present study, Component A was prepared from one colostral serum and one mature MFGM sample. The carbohydrate content of the two preparations was markedly different, but each contained much more carbohydrate than did Component C or PAS-0. The reactivity with lectins and the carbohydrate composition indicate that Component A was a mucin-like glycoprotein, as is PAS-0. Recently, Fischer *et al.* (1984) have reported that human MFGM contained a high- M_r glycoprotein (termed 'HM $_r$ -MGP') with high carbohydrate content (75%). When they analysed the HM $_r$ -MGP by SDS/polyacrylamide-gel electrophoresis on a 10–20%-(w/v)-acrylamide gel, this glycoprotein did not enter the gel and its homogeneity is unknown. However, the high carbohydrate content of HM $_r$ -MGP suggests that this glycoprotein fraction contained Component A.

EMA is a high- M_r glycoprotein immunologically defined by using conventional antisera (anti-EMA) raised against defatted human cream (Heyderman *et al.*, 1979), which was isolated from skim milk by Ormerod *et al.* (1983). Since polyclonal anti-EMA was found to react with both Component A and C, EMA should be regarded as a general term for such glycoproteins as Components A–C. Variation of the chemical composition of EMA (Ormerod *et al.*, 1983) might be due to the presence of these heterogeneous glycoproteins. However, the relationship between the EMA preparation by Ormerod *et al.* (1983) and Components A–C has not been elucidated in the present study.

Studies using various monoclonal antibodies which recognize a high- M_r glycoprotein such as PAS-0 revealed that the level of the glycoprotein in sera or in tissues was significantly different between tumour patients and healthy persons (Taylor-Papadimitriou *et al.*, 1981; Burchell *et al.*, 1983; Ceriani *et al.*, 1983, 1984; Peterson *et al.*, 1983; Hilken *et al.*, 1984a,b). This suggests that this glycoprotein has a potential use as a marker for the diagnosis or prognosis of carcinoma. However, monoclonal antibodies which recognize the high- M_r glycoprotein corresponding to Component A have not yet been reported. The preparation of monoclonal antibodies recognizing Component A could provide valuable information about the expression of such glycoprotein(s) in normal and malignant tissue.

The present study revealed that human milk serum and MFGM contained at least two high- M_r mucin-like glycoproteins. Therefore, we propose the names 'HM glycoprotein A' and 'HM glycoprotein C' for Component A and Component C (PAS-0) respectively. These glycoproteins were not observed in bovine milk, but we have found that similar glycoproteins seemed to be present in monkey milk (M. Shimizu, K. Yamauchi, Y. Miyauchi, T. Sakurai, K. Tokugawa & R. A. J. McIlhinney, unpublished work). This suggests that HM glycoproteins are specific to primate milk. The biological and physiological function of HM glycoproteins in the mammary gland and secreted milk are therefore of great interest.

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