Isolation and chemical and immunochemical characterization of the peanut-lectin-binding glycoprotein from human milk-fat-globule membranes

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Membrane glycoprotein with high M_r (HM_r-MGP) was purified from neuraminidase-treated Triton X-100-solubilized human milk-fat-globule membranes by peanut-agglutinin (PNA) affinity chromatography. The high carbohydrate content (75%), blood-group-A activity and typical monosaccharide composition (L-fucose, D-galactose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine in the proportions 0.26:1.00:1.85:1.30) indicate that the isolated HM_r-MGP is a mucinous substance. Fractionation of the oligosaccharides from alkaline-borohydride-treated HM_r-MGP on Bio-Gel P-2 suggest that the PNA-binding sites are located mainly on longer (tetra- to deca-saccharide) alkali-labile bound oligosaccharide chains. Polyclonal antibodies raised against the HM_r-MGP showed an antigenic distribution in histological sections that was comparable with the distribution of peroxidase-labelled-PNA-binding sites in both normal and malignant breast tissues. The positive immunohistological staining of some other tissue components with this antibody indicates that HM_r-MGP is not strictly breast-associated. The functional role of HM_r-MGP is unknown, but, since its expression is dependent on the differentiation state of secretory epithelial cells, it serves as a differentiation antigen that can be used for better functional characterization of breast cancers.

MFGs are derived by apocrine secretion from lactating mammary-gland cells (Bargmann *et al.*, 1961), and are surrounded by a part of the apical plasma membrane of the cells. Since the MFGMs are easy to isolate and are practically free of other cytoplasmic membrane components, they are, alongside the erythrocyte membranes, ideal models for membrane biological studies. It has been shown that the MFGMs carry antigenic determinants that are characteristic for human epithelium (Heyderman *et al.*, 1979), for the differentiation grade of breast epithelia (Ceriani *et*

al., 1977; Newman *et al.*, 1979) and also for some general membrane antigens such as HLA antigens and blood-group-like substances (Springer *et al.*, 1975; Newman *et al.*, 1980). The presence of the T (Thomsen–Friedenreich)-antigen has been demonstrated on the surface of MFGMs, on the apical plasma membranes of lactating-mammary-gland cells and even on breast-cancer cells by the use of anti-T antibodies (Springer *et al.*, 1980) and by the lectin PNA (Klein *et al.*, 1978; Newman *et al.*, 1979). This latter was shown to possess a selective affinity to the disaccharide D-galactosyl- β -(1–3)-N-acetyl-D-galactosamine, which is also the immuno-dominant group of the T blood-group antigen (Kim & Uhlenbruck, 1966; Uhlenbruck *et al.*, 1969; Lothan *et al.*, 1975; Newman & Uhlenbruck, 1977). Since the expression of free or neuraminic acid-substituted PNA-binding sites had been proved to be a useful marker for the secretory activity of breast cancer, which in turn showed a positive correlation with the susceptibility of these tumours to hormonal treatments (Klein *et al.*, 1981, 1983a,b; Vierbuchen *et al.*, 1981), there is a

Abbreviations used: HM_r-MGP, high-relative-molecular-mass membrane glycoprotein; MFGs, milk-fat globules; MFGMs, milk-fat-globule membranes; PNA, peanut agglutinin; SDS, sodium dodecyl sulphate.

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continuously growing interest in the structure and function of the PNA-binding glycoprotein components of the apical membranes of activated mammary-gland cells. Until now the existence of six or seven different glycoprotein components has been demonstrated in bovine as well as in human MFGMs (Kobylka & Carraway, 1972; Murray *et al.*, 1979) by SDS/polyacrylamide-gel electrophoresis. The three major glycoprotein components of human MFGMs, with M_r values of 155 000, 70 000 and 39 000, and a fourth, of M_r 67 000, named butyrophilin, were isolated and analysed in detail by Imam *et al.* (1981, 1982) and by Heid *et al.* (1983) respectively.

Apart from these glycoproteins, purification and chemical characterization of other glycoproteins has not been achieved. The high mannose content of the purified glycoproteins is indicative of alkali-stable asparagine-linked oligosaccharide chains. Since galactosamine was not demonstrable in them, it is very unlikely that these glycoproteins could be identical with the PNA-binding glycoproteins of MFGMs.

We now describe the isolation of a high- M_r mucus-type membrane glycoprotein (HM $_r$ -MGP) by PNA affinity chromatography and gel filtration from neuraminidase-treated human MFGMs. Investigations on the monosaccharide and oligosaccharide composition of the isolated HM $_r$ -MGP are included. We also report on the application of an antibody raised against the purified glycoprotein for the comparative histochemical study of the antigenic determinants of HM $_r$ -MGP in normal and malignant tissues.

Materials and methods

Materials

Human milk was obtained from healthy volunteers in established lactation and was stored at -20°C . Purified PNA and the immobilized lectin covalently bound to agarose beads were bought from Medac (Hamburg, W. Germany). Neuraminidase (*Vibrio cholerae*; EC 3.2.1.18) was purchased from Behringwerke (Marburg, W. Germany). Bio-Gel A1.5m and Bio-Gel P-2 were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Isolation and enzymic treatment of MFGs

A fraction enriched in MFGs was prepared as the upper cream layer resulting after the centrifugation of human milk at 2000g at 22°C for 15 min. The MFGs were washed with 3×5 vol. of 0.9% NaCl by using identical centrifugation conditions. Neuraminidase treatment was performed by suspending saline-washed MFGs in 2 vol. of phos-

phate-buffered saline (0.9% NaCl in 10 mM-sodium phosphate buffer, pH 5.6) containing 1 mM-CaCl₂ plus 0.05 vol. of neuraminidase [1 unit ($\mu\text{mol}/\text{min}$)/ml] and incubating at 37°C for 1 h with continuous gentle shaking. After washing (three times) with 0.9% NaCl, as described above, native or enzyme-treated MFGs were used directly for further experimentation.

Isolation and solubilization of human MFGMs

To saline-washed neuraminidase-treated MFGs, 1 vol. of Tris-buffered saline (0.9% NaCl in 10 mM-Tris/HCl buffer, pH 7.3) containing 1 mM-CaCl₂ was added. MFGMs were dissociated from the core lipids of the fat globules and subsequently isolated in an essentially triacylglycerol-free form by homogenization of the chilled (4°C) globule suspension in a Waring blender until the lipid coagulated followed by centrifugation of the corresponding aqueous phase, as previously described for the preparation of bovine MFGMs (Keenan *et al.*, 1977). The resulting membrane pellet was dispersed in 10 vol. of Tris-buffered saline containing 1 mM-CaCl₂ and 1% (v/v) Triton X-100, 2 mM-phenylmethanesulphonyl fluoride and 1% (v/v) ethanol (buffer A), and homogenized by ultrasonication (five bursts each of 30 s) at 0°C . The turbid homogenate was centrifuged (100 000g for 60 min). The membrane glycoproteins from the clear brown supernatant were precipitated twice with ethanol (75%, v/v) at 4°C . The precipitate was then extracted (twice) with chloroform/methanol (2:1, v/v) and finally dried in a stream of N₂. The isolated membrane glycoproteins were dissolved in Tris-buffered saline containing 1 mM-CaCl₂ and 0.1% Triton X-100 (buffer B) for further investigations.

PNA affinity chromatography

PNA-agarose gel (bed volume 10 ml) was pre-equilibrated in buffer B at 4°C . Isolated membrane glycoproteins of human MFGMs solubilized in the same buffer were applied and continuously recycled at 4°C for 16 h, a flow rate of 6 ml/h being maintained throughout. Unbound material was removed by washing with buffer B, after which the same buffer containing D-galactose (0.2 M, 100 ml, followed by 0.5 M, 50 ml) was applied. Fractions (2 ml) were automatically collected and samples (100 μl) assayed for proteins. Protein-containing fractions corresponding to specifically bound components were pooled, dialysed against 0.1 M-acetic acid at 4°C and freeze-dried. The residues were extracted three times with chloroform/methanol (2:1, v/v) to remove residual detergents, and the insoluble protein material was dried in a stream of N₂.

Gel filtration

All manipulations during this experiment were carried out at 4°C. A column (1.5 cm × 100 cm) was packed with Bio-Gel A1.5m and equilibrated with buffer B, which also served as the running buffer. A flow rate of 10 ml/h was maintained. The fractions eluted at the peaks were pooled, dialysed and freeze-dried.

Monosaccharide and disaccharide analysis

A Hewlett-Packard 7620 A gas chromatograph containing OV 17 (3% on Gas-Chrom Q) and OV 225 (3% on Gas-Chrom Q) columns (0.25 cm × 2 m) was used. The isolated glycoproteins (1 mg) were hydrolysed in aq. 3M-HCl for 4 h, and, after re-acetylation and trimethylsilylation (Sweely *et al.*, 1963), were applied to the column. The temperature of the column was raised from 120°C to 240°C (4°C/min). Isoerythritol (30 µg) was used as internal standard. Molar proportions of monosaccharides were calculated by using molar adjustment factors for each monosaccharide standard relative to isoerythritol (Clamp *et al.*, 1971).

Alkaline-borohydride treatment and analysis of the liberated oligosaccharides

HM₁-MGP (8 mg) was treated with 0.05M-NaOH containing 1M-NaBH₄ for 24 h at 50°C. β-Elimination and reduction was stopped by adding 3M-acetic acid. After Dowex 50W X8 (H⁺ form) chromatography and repeated evaporation from acidic methanol, the released oligosaccharides were dissolved in distilled water and applied on to a Bio-Gel P-2 column (136 cm × 3 cm). The flow rate was 24 ml/h. Fractions (4 ml) eluted with distilled water were assayed for carbohydrate content.

Analytical procedures

Protein was determined by the method of Lowry *et al.* (1951) or, in the presence of 1% (v/v) non-ionic detergent, by the modification described by Dulley & Grieve (1975), with bovine serum albumin as standard.

Carbohydrate content was measured by the method of Dubois *et al.* (1956).

Ouchterlony double-diffusion experiments were performed in 1% (w/v) agarose gel dissolved in buffer B and were allowed to develop at 4°C for 24 h.

Serological techniques

Agglutination assays with intact MFGs were performed as described by Farrar *et al.* (1980). Briefly, saline-washed MFGs were resuspended in phosphate-buffered saline, pH 7.3, to give an A₇₀₀ of approx. 2.0 when read against water blanks.

Solutions of PNA or antiserum (20 µl) and suspensions of MFGs (20 µl) were mixed and incubated in a humid chamber at 22°C for 30 min.

Haemagglutination-inhibition assays were performed to detect blood-group-like substances in the various fractions isolated from MFGMs. The isolated glycoproteins (2 mg/ml) and their several dilutions were preincubated with the haemagglutination titres of anti-blood-group-A and anti-blood-group-B antibodies (Biotest, Frankfurt, W. Germany) and of anti-blood-group-H phytoagglutinin (Behringwerke) for 30 min and then mixed with 2% washed, packed, human AB-group- or O-group erythrocytes.

Electrophoretic techniques

Rocket immunoelectrophoresis was carried out in 1% (w/v) agarose gel made up in a barbital/Ca²⁺ buffer system [2.8 mM-barbital (5,5-diethylbarbituric acid)/15.9 mM-sodium barbital/2 mM-calcium lactate, pH 8.8, containing 0.1% Triton X-100]. Gels containing 10–15 µl of antiserum/ml of gel were electrophoresed (2 V/cm of gel) at 4°C for 14 h.

Polyacrylamide-gradient (10–20%) gel electrophoresis was performed in the presence of SDS and 2-mercaptoethanol as described by Anderson & Anderson (1977). Gels were stained with Serva-Blue G or with the periodic acid/Schiff reagent.

Production of antiserum

Antibodies directed against HM₁-MGP of MFGMs isolated by affinity chromatography and purified by gel filtration were raised in New Zealand White rabbits by repeated subcutaneous injection at 2-week intervals of delipidated glycoprotein (200 µg) dissolved in buffer B (500 µl) and emulsified with an equal volume of Freund's adjuvant. Samples of blood were taken from the peripheral ear vein before the initial injection and at monthly intervals during the injection programme. Serum was heated at 56°C for 30 min, centrifuged at 10000g for 10 min and stored at –20°C until required.

Immunohistochemical studies

Formol-fixed paraffin-embedded and re-hydrated sections of normal and malignant breast and several other tissues were pre-incubated with bovine serum albumin (1%), washed with phosphate-buffered saline and incubated with the antiserum from rabbits at 1:100 dilution for 30 min. After repeated washing in phosphate-buffered saline, the bound antibodies were detected by incubating the sections with either fluorescein isothiocyanate- or horseradish-peroxidase-labelled anti-rabbit antibodies (Dako, Copenhagen, Denmark). Parallel sections were

labelled with non-immune rabbit serum, followed by the labelled anti-rabbit serum. The peroxidase reaction was performed with 3-amino-9-ethyl-carbazole (Sigma Chemical Co., St. Louis, MO, U.S.A.) as substrate. The specificity of the immunohistochemical reaction was confirmed by absorption of the antibody either with whole MFGs or with the purified HM_r-MGP antigen.

Results

Isolation and enzyme treatment of MFGMs

Since PNA-binding sites on MFGMs are known to be masked by terminal sialic acid residues (Newman *et al.*, 1979), the isolated intact MFGs were pretreated with neuraminidase in order to liberate all the binding sites for the lectin. The effectiveness of the treatment was checked by lectin agglutination assay (Farrar *et al.*, 1980). Although the untreated MFGs were not agglutinated by PNA, they were promptly agglutinated even at 64-fold dilution of the lectin after neuraminidase treatment. The MFGs were then homogenized (see the Materials and methods section) and the isolated membranes were solubilized with the buffer containing 1% Triton X-100. About 30–40% of the membrane proteins were dissolved by this procedure. The dissolved glycoproteins were freed from the bulk of contaminating proteins by repeated ethanol (75%) precipitation. The presence of PNA-binding glycoprotein was followed by Ouchterlony double diffusion during the whole isolation procedure (Fig. 1).

Lectin affinity chromatography, gel filtration and electrophoresis

After fractionation on the PNA column, about 5% of the Triton X-100-solubilized membrane proteins and about 20% of their carbohydrate content were specifically bound by the lectin, which could be eluted by adding 0.2M-galactose to the elution buffer (Fig. 2). Chromatography of the crude membrane proteins on Bio-Gel A1.5m produced two peaks on elution. The minor peak was eluted with the void volume, indicating an M_r of more than 500000 (Fig. 3). It contained about 75% carbohydrate and was precipitated by PNA in Ouchterlony double diffusion (Fig. 1). This component also possessed blood-group-A activity, as revealed by a titre of 1:32 in the haemagglutination-inhibition assay. An inhibitory effect on the agglutination of B and O erythrocytes could not be demonstrated. The second peak contained lower- M_r glycoproteins, which, however, were not precipitated with PNA in agarose gel. When the affinity-isolated component was applied to a Bio-Gel A1.5m column, it was eluted exclusively in the first peak. The isolated glycoprotein did not enter

Fig. 1. Ouchterlony double diffusion of the various fractions of MFGMs in agarose gel

Centre well: 2% solution of PNA. Outer wells (in clockwise order): 1, MFGMs solubilized in Tris-buffered saline, pH 7.3, containing 1% Triton X-100; 2, components soluble in 75% ethanol freeze-dried and dissolved in Tris-buffered saline; 3, fraction precipitated by 75% ethanol dissolved in Tris-buffered saline; 4, fraction eluted by 0.2M-galactose from the PNA-agarose column; 5, peak 1 (M_r over 200000) from Bio-Gel A1.5m column; 6, peak 2 (M_r under 200000) from Bio-Gel A1.5m column.

Fig. 2. Lectin affinity chromatography of MFGM glycoproteins on PNA-agarose in the presence of 0.1% Triton X-100

Human MFGM glycoprotein (250mg dry wt.), isolated from neuraminidase-treated Triton X-100-solubilized MFGMs (details are described in the Materials and methods section), was applied to a PNA-agarose column (bed vol. 10ml). Unbound membrane proteins were removed by washing with Tris-buffered saline, pH 7.3, containing 1 mM-CaCl₂ and 0.1% Triton X-100. Bound material (about 10% dry wt.) was specifically eluted by the addition of 0.2M-D-galactose (Gal) to the buffer. Subsequent elution with buffer containing 0.5M-D-galactose produced no further release of proteins. Samples (2ml) of fractions were assayed for protein by the modification of the Lowry procedure (Dulley & Grieve, 1975).

the polyacrylamide-gradient gel even under reducing conditions. The only narrow protein band, with an apparent M_r of 28 500, obviously represents subunits of PNA, which were liberated from the affinity column during the elution process.

Chemical characterization of HM_r -MGP of human MFGMs

To compare the composition of the lectin-affinity-purified HM_r -MGP of MFGMs with other glycoproteins, quantitative carbohydrate analyses were performed. The molar proportions of monosaccharides in the main fractions are shown in Table 1. Pure HM_r -MGP contained, per mg, 49.58 μ g of fucose, 158.62 μ g of galactose, 264.10 μ g of *N*-acetyl-D-glucosamine and 217.70 μ g of *N*-acetyl-D-galactosamine, corresponding to a recovery over 90% in g.l.c. analysis. The alkali-labile bound oligosaccharide chains of HM_r -MGP, which were liberated by alkaline-borohydride treatment, were separated into seven fractions by

Fig. 3. Bio-Gel A1.5m chromatography of Triton X-100-solubilized MFGMs. Fractions (2ml) were assayed for protein (A_{280} ; ----) and for carbohydrate (A_{485} ; —). For details see the text. Vertical arrows indicate the elution positions of M_r markers: Dextran Blue (V_0) and myosin (M_r 200000).

chromatography on a Bio-Gel P-2 column, which had been calibrated with a mixture of glucose oligomers ranging from one to 12 hexose units (Fig. 4). The size of oligosaccharide alditols could be estimated on the basis of their elution volumes in

Fig. 4. Bio-Gel P-2 fractionation of alkali-labile oligosaccharide chains derived from PNA-affinity-isolated HM_r -MGP of human MFGMs after alkaline-borohydride treatment. HM_r -MGP (8mg) was treated with 0.05M-NaOH containing 1 M-NaBH₄ for 24h at 50°C. β -Elimination and reduction was stopped by adding 3M-acetic acid. After Dowex 50W X8 (H^+ form) chromatography and repeated evaporation from acidic methanol, the released oligosaccharides were dissolved in distilled water and applied on to the Bio-Gel P-2 column (136cm $\times$ 3cm). Samples (4ml) of fractions were assayed for carbohydrate content by the method of Dubois *et al.* (1956). Horizontal bars numbered from 1 to 7 indicate oligosaccharide fractions with various chain length from deca- to disaccharides. The numbers on the vertical arrows indicate glucose units in the applied glucose-oligomer standards.

Table 1. Molar ratios of monosaccharides in glycoprotein fractions isolated from human MFGMs

Molar ratios were calculated by using molar adjustment factors for each of the individual monosaccharides relative to the internal standard (Clamp *et al.*, 1971). Main values from three different measurements are shown. Variation was less than 5% of each value.

	Molar proportions relative to galactose					
	Fucose	Mannose	Glucose	Galactose	Glucosamine	Galactosamine
Whole MFGMs	0.75	0.05	0.25	1.00	1.00	0.25
Fractions from PNA-agarose column:						
PNA-bound	0.26	—	—	1.00	1.85	1.30
PNA-unbound	1.95	0.20	0.31	1.00	7.40	—
Fractions from Bio-Gel A1.5m column:						
M_r over 200000	0.80	—	—	1.00	1.58	1.10
M_r under 200000	2.35	0.38	0.51	1.00	6.60	—

conjunction with their monosaccharide composition. Some of the material (fraction 1) was excluded from the gel; it comprised saccharide alditols with M_r larger than 2000. Fractions 2–7 represented saccharide alditols, the size of which ranged from three to approx. 13 hexose units (corresponding to di- to approx. deca-saccharides, if the experimentally based assumption is made that *N*-acetylhexosaminyl residues in general behave like two glucose units). In these oligosaccharide fractions L-fucose, D-galactose, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine were detected by g.l.c. *N*-Acetyl-D-galactosaminitol was present in all fractions, indicating that they were cleaved by reductive β -elimination. The high L-fucose and *N*-acetyl-D-glucosamine content of fractions 1–5 is characteristic of mucin-like carbohydrates, the structure of which should be similar to soluble fucose-containing saccharides isolated from human milk (Egge *et al.*, 1983). (Detailed data on the chemical composition of these oligosaccharides are not included in the present paper.) Fractions 6 and 7, corresponding to three to four glucose units, contained disaccharide alditols. Fraction 7 proved to be homogeneous, containing only D-galactose and *N*-acetyl-D-

galactosamine in a molar proportion of 1:0.5, and showed a relative retention corresponding to our D-galactose- β -(1-3)-*N*-acetyl-D-galactosamine standard. The amount of this disaccharide was, however, very low (1% of the carbohydrate chains of the whole HM_r -MGP).

Immunological characterization of HM_r -MGP

Antisera directed against the HM_r -MGP, which was isolated by PNA affinity chromatography and further purified by gel filtration, agglutinated the native as well as the neuraminidase-treated MFGs equally. Analysis by rocket immunoelectrophoresis revealed one marked precipitin arc with the total detergent-extracted MFGMs and the corresponding PNA-affinity-isolated HM_r -MGP. The antiserum was also used for preliminary immunohistochemical studies in order to investigate the distribution of antigenic determinants of HM_r -MGP in normal as well as in malignant breast tissues. The antibody gave a strong reaction with cells of the normal lactating mammary gland (Fig. 5), and also reacted very intensively with breast-cancer cells (Fig. 6). Moreover, some other tissue components, i.e. the apical membranes of fundic glands in normal gastric mucosa, as well as

Fig. 5. Indirect immunoperoxidase staining of lactating-mammary-gland section

The antibody directed against the PNA-affinity-purified HM_r -MGP demonstrated antigenic determinants on the membranes of secreted milk-fat globules and on the apical membranes of epithelial cells (arrows). For details of immunization and immunoperoxidase staining see the Materials and methods section. The bar represents 30 μ m.

Fig. 6. Indirect immunofluorescent staining of a moderately differentiated adenocarcinoma of the breast. The antibody against the PNA-affinity-isolated HM_r -MGP of human MFGMs shows also a selective and intensive binding to the tumour cells. Beside the apical membranes (arrows), the cytoplasm of the tumour cells also shows positive staining. Connective-tissue bundles between groups of tumour cells are completely negative. The bar represents 30 μm .

that of carcinomatous gastric tissue, the distal tubuli of human kidney and some cells of histiocytic origin, were also labelled by the antibody raised against the HM_r -MGP of MFGMs. In this respect it is specially noteworthy that these latter structures also possess an affinity for the labelled PNA lectin.

Discussion

The present study demonstrates that PNA affinity chromatography is a valuable method for the purification of a HM_r -MGP from neuraminidase-treated Triton X-100-solubilized MFGMs. This HM_r -MGP differs significantly in its M_r and carbohydrate composition from EMGP-70, EMGP-39 and EMGP-155 and also from butyrophilin, which were previously isolated in a pure form from human MFGMs (Imam *et al.*, 1981, 1982; Heid *et al.*, 1983). The HM_r -MGP is characterized by a high content of hexosamines (see Table 1). According to our investigations, all the *N*-acetyl-D-galactosamine content of MFGMs, which has been shown to be a constituent of these membranes (Glöckner *et al.*, 1976), is localized in the HM_r -MGP. The PNA-binding capability of the isolated HM_r -MGP was also emphasized by double-diffusion experiments. Since PNA is known

to bind selectively to the disaccharide D-galactosyl- β -(1-3)-*N*-acetyl-D-galactosamine (Lothan *et al.*, 1975), our findings indicate that this disaccharide is present in a high density in the isolated glycoprotein. The presence of this disaccharide among the alkali-labile oligosaccharide chains of the HM_r -MGP was demonstrated, but the amount of it was very low (less than 1% of the total carbohydrate content). Therefore it seems very unlikely that this disaccharide alone could account for the intensive PNA binding of MFGMs. Since both galactose and *N*-acetyl-D-galactosamine were also present in the tri- to deca-saccharide chains of HM_r -MGP, it should be assumed that similar disaccharides exist in them in the terminal position. Mass-spectrographic studies of the isolated oligosaccharides may help to elucidate this question.

Some of the biochemical and immunological properties of HM_r -MGP suggest that it may be identical with the epithelial membrane antigen (EMA) reported by Heyderman *et al.* (1979), Sloane & Ormerod (1981) and Ormerod *et al.* (1982). By using the antibody directed against the pure HM_r -MGP, antigenic determinants were demonstrated in some other epithelial tissues, which were also shown for the distribution of EMA (Sloane & Ormerod, 1981). The antibody against

the HM_r-MGP also reacted very intensively with activated gastric epithelial cells and with the apical membrane surfaces of gastric cancers, from which a high-*M_r* mucus with similar chemical and immunological properties has been isolated by the same method (Fischer *et al.*, 1984a,b).

Another very similar glycoprotein, the Ca 1 antigen, has been isolated via wheat-germ agglutinin affinity chromatography from a laryngeal carcinoma cell line (Ashall *et al.*, 1982; McGee *et al.*, 1982), and the antibody against it was suggested to be useful as a general carcinoma marker (McGee *et al.*, 1982). This hope was, however, disappointed by more recent investigations, showing that the antigenic determinants are also present on some activated normal and benign tissue samples (Faradinas *et al.*, 1984; Lloyd *et al.*, 1984; Fergusson & Fox, 1984), among others including breast tissues (Clough *et al.*, 1984).

Similarly, a high-*M_r* sialoglycoprotein was isolated from rat ascites mammary-adenocarcinoma cell lines by using density-gradient centrifugation by Sherblom *et al.* (1980). The carbohydrate composition of the purified compound shows striking similarities to our HM_r-MGP. Moreover, Steck & Nicolson (1983) reported a 580000-*M_r* glycoprotein with PNA-binding ability in rat mammary adenocarcinoma, the appearance of which correlated with the metastatic potentials of the various cell clones.

The functional role of these HM_r-MGPs of human MFGMs and rat mammary-carcinoma cell lines is still unknown, but it has been shown by histological techniques that the distribution of free and sialic acid-masked PNA-binding sites correlate well with the functional degree of mammary development and with the secretory activity of rat mammary cell lines (Newman *et al.*, 1979; Klein *et al.*, 1979). Investigations on a larger scale of human and of *N*-nitrosomethylurea-induced rat mammary carcinomas revealed evidence that the amount of PNA-binding sites depends on the secretory status of the tumours and is closely related to the expression of hormone receptors on them. Therefore the appearance of PNA-binding sites may be indicative of the hormone-susceptibility of the individual tumours (Klein *et al.*, 1983a,b; Vierbuchen *et al.*, 1983). These studies, however, could not detect a clear-cut relationship between the existence of PNA-binding sites and the metastatic potential of the tumours, which was suggested by Steck & Nicolson (1983) from their studies on 1376NF mammary-adenocarcinoma clones. The occurrence of the PNA-binding HM_r-MGP in MFGMs derived from normal lactating-mammary-gland cells, and probably also in some other secretory epithelial cells, as indicated by the positive immunohistochemical reactions with the

anti-HM_r-MGP, suggest a special function of this glycoprotein in the secretion process.

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