Preferential Expression of a M, 155,000 Milk-Fat-Globule Membrane Glycoprotein on Luminal Epithelium of Lobules in Human Breast

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ABSTRACT

An integral membrane glycoprotein with an apparent molecular weight of 155,000 and isoelectric points ranging from 7.2 to 7.6 has been found to be predominantly expressed on the apical plasma membrane of luminal epithelial cell lining the lobules and terminal ducts in breast. The glycoprotein was purified to homogeneity from human milk-fat-globule membrane and was termed MFGM-gp 155. Polyclonal antibodies were raised which specifically reacted to a single component that electrophoretically comigrated with this glycoprotein in immunoblotting experiments. These antibodies appear to recognize epitopes which are expressed on the protein segment of the glycoprotein. Using an indirect immunohistological method, the glycoprotein was localized predominantly on the apical plasma membrane of luminal epithelial cells lining the alveoli of normal breasts. The expression of the antigen was maintained in both morphologically well- and poorly differentiated lobular carcinoma cells. The antigen was weakly detectable on normal epithelial cells lining the terminal ducts and malignant cells of infiltrating ductal and medullary carcinomas. Expression of the glycoprotein is not organ specific as it is detectable in normal epithelial cells of kidney, pancreas, salivary gland, and stomach and in malignant cells of colon and stomach. The antibodies did not react with large ductal, myoepithelial, stromal, endothelial, epidermal squamous epithelial cells, melanocytes, eccrine sweat glands and ducts, sebaceous glands, erythrocytes, and lymphocytes in breast and skin tissues. Thus, antibodies to this glycoprotein appear to be useful phenotype markers to study differentiation of mammary epithelial cells and the pathogenesis of different histological types of mammary carcinomas, including Paget’s disease and signet-ring cell carcinoma of mammary gland.

INTRODUCTION

Mammary gland consists of various types of epithelial cells that include luminal epithelial cells lining the ducts and lobules and myoepithelial cells (1). The luminal epithelial cells are separated from the stromal matrix by a layer of myoepithelial cells in normal mammary gland. Distinction of various epithelial cell types in the gland rests upon judicious application of morphological criteria and the location of these cells with respect to each other. The application of monoclonal antibodies to cytokeratin, myosin, collagen type IV, laminin, and vimentin has shown usefulness in identifying certain epithelial and mesenchymal cell types and their relationship during development of rat mammary gland (2, 3). In humans, both polyclonal and monoclonal antibodies to components of human MFGM, which is said to be derived mainly from apical plasma membrane of lactating mammary epithelial cells, have been used to distinguish luminal from myoepithelial cells (4); however, they can not discriminate luminal epithelium in ducts, terminal ductules, or lobules (5-9). We report the production of antibodies to an integral membrane glycoprotein with an apparent molecular weight of 155,000 which is preferentially expressed on the surface of luminal epithelial cells of terminal ductules in lobules of human breast.

MATERIALS AND METHODS

Materials. Affinity-purified immunoglobulin G fraction of swine antirabbit immunoglobulin G and rabbit PAP complexes were purchased from Cappel Laboratories, Melvern, PA. Reagents for electrophoresis were bought from Bio-Rad, Richmond, CA. Endo-β-N-acetylglucosaminidase H (approximately 25 units/mg of enzyme protein) was obtained from Boehringer Mannheim Biochemicals. Pepsin and the rest of the chemical reagents used were of the highest purity available from Sigma Chemical Co., St. Louis, MO.

Purification of Human MFGM-gp 155. The MFGM-gp 155 was purified from the human milk-fat-globule membrane. Purification procedures consisted of extraction of MFGM with magnesium chloride followed by lectin affinity chromatography and gel filtration in the presence of protein-disaggregating agents (10). The glycoprotein yields a single band under reducing conditions with an estimated molecular weight of 155,000 on SDS-polyacrylamide slab gel electrophoresis. It contains 21% carbohydrate by weight, with aspartic and glutamic acid and serine as the major amino acid residues. Quantitative analyses of carbohydrate components suggest that the glycoprotein, with the absence of N-acetylgalactosamine, has N-acetylgalcosamines linked by N-glycosidic bonds to asparagine residues (Table 1; Ref. 10).

Analysis of MFGM-gp 155 by Two Dimensional Gel Electrophoresis. The purified glycoprotein was solubilized by dissolving it in 0.05 M Tris-HCl buffer, pH 6.8, containing SDS detergent (SDS/membrane weight of 155,000; SDS, sodium dodecyl sulfate; PBS, 0.02 M sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl; NP-40, Nonidet P-40; PA, peroxidase-antiperoxidase; PB, sodium phosphate buffer; TCA, trichloroacetic acid; MFGM-gp 70, milk-fat-globule membrane with a molecular weight of 70,000).
Following the application of sample, the column was washed with PB, and the immunoglobulin fraction was eluted with the buffer containing a gradient of 5 to 100 mM NaCl. Two-mI fractions were collected, and the A280 of each sample was measured on a spectrophotometer (Beckman Instruments, Palo Alto, CA). The presence of rabbit IgG and rabbit anti-MFGM-gp 155 in fractions was detected by setting it against goat antirabbit IgG and the immunogen (MFGM-gp 155), respectively, in an Ouchterlony immunodiffusion plate. The rabbit anti-MFGM-gp 155 antibodies thus prepared were used for subsequent experiments.

Western Immunoblotting of Protein from Milk-Fat-Globule Membrane. Following the separation of the whole MFGM protein components, bands were electrophoretically transferred to nitrocellulose filter paper as described by Towbin et al. (13). Each lane was cut from the filter paper, washed with PBS containing 1.0% (w/v) bovine serum albumin (radioimmunoassay grade) and 0.05% (v/v) Tween 20, and incubated overnight at 4°C with an appropriate dilution of IgG fraction of either antibodies to MFGM-gp 155 or preabsorbed specific antibodies with MFGM-gp 155. After the incubation, the strips of filter paper were washed thoroughly as above and incubated with peroxidase-conjugated goat antirabbit immunoglobulin in an appropriate dilution for 2 h. The strips of filter paper were then washed as above. Finally, the color was developed by incubating the strip with a solution containing 4-chloronaphthol and 0.03% hydrogen peroxide.

Determination of Epitopes Recognized by Anti-MFGM-gp 155 Antibodies. Investigation was made to determine whether the antibodies were directed to the protein and/or the carbohydrate portion of MFGM-gp 155.

Treatment with Endo-β-N-acetylglucosaminidase H. To monitor the cleavage of intact glycoprotein with endo-β-N-acetylglucosaminidase H, 0.25 mg of the glycoprotein in 100 μl of 0.1 M sodium citrate buffer, pH 5.5, containing 50 milliunits of the enzyme was incubated at 37°C for 18 h as described by Tarentino et al. (14). Following incubation, the reaction mixture was mixed with an equal volume of cold 12.5% (w/v) TCA for 15 min at 4°C. The mixture was centrifuged at 12,000 × g and 4°C for 15 min, and the supernatant was removed and dialyzed with several changes of PBS at 4°C. The pellet was dissolved in 100 μl of PBS and dialyzed. To ensure a complete precipitation, an appropriate control containing only the glycoprotein and buffer in the absence of the enzyme was included.

Treatment with Pepsin. Two hundred fifty μg of the glycoprotein were dissolved in 100 μl of 0.07 M acetate buffer, pH 4.0, containing 0.05 M NaCl and 15 μg of pepsin, and the reaction mixture was incubated at 37°C in a water bath for 18 h. At the end of the digestion period, the pH of the solution was adjusted to 8 with NaOH and was dialyzed against several changes of PBS.

Treatment with Heat. Two hundred fifty μg of the glycoprotein were dissolved in 100 μl of PBS, and the mixture was heated at 100°C for 15 min.

Antibody Absorptions. The supernatants and the TCA-precipitable fractions resulting from treatments with endo-β-N-acetylglucosaminidase H, pepsin, or heat were individually mixed with an equal volume of the antibody to MFGM-gp 155 solutions. Untreated MFGM-gp 155 samples served as controls. The mixtures were incubated at 4°C for 16 h. Following incubation, the mixtures were centrifuged at 100,000 × g and 4°C for 30 min, and the absorbed antibodies’ solutions were subsequently used for the immunostaining.

Absorption of Antibodies to MFGM-gp 155. The antibodies (1 mg of protein per ml) were separately mixed and incubated with 1 mg of protein of each of α-lactalbumin, caseins, albumin, secretory IgA, β2-microglobulin, α1-acid glycoprotein, α1-antitrypsin, antichymotrypsin, erythrocyte, lymphocytes, keratins, and 0.5 mg of each of carcinomaembryonic antigen or mouse mammary tumor virus glycoproteins immobilized to Sepharose 4B. Following an overnight incubation, the mixtures were centrifuged at 100,000 × g and 4°C for 30 min. The supernatants containing absorbed antibodies were removed and subsequently applied to tissue sections for their immunostaining analysis.

Preparation of Tissue Sections. Normal and neoplastic tissues were obtained from the surgical pathology files of the University of Southern California School of Medicine, Los Angeles County Medical Center. The tissues were fixed in 10% buffered formalin, dehydrated, cleared, and embedded in paraffin. The tissue sections were cut at 5 μm for both hematoxylin/eosin and immunoperoxidase staining.

An indirect Immunoperoxidase Staining Method. The three-layered unlabeled antibody method described by Taylor (15) was used for localizing tissue antigen with the specific antibodies. Swine antirabbit was used as the bridge antibody between the specific antibodies and the rabbit PAP complex.

The immunoglobulin G fraction of polyclonal rabbit antibodies to MFGM-gp 155 was used to localize the antigen in tissue sections. Throughout this study, primary, secondary, and tertiary antibodies were used at a constant concentration as determined to be optimal by titration analyses. The visual estimates of intensities of staining were scored as follows: −, absence; ±, borderline; 1+, weak; 2+, moderate; and 3+, intense. To account for case-to-case variations in the degree of intensity of staining, any given tumor specimen was evaluated relative to a tissue section containing normal mammary epithelium which was scored as 3+, and it served as a positive control. The visual estimates of the percentage of cells stained were determined by examining, at high magnification (×400), 5 different and random fields on every tissue section. The mean of counts from the fields examined was used as the percentage of cells with staining.

For each experiment, 2 different controls were performed to ensure the specificity of the reaction. These included the use of specific antibodies absorbed with the antigen (1 mg of MFGM-gp 155 per ml of antibodies’ solution) and preimmune rabbit serum.

RESULTS

The Analysis of MFGM-gp 155 by Two-Dimensional Gel Electrophoresis. The purified glycoprotein was further analyzed by two-dimensional gel electrophoresis by the method of O’Farrell (16). It was resolved into several closely related isotypes of similar molecular weights and with the apparent isoelectric points that ranged from 7.2 to 7.6 (Fig. 1). No other component was detected in the purified preparation. The physiochemical properties of the glycoprotein are summarized in Table 1.

Immunoblotting of MFGM-gp 155. All the major glycoprotein components of MFGM were successfully transferred to nitrocellulose filter paper and stained with amido black (Fig. 2, Lane A), which corresponded to the previously identified components.
PREFERENTIAL EXPRESSION OF MFGM-gp 155 ON HUMAN BREAST LOBULES

Fig. 2. Immunoblotting of MFGM-gp 155. Following the separation of proteins from human milk-fat-globule membrane by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the protein bands were electrophoretically transferred to nitrocellulose filter paper. Each lane containing the transferred bands was cut and incubated with either polyclonal antibodies to MFGM-gp 155 or preimmune rabbit serum as described in the text. Lane A, proteins extracted from MFGM electrophoretically transferred to nitrocellulose filter paper and stained with amido black; Lane B, like Lane A, immunoblotted with the specific antibodies; Lane C, like Lane A, immunoblotted with preimmune rabbit serum.

Table 2 Determination of the nature of epitopes recognized by antibodies to MFGM-gp 155

<table>
<thead>
<tr>
<th>Treatment of MFGM-gp 155</th>
<th>Binding properties of antibodies to MFGM-gp 155 absorbed with treated MFGM-gp 155 to cellular antigen in tissue sections</th>
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<tr>
<td>Endo-β-N-acetylglycosaminidase H</td>
<td>Reactive (TCA-soluble fraction) Nonreactive (TCA-precipitable fraction)</td>
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<tr>
<td>Pepsin</td>
<td>Nonreactive</td>
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<td>Heat</td>
<td>Nonreactive</td>
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designated 3, 12, 16, 18, and 19 (16). A component in the filter strip specifically reacted to antibodies to MFGM-gp 155 and migrated in the gel to the same position as did MFGM-gp 155 (Fig. 2, Lanes A and B), establishing the specificity of the antibodies. No reactivity was obtained when the filter strip was incubated with the preabsorbed antibodies to MFGM-gp 155 (Fig. 2, Lane C).

Determination of Epitopes on MFGM-gp 155. The antibodies absorbed with TCA-precipitable fractions, resulting from endo-β-N-acetylglycosaminidase H, pepsin, and heat treatment of the glycoprotein, lead to a complete abolishing of immunostaining of cells. However, the absorption of the antibodies with the TCA-soluble fraction of the endoglycosidase-treated supernatants had no effect on the intensity of the immunostaining (Table 2).

Immunohistological Analysis of the Absorbed Antibodies. Both unabsorbed and absorbed antibodies with milk whey proteins, serum proteins, erythrocytes, lymphocytes, keratins, carcinoembryonic antigens, or mouse mammary tumor virus glycoproteins showed undistinguishable intensity of immunohistological staining of cells in tissue sections, suggesting that MFGM-gp 155 is immunologically distinct from these proteins (Table 3).

Table 3 Immunohistological analysis of adsorbed antibodies

<table>
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<tr>
<th>MFGM-gp 155 is not immunologically related to</th>
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<tr>
<td>Milk whey proteins</td>
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<tr>
<td>α-Lactalbumin</td>
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<td>Caseins</td>
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<td>Serum proteins</td>
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<td>Albumin</td>
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<td>Secretory IgA</td>
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<td>β2-Microglobulin</td>
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<td>α1-Acid glycoprotein</td>
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<td>α1-Antitrypsin</td>
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<td>Antichymotrypsin</td>
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<tr>
<td>Keratins</td>
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<tr>
<td>Oncofetal antigens</td>
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<td>Carcinobryonic antigen</td>
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<td>Lymphocyte antigens</td>
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<tr>
<td>Blood group antigens</td>
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<tr>
<td>Viral proteins</td>
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<td>Mouse mammary tumor virus (gp 28)*</td>
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<td>Mouse mammary tumor virus (gp 47)</td>
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<td>Mouse mammary tumor virus (gp 52)</td>
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* gp 28, M, 28,000 glycoprotein; gp 47, M, 47,000 glycoprotein; gp 52, M, 52,000 glycoprotein.
Fig. 3. An indirect immunoperoxidase staining of formalin-fixed and paraffin-embedded tissue sections with IgG fraction of rabbit antibodies to MFGM-gp 155. a, normal breast. Luminal epithelial cells of terminal ductules in lobules showing reactivity predominantly on the luminal membrane with the antibodies (see thin arrows). Note the absence of reactivity with the luminal epithelial cells lining large or medium-sized ducts as indicated by thick arrows, myoepithelial cells, and stromal cells. Original magnification, × 150. b, same as in a with higher magnification showing strong reactivity with apical plasma membrane of luminal epithelial cells of lobules. Original magnification, × 500. c, poorly differentiated lobular carcinoma cells of breast showing heterogeneous reactivity with the antibodies. Original magnification, × 312. Following immunostaining, the sections were counterstained with Mayer's hematoxylin. Controls included the omission of either the primary, secondary, or tertiary antibodies, and replacement of primary antibodies by either preimmune rabbit serum or specific antibodies which were absorbed with the corresponding antigen (1.0 mg/ml of antibodies' solution).

cells. However, patterns of expression of the antigen in well- to poorly differentiated malignant cells were similar to those obtained with lobular carcinoma. The results of immunohistochemical localization of the antigen in normal, benign breast tissue and three major histological types of carcinomas are summarized in Table 4. Absorption of the specific antibodies with the immunogen (1 mg of MFGM-gp 155 per ml of antibodies' solution) led to a complete abolition of staining of the cells.

The glycoprotein was not detected in the normal cells of liver, skin, and prostate and in carcinomas of kidney, squamous cell and adenocarcinoma of lung, melanoma, and prostate (Table 5). However, the antigen showed variable degrees of expression in normal epithelial cells lining the collecting tubules of kidney, acinar cells of pancreas, surface epithelium of colon, cells lining to bronchioles of lung, columnar epithelial cells of stomach, acinar cells of pancreas, and in carcinoma cells of colon, lung (small cells), and stomach (Table 5).

DISCUSSION

The purified MFGM-gp 155, which yields a single band under reducing conditions on slab gel electrophoresis, migrated as closely related multiple isotypes of similar molecular weights and with the apparent isoelectric points that ranged from 7.2 to 7.6. The microheterogeneity might be attributed to various extents of glycosylation and/or phosphorylation. A further study is required to establish the molecular basis of the heterogeneity.

The glycoprotein was treated with endo-β-N-acetylgalactosaminidase H, pepsin, and heat to ascertain the nature of epitopes recognized by the antibodies. The results suggest that most of the epitopes recognized are expressed on the protein portion of MFGM-gp 155. However, only the neutral oligosaccharides are released by endo-β-N-acetylgalactosaminidase H treatment of glycoproteins containing both complex and neutral oligosaccharides, such as IgM, porcine RNase, and thyroglobulin. Conversely, glycoprotein devoid of neutral oligosaccharides of the type present in DNase A, RNase B, transferrin, fibrinogen, α1-acid glycoprotein, and IgG is unaffected by the endoglycosidase (13). Since MFGM-gp 155 contains neutral oligosaccharides (10), it was anticipated that all the epitopes present on such oligosaccharides would have been released. Within the limitation of the technique, no decrease was observed in the binding capacity of the antibody to the endoglycosidase-treated component. However, the binding property of the specific antibodies was adversely affected when the glycoprotein was treated with pepsin, cleaving the protein into small fragments. The denaturation of the glycoprotein by heat similarly affected the recognized epitopes. These observations are consistent with the interpretation that the major epitopes of MFGM-gp 155 are expressed on protein domain. However, more detailed structural studies are required to define their exact molecular nature.

The immunohistological analysis of absorbed antibodies clearly indicates that MFGM-gp 155 is not immunologically related to milk whey proteins, serum proteins, blood group antigens, lymphocyte antigens, keratins, carcinoembryonic antigen, or mouse mammary tumor virus glycoproteins.

The specificity of the antiserum was established by demonstrating that only MFGM-gp 155 in the whole extract of MFGM reacted with the antibodies. An interesting observation was the preferential expression of MFGM-gp 155 on the apical plasma membrane of luminal epithelial cells lining the terminal ductules and lobules of normal breast. The appearance of MFGM-gp 155-positive cells at the junction of the terminal
ducts and ductules suggests that the cells at the extralobular terminal ducts probably differentiate to form luminal cells in the intralobular ductules. The common origin of various luminal epithelial cell types has been suggested earlier, owing to the expression of a common glycoprotein, MFGM-gp 70, on the apical plasma membrane of cells lining both the large and small ducts, and the ductules (9). MFGM-gp 155 was readily detectable on the cells in tissues that were processed in buffered formalin. Therefore, it seems unlikely that the lack of MFGM-gp 155 expression on the epithelial cells lining the large ducts was due to such processing of tissues.

Among the three major classes of mammary carcinomas, malignant cells of lobular carcinoma exhibited more intense staining than those of infiltrating ductal and medullary carcinomas. The observation suggests a possible quantitative antigenic difference between lobular and the latter two types of carcinomas. In normal and benign mammary epithelial cells, the glycoprotein was demonstrated to be predominantly expressed on the surface of apical plasma membrane. By contrast, in poorly differentiated tumor cells at both primary and metastatic sites, the antibodies were shown to localize component mainly in the cytoplasm. Indeed, a correlation has been observed between the patterns of expression of the antigen and the degree of differentiation of the cells. The altered expression of other membrane glycoproteins in malignant mammary and nonmammary epithelial cells has been widely reported (5, 7, 9, 17–20). Such altered expression of normal glycoproteins may be the result of critical modifications in their synthesis, processing, or assembly in the malignant cells.

An apparent trend of decreasing expression of this component was observed from well- to poorly differentiated cells in tissue sections. The antigen-negative cells were least frequent in sections from well-differentiated cells of lobular carcinoma and represented 5 to 10% of the population. In poorly differentiated cases, such cells comprised 20 to 40% of the total cell population. This trend was also observed in infiltrating ductal carcinoma. A further study is warranted to determine the extent of antigenic heterogeneity among the metastatic cells in distant organs. Such a study may help to ascertain whether any selective advantage is gained by those cells lacking in the expression of the glycoprotein in the process of metastasis.

Both normal and neoplastic squamous epithelial cells failed to react with the antibodies. However, the antibodies reacted with variable intensity to normal and neoplastic glandular epi-
thelial cells from various organs. A possible explanation for such an occurrence may be attributed to the common functional and/or structural properties of the antigen in those cells, as they all are involved in secretion. With respect to the kidney, it is possible that the antigen shed from breast cells may be passively absorbed on to the apical plasma membrane of the epithelial cells lining the collecting tubules of kidney. However, it should be recognized that the demonstration of immunohistological staining of tissue of different organs is not absolute evidence of identity of the immunoreactive molecules. Hence, isolation and characterization of recognized antigens from these organs would facilitate a meaningful comparison between these immunoreactive molecule(s).

Although the function of MFGM-gp 155 has not yet been established, its synthesis appears to be controlled by differentiation. The molecule may have an essential function for those cells that maintain the phenotype of lobular epithelial cells, since it remains expressed in well-differentiated malignant cells. Hence, antibodies to the MFGM-gp 155 appear to be useful phenotype markers to study the ontogeny and gene regulation in mammary gland and the pathogenesis of different histological types of mammary carcinomas, including Paget's disease and signet-ring cell carcinoma. In addition, the antibodies may be useful in characterizing mammary epithelial cells in culture.

REFERENCES

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