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Membrane Glycoprotein Differences between Normal Lactating Mammary Tissue and the R3230 AC Mammary Tumor[1]

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SUMMARY

Membrane glycoproteins have been studied in the normal lactating mammary gland and R3230 AC mammary tumor of the rat. Plasma membrane-enriched fractions were obtained from these tissues by discontinuous sucrose gradient centrifugation of a microsomal preparation from the tissue homogenates. The lightest membrane fractions (F-1 and F-2) have the greatest enrichment of plasma membrane markers, with a 14- to 20-fold purification of 5'-nucleotidase and Na^+-K^+-adenosine triphosphatase over the homogenate values in both tumor and normal tissues for F-1. Electron microscopy shows smooth membrane vesicles for these fractions. Polypeptide analysis by acrylamide gel electrophoresis shows essentially the same patterns for F-1 and F-2 and only relatively minor differences between membrane components of tumor and normal tissues. Glycoprotein analysis of the polyacrylamide gels by periodate-Schiff staining indicates more dramatic differences. Membrane Fraction F-1 from normal tissue contains two major glycoproteins, GP-II and GP-III, while Fractions F-2 and F-3 contain an additional glycoprotein, GP-I, with a higher apparent molecular weight. In the tumor, the component corresponding to GP-III is decreased or absent and a new component GP-IV is seen at a lower apparent molecular weight.

INTRODUCTION

The role of the cell surface in processes involving cellular growth and neoplastic transformation has been subjected to increased scrutiny in recent years (8). Particular attention has been paid to changes in virus-transformed cultured cells and their revertants or temperature-sensitive analogs (6, 12, 23, 31, 33, 35, 37, 39). Lectin agglutinability (2, 16), glycopeptide characterization (1, 38), glycolipid analysis (24, 30), and enzymatic surface labeling (12, 14, 15) all have indicated observable differences between the normal and transformed cultured cells. Extension of these kinds of experiments to normal and neoplastic tissue cell types is difficult because of the physical state of the cells, heterogeneity of cell populations, and extensive dedifferentiation of the cells. However, it should be possible to obtain some information about variations of cell surface components in tissues if plasma membranes are isolated from carefully selected tissue types. We have investigated this problem by comparing plasma membrane-enriched fractions from the normal lactating mammary gland and a minimal deviation adenocarcinoma which has maintained characteristic mammary gland function. The most striking observation was a difference in glycoprotein components of the membranes.

MATERIALS AND METHODS

Tissue Preparation. Normal lactating mammary glands were excised from lactating rats (Fischer F344/CR, 2 weeks postpartum) after pups had been taken away the night before. Excised glands were washed once with buffered sucrose [250 mM sucrose-20 mM Tris-HCl (pH 7.4)], minced into small pieces with scissors, and suspended in 4 volumes of buffered sucrose. All operations were at 4°.

The R3230 AC tumors were obtained from the Mason Tumor Bank (Worcester, Mass.) and were transplanted by a sterile trocar technique. Tumors were excised from tumor-bearing Fischer rats about 3 weeks after tumor implantation. At this stage, the tumors show little or no necrosis inside the tumor, and tumors with visible necrosis were rejected. Tumor capsules were sliced open and the inner soft portion of the tumor was scraped away with a razor blade, cut into small pieces with scissors, washed twice with 4 volumes of buffered sucrose, and suspended in 4 volumes of buffered sucrose.

Membrane Isolation. Isolation of plasma membrane-enriched fractions followed the scheme shown in Chart 1. Tissue samples (normal and tumorous) in buffered sucrose were homogenized by a Sorvall Omni-Mixer (Speed 5, twice for 30 sec) in an ice bath and filtered through 4 layers of cheesecloth. The filtrate was centrifuged at 750 to 1000 × g for 10 min and the pellet was suspended in 1 volume of buffered sucrose. After 3 strokes in a loose Dounce homogenizer (Kontes Glass Co., Vineland, N. J.) the sample was centrifuged. The washed pellet (P-1) contained primarily naked nuclei, as shown by phase contrast micros-

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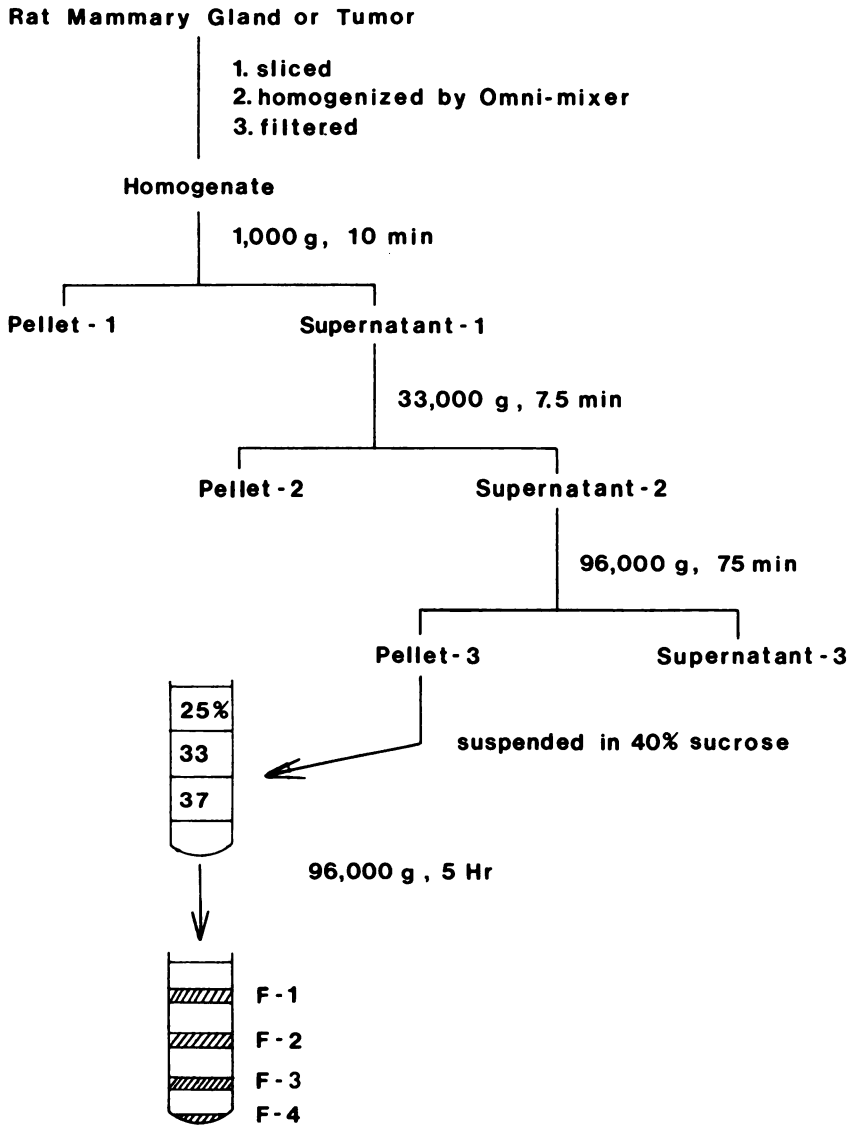

Chart 1. Membrane isolation scheme for normal and tumor tissues.

copy. The pooled supernatant solutions (S-1) were centrifuged at 33,000 × *g* for 7.5 min, and the pellet was washed with 0.5 volume of buffered sucrose to give a fraction (P-2) enriched in mitochondria. The remaining supernatants were combined (S-2) and centrifuged at 96,000 × *g* (SW 27 rotor) for 75 min to give a microsomal pellet (P-3) and soluble fraction (S-3).

The microsomal fraction was suspended in 0.5 volume of buffered sucrose by 3 strokes of the loose Dounce homogenizer and mixed with 1.1 volumes of 69% (w/w) sucrose. The final sucrose concentration was adjusted to 40% (w/w), as determined by refractometry. All sucrose solutions contained 10 mM Tris-HCl (pH 7.4). A discontinuous gradient of 9 ml each of 40% (containing sample), 37, 33, and 25% sucrose was prepared, and the sample was fractionated by flotation through this gradient at 96,000 × *g* and 4°. The materials at the gradient interfaces were collected, diluted with 3 volumes (or greater) of 10 mM Tris-HCl (pH 7.4), and centrifuged for 2 hr at 96,000 × *g*. Samples were suspended in a small volume of buffered sucrose and divided into aliquots to be used for analysis. Some samples were frozen at −20° until assays were performed.

Samples of each membrane fraction were prepared for electron microscopy as described previously (32) and examined in a Phillips 200 electron microscope.

Golgi membranes were prepared by the method of Morré *et al.* (25).

Analytical Procedures. Protein was determined by the method of Lowry *et al.* (21). P_i released during enzyme assays was measured by the method of Lazarus and Chou (19), except that in the final step phosphomolybdate was extracted by mixing the final reaction mixture with 1 volume of isoamyl alcohol. After centrifugation, the absorbance of the alcohol phase was measured at 740 nm. The 5'-nucleotidase and succinate dehydrogenase [succinate 2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium reductase] were assayed as described by Morré (25). Mg^{++}-

and Na^+-K^+-ATPase were measured as described previously (32) and NADPH-cytochrome *c* reductase was assayed by the method of Ragnotti *et al.* (28). Galactosyltransferase was assayed by the method of Fitzgerald *et al.* (11), using *N*-acetylglucosamine as the galactosyl acceptor.

Sodium Dodecyl Sulfate Acrylamide Gel Electrophoresis. Membrane samples were dissolved by boiling for 10 min in 4% sodium dodecyl sulfate-2% mercaptoethanol-5 mM EDTA-40 mM phosphate (pH 7.4) at a protein concentration of about 3 mg/ml. One-fourth volume of 50% glycerol-0.05 mg pyronin Y per ml-50 mM phosphate (pH 7.4) was added, and a sample containing 50 μg of protein per gel was applied (3). Gels were comprised of a 15-mm stacking gel (total acrylamide concentration, 3.2%; ratio of cross-linker to monomer, 0.065) over a 100-mm separating gel (concentration, 6%; ratio, 0.026). After the samples were loaded onto the stacking gels, the current was applied at 2 ma/gel for about 1 hr to move the tracking dye into the separating gel. The current was then raised to 8 ma/gel until the dye reached the end of the gel. Gels were stained for protein and carbohydrate by the method of Fairbanks *et al.* (10). Gel profiles were obtained by scanning with a Gilford 240 spectrophotometer equipped with a gel transport apparatus.

RESULTS

In order to study cell surface properties in tissues, a method of obtaining plasma membranes is required. In the course of this work, a number of membrane isolation techniques have been investigated, involving different methods of cell disruption or centrifugation. Membrane fractions were assayed for enrichment in 5'-nucleotidase and Na^+-K^+-ATPase as a preliminary means of evaluating plasma membrane purification. Chart 1 shows the procedure chosen for the experiments reported here. It involved isolation of a microsomal fraction by differential centrifugation and fractionation of the microsomes by sucrose density centrifugation. The homogenization and isolation steps were monitored by phase contrast microscopy and enzyme analyses, and selected samples were examined by electron microscopy. Enzyme-specific activities and relative enrichments for the various fractions are shown for typical preparations of the normal lactating mammary gland and the tumor (Table 1). Over a series of 7 preparations (4 normal, 3 tumor), the 5'-nucleotidase-specific activities varied from 1.8 to 4.1 μmoles/hr/mg protein for the normal homogenates, 1.3 to 3.3 for tumor homogenates, 41 to 64 for normal plasma membrane fraction, and 25 to 49 for the tumor plasma membrane fraction. A recovery of 15 to 20% of the total homogenate 5'-nucleotidase for F-1 plus F-2 is customary. The other enzyme markers exhibit similar variability. Thus the data show an enrichment of plasma membrane markers 5'-nucleotidase and Na^+, K^+-ATPase in the lighter fractions. The mitochondrial marker succinate dehydrogenase is found primarily in the P-2 fraction, and the marker for endoplasmic reticulum, NADPH-cytochrome *c* reductase, resides primarily in the heavier fractions on the sucrose gradient.

The general conclusions from these data are supported by electron microscopic observations. Fractions 1 and 2 of the sucrose gradient contain smooth, small-membrane vesicles, granules, and rough membranes. Samples P-1 and P-2 are rich in nuclei and mitochondria, respectively. In an effort to localize the Golgi apparatus, the fractions were assayed for galactosyltransferase. The activity was localized with the plasma membrane markers on sucrose density gradient centrifugation, showing a 15- to 25-fold enrichment in F-1. However, no evidence for Golgi structures was seen by electron microscopy, possibly due to Golgi disruption during homogenization.

As a further characterization and comparison of the membranes, the electrophoretic profiles of the polypeptides on sodium dodecyl sulfate acrylamide electrophoresis was examined. Chart 2 shows the patterns for the F-1 fractions for the normal and tumorous membranes. The major difference between these is in the high-molecular-weight region. The tumor has a pair of bands with molecular weights greater than 200,000. The normal membranes have a similar pair of bands, but these are obscured by the presence of a heavier band in the same region. The latter does not appear to be a membrane component. It is found primarily in the soluble fraction and appears to be decreased

Table 1

Enzyme-specific activities and enrichment of membrane fractions of normal mammary tissue and the R3230 AC tumor

Tissue	Enzyme	Activity (μmoles/hr/mg protein)				
		Homogenate	F-1	F-2	F-3	F-4
Normal	5'-Nucleotidase	2.8	43 (15)[a]	32 (12)	5.1 (1.8)	0.6 (0.2)
	Na^+-K^+-ATPase	0.39	6.4 (17)	4.1 (10)	0.5 (1.3)	0 —
Tumor	5'-Nucleotidase	1.9	25 (14)	25 (14)	7.9 (4.2)	1.0 (0.5)
	Na^+-K^+-ATPase	0.44	9.6 (22)	7.1 (16)	1.1 (2.5)	0.3 (0.6)

[a] Values in parentheses, relative specific activities expressed as the ratio of the value for the particular fraction to that of the homogenate.

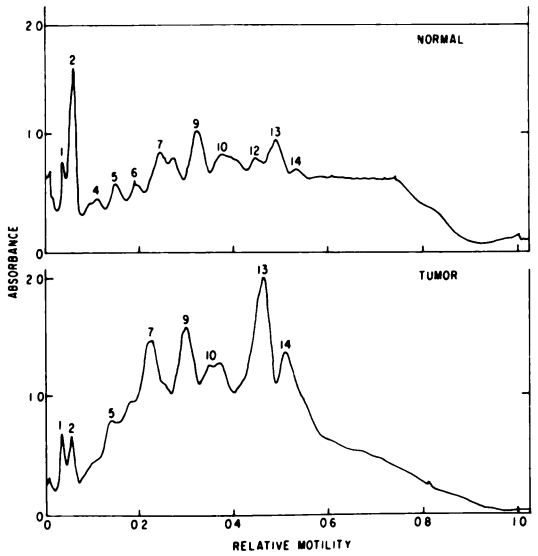

Chart 2. Polypeptide profiles of F-1 fractions of membranes from normal lactating mammary gland and the R3230 AC tumor. Electrophoresis was performed as described in "Materials and Methods," and gels were stained with Coomassie blue. *Numbers*, pattern of 14 corresponding bands for the 2 tissue types as shown by direct comparison of gels or slab gel electrophoresis. Not all bands have been numbered. The gel profiles do not correspond exactly because they are from 2 different electrophoretic runs. The estimated molecular weight values for selected bands are: Band 2, 220,000; Band 9, 87,000; Band 14, 41,000.

in those plasma membrane fractions with the highest specific activities of marker enzymes. One possibility is that this protein is myosin from myoepithelial cells of mammary tissue which has adsorbed to different membrane fractions during homogenization. There are some other differences in the normal and tumor patterns, but these are primarily quantitative in nature and reflect variations in the minor components. Examination of polypeptide patterns of other fractions indicate that F-1 and F-2 are essentially the same. They differ in significant respects from F-3 and F-4, and quite markedly from S-1, P-1, and P-2. These results were obtained by direct comparisons of electrophoresis runs on tube gels or slab gels from a number of samples of both normal and tumor-tissue membranes. Although there are definite differences in the patterns between the normal and tumorous samples, as shown in Chart 2, we have not emphasized these differences because they are variable among different samples. For the protein bands, the variability between tumor and normal has not appeared more significant than the variability between different tissues. Although the same pattern of about 14 major bands was observed, the intensities of these bands were variable. This variability between samples probably resulted from differences in the purity of the membrane fractions.

Glycoprotein patterns of the various membrane fractions were observed by periodate-Schiff staining of the acrylamide gels. Only the microsomal fractions showed significant glycoprotein bands, and the intensities were greatest for F-1 and F-2. F-3 showed a virtually identical pattern to F-2 but with less intense bands, indicating some cross-contamination of the fractions. Comparisons of the normal and tumor-membrane glycoproteins are shown in Chart 3. Two major glycoproteins (GP-II and GP-III) are observed for the F-1 fraction of the normal mammary gland. A 3rd glycoprotein (GP-I) with a higher apparent molecular weight is found in F-2 and F-3. The tumor shows a different pattern. A glycoprotein peak corresponding to GP-II is present in all 3 sucrose density fractions, but GP-III is greatly reduced and a new peak is apparent at much lower apparent molecular weight, particularly in the F-1 fraction. There also appears to be a difference in the amount and position of the slowest migrating component (GP-I) in tumor for this experiment. The glycoprotein patterns for the normal mammary gland have been quite reproducible over a series of preparations. Variability of the tumor glycoproteins is somewhat greater, with small variations in the band positions, but the absence or decrease of a component corresponding to GP-III and the presence of a low-molecular-weight component (GP-IV) have been observed in all cases.

The presence of galactosyl transferase in the membrane fractions suggests the presence of Golgi membranes. Therefore, Golgi were isolated by an alternative procedure (26) from normal lactating mammary gland. Electrophoresis and periodate-Schiff staining showed only 1 faint band that did not correspond to the bands observed in the previously described experiments. Therefore, it seems unlikely that the glycoprotein results observed are due to changes in the Golgi apparatus. Thus, it appears that the patterns of Charts 2 and 3 result from proteins of both plasma membranes and Golgi, but that the Golgi contribution does not significantly influence the glycoprotein patterns.

DISCUSSION

Comparison of normal and neoplastic tissues is often difficult because of the dedifferentiation and loss of function of tumors. The R3230 AC is a slow-growing tumor derived from a spontaneous carcinoma (13). The tumor line was originally described by Dunning (5). The tumor elaborates a

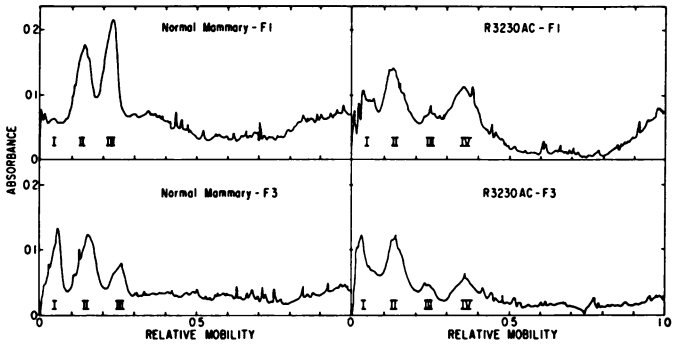

Chart 3. Carbohydrate profiles of F-1 and F-3 fractions of membranes from normal lactating mammary gland and the R3230 AC tumor. Gels were stained by the periodate-Schiff procedure. Apparent molecular weights of the bands were estimated as: Band I, 228,000; Band II, 170,000; Band III, 110,000; Band IV, 70,000.

milk-like fluid which contains lactose, fatty acids, and casein-like proteins characteristic of milk. Hilf *et al.* (13) have performed an extensive series of biochemical studies characterizing the hormonal responsiveness of the tumor. Many of the properties are similar to those of normal mammary tissue. Although the tumor is deficient in estrogen-binding receptors, it contains specific higher-affinity prolactin-binding sites (4, 36). The specificity and affinity of these sites are similar to those found in normal tissues (4), but the number of sites may be lower in the tumor (36). These similarities suggest that this tumor system will be a useful one for membrane comparisons which might shed some light on the role of the plasma membrane in neoplastic phenomena. The validity of these comparisons is reinforced by the comparisons on the membrane fractions isolated in this work. Although there are some differences between the normal and tumor membranes, the similarities far outweigh the differences for the enzyme analyses and polypeptide distributions.

Since the primary interest in this research is in cell surface phenomena, it is important to demonstrate the presence of plasma membranes in these preparations. Both the plasma membrane enzyme markers and electron microscopy indicate significant enrichment of plasma membranes in the F-1 and F-2 fractions. One puzzle is the large enrichment in galactosyltransferase, which is usually considered a marker for the Golgi apparatus (27). Although this result suggests the presence of Golgi contamination in these membrane fractions, it is possible that the secretion mechanism and plasma membrane biosynthesis in the mammary cell may yield plasma membranes containing significant quantities of the galactosyltransferase (34). At any rate, the Golgi do not appear to contribute to the glycoprotein differences observed.

The concentration of the glycoproteins in the F-1 and F-2 fractions offers further evidence of the presence of cell surface materials. The method for glycoprotein staining is biased toward the detection of sialic acid-containing glycoproteins (17), and sialic acid is primarily, although not exclusively, a cell surface constituent (18). Thus, the bulk of the evidence supports the contention that these glycoproteins are cell surface components. The segregation of different surface glycoproteins in different membrane fractions is not particularly surprising, since the membranes may be fragmented into different domains during cell homogenization. Differences among the apical, basal, and lateral surfaces of secretory mammary cells have been postulated (20), and separation of different functional domains or activities of both plasma membranes (9) and the Golgi apparatus (7) has been demonstrated.

The glycoprotein nature of the bands shown by periodate-Schiff staining is indicated by their behavior on acrylamide gel electrophoresis (17) and their extraction by the lithium diiodosalicylate-phenol procedure (22). Differences in cell surface molecules have been observed between normal and transformed cells in culture (12, 14, 15). Some of these differences have been related to glycoproteins at the cell surfaces (12). Our observation of similar differences between normal and tumorous tissues reinforces the concept of the importance of these molecules in neoplastic behavior. It has been suggested that changes in cell surfaces may result from proteolysis of the cells (15, 29). Although this hypothesis would also serve to explain our observations, attempts to reproduce the alterations by protease treatment of normal tissue samples have not been successful. Further study of the nature of these glycoprotein molecules is necessary to understand their role in neoplastic phenomena.

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