Establishment of Plasma Membrane Domains in Hepatocytes.

I. Characterization and Localization to the Bile

Canaliculus of Three Antigens Externally Oriented in the

Plasma Membrane

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ABSTRACT   A membrane fraction denoted N_{2upper} was isolated from homogenates of rat liver by sucrose gradient centrifugation. This fraction, which was enriched 65-fold over the homogenate in 5'-nucleotidase activity, was used as an immunogen in goats. The antisera obtained contained antibodies to three predominant polypeptides in the N_{2upper} membrane fraction, as shown by crossed immunoelectrophoresis. These polypeptides had molecular weights of 105,000, 110,000, and 160,000 after recovery from the crossed immunoelectrophoretic gels and are denoted PM105, PM110, and PM160. Each was a distinct polypeptide, as shown by the distinct peptide patterns resulting from limited proteolysis in the presence of detergents. The three polypeptides were synthesized by primary cultures of hepatocytes and were externally oriented at the surface of these cells, as shown by their accessibility in situ to iodination catalyzed by lactoperoxidase. They were not detectable in the serum by crossed immunoelectrophoresis. The three antigens were present at very low (PM110) or nondetectable (PM105, PM160) concentrations in intracellular membrane fractions derived from the Golgi and smooth and rough endoplasmic reticulum of liver. The antigens also were reduced in concentration in a plasma membrane fraction most likely derived from the sinusoidal surface of the hepatocyte. The three membrane antigens bind to concanavalin A; hence, they are probably glycoprotein constituents of a discrete domain of the hepatocyte plasma membrane. Immune complexes were isolated after crossed immunoelectrophoresis and injected into rabbits. Each of the antisera obtained was reactive to one of the membrane polypeptides. Sections of fixed rat livers were reacted with each of the antibodies and then the primary antibody was localized by indirect immunocytochemical methods using horseradish peroxidase or colloidal gold as labels. Each of the three antigens was localized by this method to the bile canalicular domain of the hepatocyte plasma membrane.

Recent studies from our laboratory (1, 2) have been concerned with analyses of the composition of the plasma membrane particularly with respect to the externally oriented proteins and glycoproteins and with their route of biogenesis in mammalian cells. We have used for these studies primarily tissue culture cell lines derived from rat hepatomas because the externally oriented membrane proteins of these cells are easy to identify by labeling cells in situ by either lactoperoxidase-catalyzed iodination or by reduction of galactose oxidase-treated cells with tritiated sodium borohydride (3–5). Further,

the cell culture lines have a relatively simple plasma membrane that for the most part lacks the regional specializations that characterize the plasma membrane of cells in tissues within the animal. For example, the plasma membrane of the hepatocyte within the liver consists of at least a sinusoidal or perisinusoidal region where exchanges between the hepatocyte and tissue fluids and the circulation take place. This domain of plasma membrane would be expected to contain receptors that facilitate these exchanges, such as the specific receptors for insulin, other hormones, and other large and small molec-

ular weight material in the blood. The lateral or intercellular domain of the plasma membrane might be expected to contain different proteins, perhaps proteins or protein channels that facilitate exchange between cells. Similarly, the membrane domains of the bile canaliculus, the tight and gap junctional complexes, and the coated pit regions would be expected to contain at least some proteins and glycoproteins that differ from each other and from the other domains of the membrane. These specific proteins presumably would endow these membrane domains with the specific functions they perform. The mechanism(s) used by the hepatocyte to establish the differentiated state of its plasma membrane and the mechanism(s) used to modulate the composition of these membranes in response to different cues in the environment are not well understood.

One goal of research in our laboratory is to identify the proteins that make up the different domains of the plasma membrane of the hepatocyte, to assess their distribution in the different membrane systems of the hepatocyte, and to follow and compare the route of biogenesis and the metabolic turnover of a set of proteins in each domain (6). Toward this goal, we have used in this study a method of liver cell membrane fractionation that yields a fraction heavily enriched in 5'-nucleotidase activity. We then used this membrane fraction as an immunogen and obtained an antiserum that reacts with externally oriented proteins of the hepatocyte, as shown by the accessibility of these protein antigens in primary hepatocyte cultures to iodination catalyzed by lactoperoxidase. We characterized the plasma membrane antigens by the technique of crossed immunoelectrophoresis. We reisolated the membrane antigens using the crude antisera and made antisera to each of three antigens in rabbits and then determined the localization of these antigens in various membrane systems of the hepatocyte. The polypeptide antigens of 160,000, 110,000, and 105,000 M_r identified by this approach are present in the bile canalicular domain of the hepatocyte plasma membrane.

MATERIALS AND METHODS

Isolation of Plasma Membrane and Golgi Functions: A plasma membrane fraction denoted P$_2$[1] was obtained from Sprague-Dawley rats by a modification (7) of the method of Aronson and Touster (8). The membrane fraction denoted N$_2$ was isolated by a further modification of the original method in which N$_2$, which is normally isolated on a layer of 37.2% sucrose, was separated into N$_{2upper}$ (N$_{2u}$), which floated on 32% sucrose, density 1.14 at 5°C, and N$_{2lower}$ (N$_{2l}$), which floated on 37.2% sucrose, density 1.17 at 5°C. Golgi fractions were prepared from homogenates of rat liver exactly as described by Ehrenreich et al. (9). Proteins in isolated membrane fractions were iodinated in vitro using a chloramine T procedure as described elsewhere (10).

Immunization: A goat was immunized with 1 mg of freshly prepared N$_{2u}$ membrane, which was washed by centrifugation in 0.14 M NaCl, 0.01 M Tris, pH 8, and 0.001 M EDTA, resuspended, and sonicated in 0.5 ml of 2% Triton X-100 and 0.01 M Tris, pH 7.8, followed by mixing with 0.5 ml of complete Freund's adjuvant for the initial injection and incomplete Freund's for the subsequent injections. The goat was starved overnight and injected subcutaneously every 2 wk. After 10 wk, serum was harvested and used for these experiments. Monospecific antibody was prepared to the hepatocyte receptor for galactose-terminated proteins purified from rat liver as previously described (6). Receptor binding activity for iodinated asialo-orosomucoid was also assayed as described previously (6).

Immunoelectrophoresis: Crossed immunoelectrophoresis was done at 15°C with a buffer containing 0.0056 M barbital, 0.031 M sodium barbital, 0.090 M glycine, and 0.045 M Tris base at a final pH of 8.7 (11). The agarose

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[1] *Abbreviations used in this paper:* GF$_1$, GF$_2$, GF$_3$, Golgi fractions 1, 2, and 3; N$_{2u}$, upper N$_2$ membrane fraction; N$_{2l}$, lower N$_2$ membrane fraction; P$_2$, plasma membrane fraction; SDS, sodium dodecyl sulfate.

was dissolved in this buffer with the addition of 1% Triton X-100 and was poured on gel bond film from Marine Colloids Division, FMC Corp., Rockland, ME. Electrophoresis in the first dimension for 3–6 h at 5 V/cm was followed by electrophoresis in the second dimension for 16–22 h at 2 V/cm. Electrophoresis in a SDS acrylamide gel (12), followed by immunoelectrophoresis in the second dimension, was done as described by Chua and Blomberg (13). Antigens identified after crossed immunoelectrophoresis by autoradiography were cut off the dried immunoelectrophoresis plate and eluted from the agarose by heating at 100°C for 3 min in sample buffer (12) containing 1.0% SDS, and the eluted sample was applied to an SDS acrylamide gel. This same material also was injected into rabbits for immunization using a protocol similar to that described above for goat immunization. In this way, antisera were obtained that were specific to the resolved antigens in the N$_{2u}$ membrane fraction. These antisera were used to localize the antigens in sections of glutaraldehyde-formaldehyde-fixed livers of rats as denoted in the legends to the appropriate figures. The antisera made in rabbits to the immune complexes contain antibodies to goat IgG in addition to antibodies to the rat plasma membrane antigens.

The rabbit IgG fractions were absorbed with goat serum containing preimmune goat IgG before use. To prevent nonspecific staining of liver sections in the indirect immune localization studies, the sections were incubated for 30 min in 5% normal goat serum in Tris-buffered saline (5 mM Tris, pH 7.6). The liver sections then were rinsed several times in Tris-buffered saline before the addition of primary rabbit IgG directed against rat plasma membrane proteins. All IgG fractions, including primary and secondary IgG fractions, and linker fractions when used, were also made 5% in normal goat serum. When this regimen for localization was followed (see Figs. 11 and 12), there was no detectable nonspecific immune labeling of tissue sections. The IgG fraction of goat anti-rabbit IgG was conjugated to colloidal gold as follows (14, 15). Gold colloid was prepared by the citrate reduction method of Frens (16), yielding particles of ~15 nm in diameter. An appropriate amount of IgG to be labeled was mixed with 1 ml of pH-adjusted colloid. The conjugate was centrifuged to remove large aggregates and then was washed free of unbound gold by high-speed centrifugation. The sedimented conjugate was resuspended in 50 mM Tris-buffered saline containing 0.01% polyethylene glycol, 20,000 M_r, and centrifuged again. The colloidal conjugate was resuspended in Tris-buffered saline and stored at 4°C.

Primary Hepatocyte Cultures: Hepatocytes were prepared by a modification (6) of the method of Seglen (17) involving perfusion of 200–300-g male Sprague-Dawley rats with collagenase, centrifugation of the cells at 50 g for 4 min, and plating at 5 × 10^6 hepatocytes/60-mm culture dish (Lux Scientific Corp., Thousand Oaks, CA) in Williams' medium E with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 μM dexamethasone (18). After 1 h of attachment, the cells were washed to remove dead cells and fresh medium was added. Greater than 95% cell viability, judged by exclusion of 0.4% trypan blue, was obtained. Cells either in suspension or in monolayer were iodinated in situ via lactoperoxidase-catalyzed iodination as described by Warren and Doyle (6). Total cell protein was labeled with [^{35}S]methionine also as described elsewhere (6).

Assays for Enzymes and Protein: Protein was assayed by the method of Lowry et al. (19) with bovine serum albumin as a standard. 5'-Nucleotidase was assayed by following the hydrolysis of 0.01 M 5'-adenosine monophosphate at pH 8.5, 37°C, for 20 min (20) by measuring the release of inorganic phosphate by a modification (21) of the method of Chen et al. (22). A blank reaction with 3'-adenosine monophosphate was used as a control for alkaline phosphatase. Succinate-cytochrome c reductase was measured spectrophotometrically by following the reduction of cytochrome c (23). Glucose-6-phosphatase was assayed by following the hydrolysis of glucose-6-phosphate (24) in the presence of EDTA and potassium fluoride to inhibit alkaline and acid phosphatase (25).

Peptide Mapping: Peptide mapping of iodinated antigens was done using a modification of the method of Cleveland et al. (26) and is described in detail elsewhere (6).

RESULTS

Enzyme Characterization of the Membrane Fractions Isolated from Rat Liver

The relative specific activity value of 23 for 5'-nucleotidase in the P$_2$ fraction of rat liver (Table I) is similar to that found by Touster et al. (27), and is in the range that is usually expected for a relatively pure fraction of plasma membrane. The P$_2$ fraction of rat liver is enriched in markers that characterize the sinusoidal front of the hepatocyte plasma mem-

TABLE I
Enzyme Characterization of Plasma Membrane Fractions

Fraction	Protein		5'-nucleotidase		Succinate-cytochrome c reductase		Mitochondrial protein
	mg/g wet wt of liver	Percent of total	moles Pi/ mg protein·h	Relative specific activity	nmol cyt. c reduced/mg protein/min	Relative specific activity	Contaminating percent fraction
Homogenate	180	100	4.2	1.0	13	1.0	15
N_{2u}	0.04–0.08	0.02–0.04	270	64	≪0.8*	≪0.06	0
N_{2l}	0.03–0.05	0.02–0.03	46	11	17	1.3	2.0
P_2	0.9–1.7	0.5–0.9	98	23	0.16	0.01	0

* The high background rate in the absence of the substrates prevented assay of low concentration.

brane, including the hepatic binding protein for serum galactose terminated glycoproteins and the receptor for insulin (7). The N_2 membrane fraction separated into two bands of slightly different densities after sucrose gradient centrifugation. The lighter band, N_{2u}, contained approximately sixfold more 5'-nucleotidase activity than the more dense band. The extremely high relative specific activity of 64 for 5'-nucleotidase in N_{2u}, as well as the amount of binding activity for the serum galactose-terminated glycoproteins that it contains relative to P_2 (7), suggests that it is derived primarily from a domain of the plasma membrane other than the sinusoidal domain. There was very little contamination of the plasma membranes by inner mitochondrial membranes, as shown by the low activity of succinate-cytochrome c reductase in both P_2 and N_{2u}.

Assays for glucose-6-phosphatase gave variable results. The instability of this enzyme in isolated subcellular fractions is a major problem because an overnight centrifugation step was required to isolate the plasma membranes. The homogenate gave a 1.4–2.8-fold-higher activity for glucose-6-phosphatase when it was stored at −20°C than when it was stored at 0°C overnight, thereby making calculation of the relative specific activities difficult. The storage time before the assay of the fractions and the concentration of proteins in the different fractions also contributed to variation in enzyme activity. The presence of glucose-6-phosphatase in the Golgi fractions, as well as in the endoplasmic reticulum, and its differential rate of inactivation in the two membranes (28) led us to abandon use of it as a specific marker for endoplasmic reticulum.

It is likely that N_{2l} contains nuclear membrane because it has the same density as one subfraction of rat liver nuclear envelopes. The presence of 5'-nucleotidase in the nuclear envelope has been reported (29). However, the 5'-nucleotidase activity in the nuclear envelope fraction could also be due to plasma membrane sticking to other membranes at this density.

On the basis of the high specific activity of 5'-nucleotidase in the N_{2u} fraction, the reproducibility in yield of this fraction and the low concentration in N_{2u} of marker constituents characteristic of other liver cell fractions, we used N_{2u} as an immunogen in goats in the hope of producing antibodies specific for proteins of a domain of the hepatocyte plasma membrane different from the sinusoidal domain.

Characterization of the Antigens in the Plasma Membrane Fraction N_{2u} by Crossed Immunoelectrophoresis of Rat Liver

The crossed immunoelectrophoresis pattern of proteins from N_{2u}, which were dissolved in Triton X-100 at 3 mg/ml
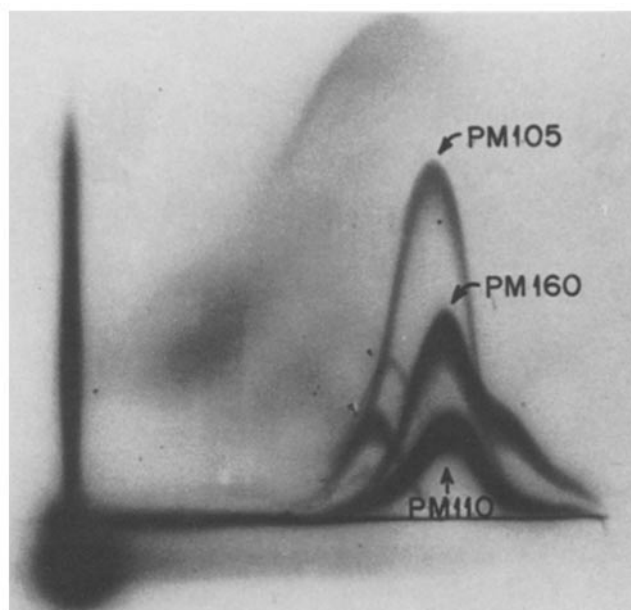

FIGURE 1 Autoradiogram of crossed immunoelectrophoresis of N_{2u} membrane fraction. Electrophoresis of 48 µg of [125I]-labeled N_{2u} for 4.8 h at 5 V/cm in the first dimension was followed by electrophoresis into 5% antiserum prepared against the N_{2u} fraction in goats. The immunoprecipitin lines are denoted as PM105, PM110, and PM160 to indicate plasma membrane antigens with approximate M_r 105,000, 110,000, and 160,000 (see Fig. 2).

and reacted with the antisera to this fraction produced in goats, is shown in Fig. 1. Although the exact mobility of the antigens resolved in the fraction of N_{2u} plasma membrane varies slightly with experimental conditions such as antigen concentration, they are given the same number designation in all the following figures. Antigens denoted PM105, PM110, and PM160, which were the only antigens observed by crossed immunoelectrophoresis when the N_{2u} preparation was diluted 10-fold from that in Fig. 1 or when the antibody concentration was decreased, were the major antigens in the N_{2u} fraction and were further characterized.

The apparent molecular weights, determined by SDS PAGE (Fig. 2) of the antigens from the crossed immunoelectrophoresis gel, were 105,000, 110,000, and 160,000. PM160 appears to exist as an aggregate with a shoulder in crossed immunoelectrophoresis (see Fig. 1). However, the shoulder is usually absent from freshly prepared plasma membrane, and the area under its peak increases with the length of storage of the membrane preparation. Hence, since it has a molecular weight (see Fig. 2) and other properties similar to those of the main
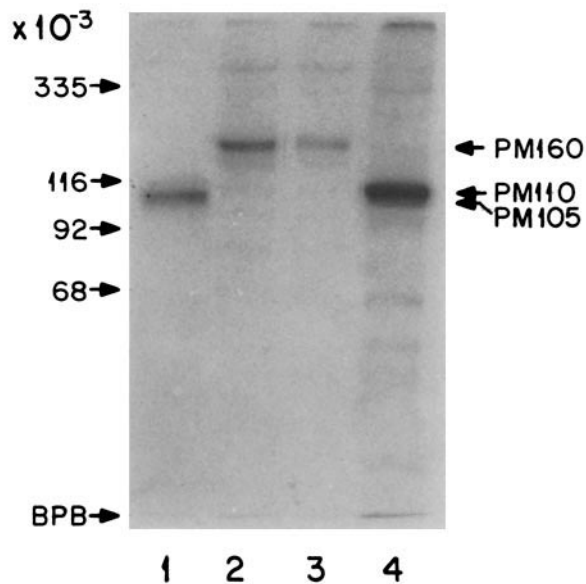
FIGURE 2   Autoradiogram of a SDS 7.5% PAGE of peaks cut out and eluted from crossed immunoelectrophoresis of ^{125}I-labeled preparations of N_{2u} membranes (Fig. 1). (Lane 1) Peak PM105; (lane 2) peak PM160; (lane 3) right shoulder of PM160; and (lane 4) PM110. BPB, bromophenol blue. Location of protein standards of known molecular weight are indicated on the left of the figure.

antigen in PM160, it is likely that the shoulder antigen is some aggregate form of the main PM160 antigen.

Peptide Mapping of the Antigens in the N_{2u} Fraction of Rat Liver

Peptide mapping by limited proteolysis in the presence of detergent was done to determine whether the antigens in N_{2u} resolved by crossed immunoelectrophoresis were structurally related to each other. The proteins present in the membrane fraction N_{2u} were iodinated by the chloramine T method in the presence of Zwittergent 314 in order to increase the accessibility to iodination of any tyrosine residues located within the membrane bilayer or inside closed membrane vesicles in the fraction. Zwittergent 314 had very little effect on the pattern of antigens resolved by crossed immunoelectrophoresis. The antigens were separated by crossed immunoelectrophoresis at low antigen and antibody concentrations (as shown in Fig. 1), eluted, and further purified by electrophoresis in SDS 7.5% polyacrylamide gels. The resolved iodinated components were cut out from the latter gel and were partially digested with proteolytic enzymes in another polyacrylamide gel. The digest was then electrophoresed into an SDS 15% polyacrylamide gel for analysis. As shown in Fig. 3, all three antigens (PM105, PM110, and PM160) gave different peptide patterns after digestion with α-chymotrypsin and Staphylococcus aureus protease, which indicates that the antigens in the immunoprecipitin peaks of Fig. 1 are different polypeptides.

N_{2u} Antigens Are Present in Hepatocytes

To show that the plasma membrane of hepatocytes contains the three antigens denoted PM105, PM110, and PM160, hepatocytes in primary culture were labeled by lactoperoxidase-catalyzed iodination and the labeled plasma membrane proteins were analyzed by crossed immunoelectrophoresis,
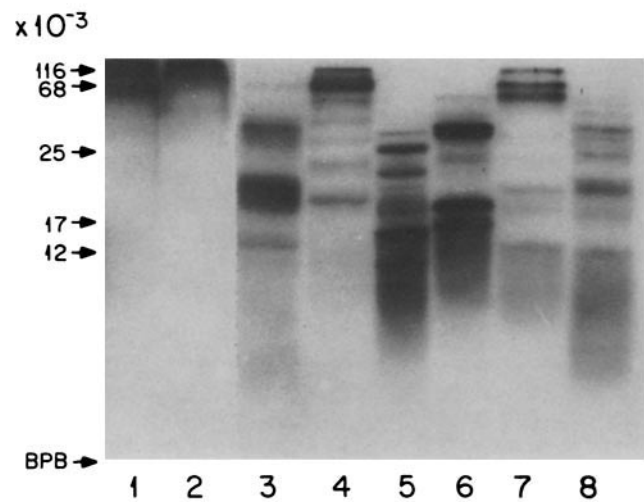

FIGURE 3   Autoradiogram of a polyacrylamide gel for peptide mapping of the antigens in N_{2u}. The iodinated antigens were isolated by crossed immunoelectrophoresis (Fig. 1) and were further fractionated by SDS 7.5% polyacrylamide gel electrophoresis. The subunits of M_r 105,000, 110,000, and 160,000 were eluted and partially digested with enzymes in the presence of 0.5% SDS; electrophoresis was done on an SDS 15% polyacrylamide gel. No enzyme treatment: (1) PM110, (2) PM160. Digestion with α-chymotrypsin; (3) PM160; (4) PM105; (5) PM110. Digestion with S. aureus protease; (6) PM110; (7) PM105; (8) PM160.
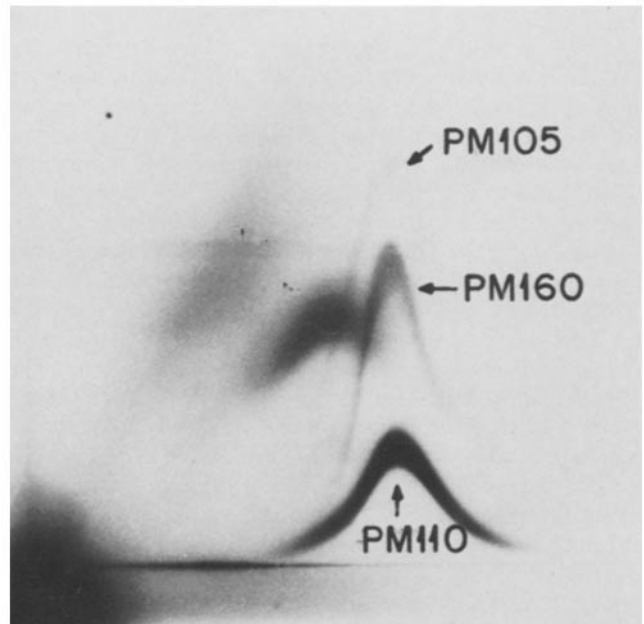

FIGURE 4   Autoradiogram of a crossed immunoelectrophoresis gel of a membrane extract from ^{125}I-labeled hepatocytes and unlabeled N_{2u} fraction of rat liver. The hepatocytes were labeled with ^{125}I by the lactoperoxidase-catalyzed method after they had been in primary tissue culture for 6 h. The sample contained 43.2 μg of total hepatocyte membranes and 4.8 μg of purified N_{2u} as carrier. Electrophoresis in the first dimension was for 6 h at 5 V/cm and was followed by electrophoresis into 1% antiserum.

followed by SDS PAGE. Crossed immunoelectrophoresis of the labeled hepatocyte membranes with the plasma membrane fraction N_{2u} added as a carrier antigen revealed the presence of an additional antigen to the left of PM160 that was not seen in N_{2u} (Fig. 4). PM105, PM110, and PM160 were iodinated in situ in the hepatocytes (Fig. 4) and could

FIGURE 5 Autoradiogram of an SDS 7.5% polyacrylamide gel after electrophoresis of antigens present in ^{125}I-labeled hepatocytes. Crossed immunoelectrophoresis was done with a sample consisting of 43.2 μg of membranes from hepatocytes labeled with ^{125}I and 4.8 μg of N_{2u} as carrier (see Fig. 4 for a typical analysis). Electrophoresis for 6 h at 5 V/cm was followed by electrophoresis into 1% antiserum. The antigens were eluted for electrophoresis on a dodecyl sulfate 7.5% polyacrylamide gel. Lanes: 1) total membranes from hepatocytes labeled with ^{125}I; 2) ^{125}I-labeled N_{2u}. Antigens from crossed immunoelectrophoresis of ^{125}I-hepatocyte membranes: lanes: (3) PM105, (4) PM160, (5) PM110, and (6) immunoprecipitin line to the left of PM160 in Fig. 4. Standards from crossed immunoelectrophoresis of N_{2u}: lanes: (7) PM105, (8) PM160, and (9) PM110.
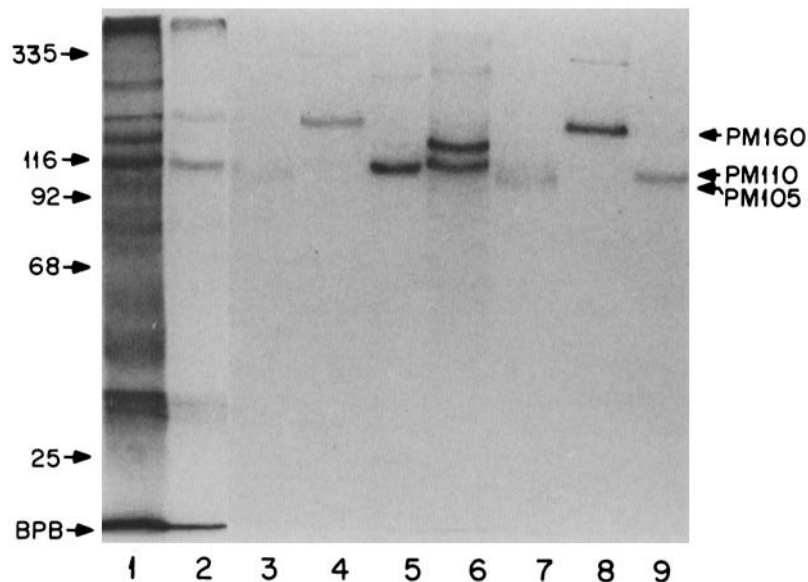
be isolated after crossed immunoelectrophoresis and analyzed further by electrophoresis on an SDS 7.5% polyacrylamide gel (Fig. 5). Membrane polypeptides with molecular weights of 105,000, 110,000, and 160,000 were present as antigens in the ^{125}I-labeled hepatocytes. Hence, these antigens are externally oriented on the primary culture of rat hepatocytes.

That the membrane antigens in hepatocytes accessible to iodination were indeed synthesized by the hepatocyte was shown by labeling hepatocytes in primary culture with [35S]methionine and analyzing the membranes by tandem-crossed immunoelectrophoresis (Fig. 6). Antigens PM105, PM110, and PM160 were labeled in the immunoelectrophoresis pattern. Also observed was an additional peak to the right of the PM160, PM110, and PM105 areas. SDS PAGE of these peaks confirmed the labeling of antigens PM105, PM110, and PM160 with [35S]methionine. In the electrophoresis of the homogenates of the hepatocyte membranes, bands with the molecular weights of antigens PM105, PM110, and PM160 could be distinguished in the membranes from iodinated hepatocytes but were present in too low a concentration to be seen in the [35S]methionine-labeled pattern (data not shown).

Antigens with Molecular Weights of 105,000, 110,000, and 160,000 Are Not Serum Proteins

Tandem-crossed immunoelectrophoresis with ^{125}I-labeled N_{2u} in the left sample hole and either 0.14 M NaCl as a control or an excess of rat serum in the right sample hole (Fig. 7) for crossed immunoelectrophoresis showed that the rat serum proteins did not cause any deflection of the precipitin lines. This rules out the possibility that antigens PM105, PM110, and PM160 are present in significant concentrations in serum.

Analyses of Other Rat Liver Fractions for Plasma Membrane Antigens Present in N_{2u}

The N_{2u} plasma membrane fraction is enriched in the concentrations of antigens PM105, PM110, and PM160 both in comparison with N_{2l} (sixfold; not shown) and P_2 (three- to fourfold; data not shown). Glycoproteins were isolated from the membrane fractions by chromatography on concanavalin
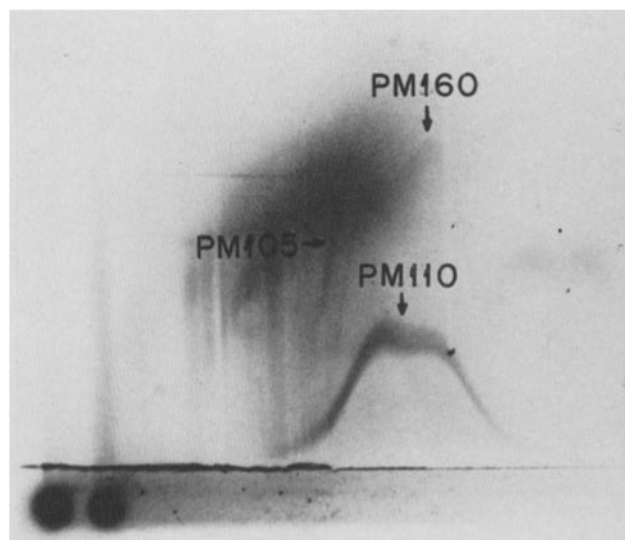

FIGURE 6 Autoradiogram of tandem crossed immunoelectrophoresis of [35S]methionine-labeled hepatocyte membranes mixed with the N_{2u} fraction. Each sample hole was filled with 4.8 μg of N_{2u} and 43.2 μg (7.8 × 10⁵ cpm) of membrane from hepatocytes labeled in primary culture with [35S]methionine. Electrophoresis in the first dimension was for 6 h at 5 V/cm and was followed by electrophoresis into 1% antiserum.

A-Sephadex. Antigens PM105, PM110, and PM160 were all enriched in the concanavalin A-bound fraction and were specifically eluted with 0.5 M α-methyl mannoside.

The three Golgi fractions isolated from rat liver by the method of Ehrenreich et al. (9) were analyzed by crossed immunoelectrophoresis to determine if the antigens denoted PM105, PM110, and PM160, which were present in the plasma membrane of hepatocytes, were also present in the Golgi apparatus. In addition, the pellet and the load from the Golgi fraction, which were enriched in rough and smooth endoplasmic reticulum, respectively, were also analyzed by crossed immunoelectrophoresis. These membrane fractions were iodinated in the presence of Zwittergent 314 so that proteins on both sides of the vesicles would be iodinated. Crossed immunoelectrophoresis of 48 μg of protein from each of the five fractions was done into both 1 and 4% antiserum
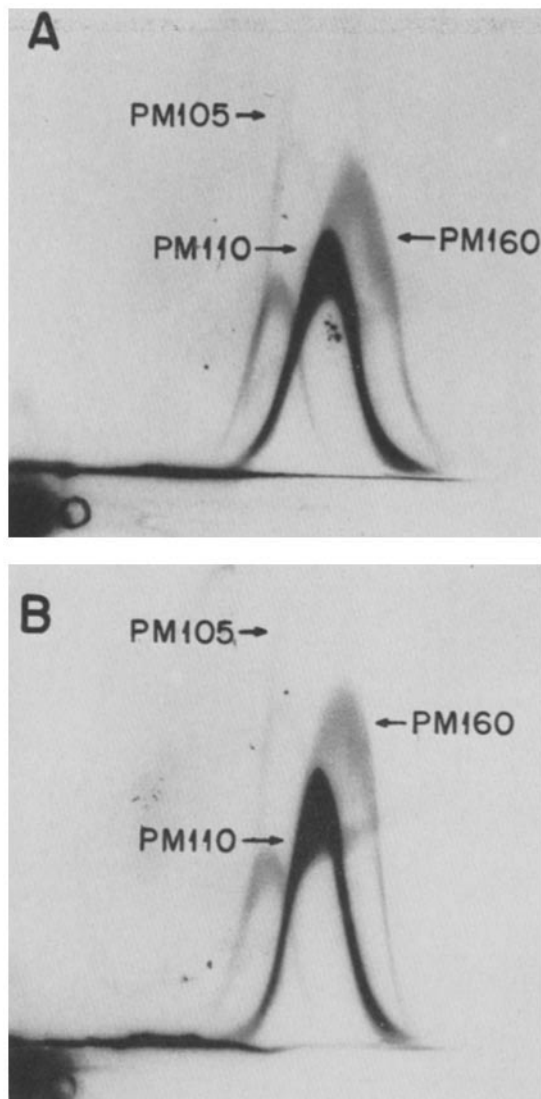
FIGURE 8 Crossed immunoelectrophoresis of Golgi fractions, 48 μg of Golgi fraction (2) (GF$_2$) into 4% antiserum (A); Golgi fraction 3 (GF$_3$), 48 μg into 1% antiserum (B); longer exposure of B (C); Golgi fraction 1 (GF$_1$), 48 μg into 1% antiserum (D); Golgi fraction 1, 48 μg into 4% antiserum (E); N$_{2u}$, 4.8 μg into 1% antiserum (F). Electrophoresis was for 4 h at 5 V/cm followed by immunoelectrophoresis for 22 h at 2V/cm.

FIGURE 7 Autoradiogram of tandem crossed immunoelectrophoresis of N$_{2u}$ and rat serum. The left hole in both A and B contained 48 μg of ^{125}I-N$_{2u}$, whereas the right hole contained 0.14 M NaCl as a control in A and 600 μg protein of rat serum (unlabeled) in B. Electrophoresis for 5 h at 5 V/cm was followed by electrophoresis into 4% antiserum.

in the second dimension. In N$_{2u}$, the plasma membrane fraction, the antigens denoted PM105, PM110, and PM160 could easily be seen when 48 μg of N$_{2u}$ was reacted by crossed immunoelectrophoresis with 4% antiserum or when 4.8 μg of N$_{2u}$ was reacted with 1% antiserum (Fig. 8F). In most N$_{2u}$ preparations there was a complete separation of PM160 and PM110 precipitin lines, but in this particular preparation there was an unusually low concentration of the PM160 precipitin line.

In the rough endoplasmic reticulum, no peaks with a mobility in the range of the precipitin lines formed by PM105, PM110, and PM160 were seen (data not shown). Similarly, in the smooth endoplasmic reticulum, the antigen concentrations were much too low to form clear precipitin lines (data not shown). In the immunoelectrophoresis of Golgi fraction 3 (GF$_3$) into 1% antiserum, a peak provisionally denoted PM110, was seen (Fig. 8, B and C). With 4% antiserum (not shown), PM110 could be seen and showed the dark staining
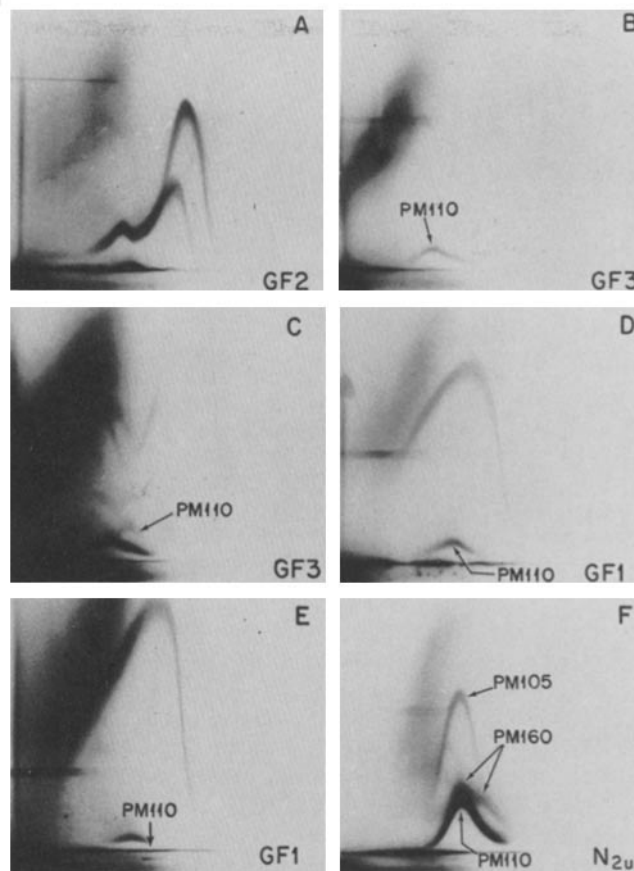
pattern characteristic of PM110 in the plasma membrane fraction N$_{2u}$. Two extremely light precipitin lines appeared above PM110 (Fig. 8, B and C) in the GF$_3$ fraction, and it is possible that these correspond to PM105 and PM160 in the plasma membrane fraction. Other peaks also appeared in GF$_3$, and the gel from this fraction appeared more complex than those of GF$_2$ and GF$_1$. GF$_1$ contained an immunoprecipitin line in the region of PM110, which was stained darkly by Coomassie Blue in a gel with 4% antiserum (Fig. 8, D and E). In addition, there was a very broad immunoprecipitin line above the peak with mobility similar to antigen PM110. Immunoelectrophoresis of GF$_2$ into 4% antiserum (Fig. 8A) revealed two precipitin lines, both of which appeared to fuse completely to a third precipitin line. None of these had the characteristics of antigens PM105, PM110, and PM160.

SDS PAGE of the Antigens Present in the Golgi Fractions

The peaks in the Golgi fractions, which had mobility resembling that of antigen PM110 in the plasma membrane fraction, were eluted from the crossed immunoelectrophoretic gels to determine their apparent molecular weights by SDS polyacrylamide gel electrophoresis. All of these peaks had an apparent molecular weight of 110,000, which is identical to
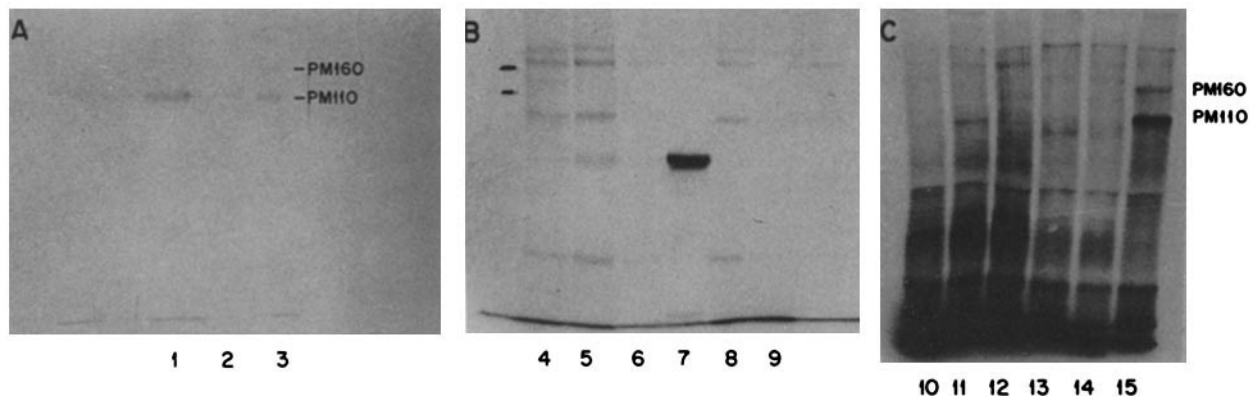
FIGURE 9 Autoradiogram of SDS 7.5% PAGE of antigens eluted from crossed immunoelectrophoresis of Golgi fractions (A and B) and of homogenates of membrane fractions (C). (A) Antigens eluted from the crossed immunoelectrophoresis of GF₃ (from Fig. 8): lanes: (1) immunoprecipitin line PM110 from the crossed immunoelectrophoresis of GF₃ (Fig. 8, B and C); (2) immunoprecipitin line corresponding to PM110 from the crossed immunoelectrophoresis of GF₁ (Fig. 8E). Although not readily apparent in the figures, a faintly staining band at the M_r 110,000 position can be detected in the original experiment; (3) peaks PM110 and PM160 from the crossed immunoelectrophoresis of N₂ᵤ (Fig. 8F). (B) Antigens eluted from the crossed immunoelectrophoresis of GF₂ into 4% antiserum (see Fig. 8A): lanes: (4) left immunoprecipitin line; (5) antigens in the line joining left precipitin line to double right precipitin line; (6) lower right precipitin line; (7) upper right precipitin line of GF₂. Antigens eluted from the crossed immunoelectrophoresis of GF₁ into 1% antiserum (Fig. 8D): lanes: (8) left side of major precipitin line; (9) right side of major precipitin line. Mobilities of M_r 110,000 and 160,000 are indicated. (C) lanes: (10) Pellet obtained during the Golgi fractionation and enriched in rough endoplasmic reticulum; (11) load obtained during the Golgi fractionation and enriched in smooth endoplasmic reticulum; (12) GF₃; (13) GF₂; (14) GF₁; and (15) N₂ᵤ.

that of the plasma membrane antigen PM110 (Fig. 9A). These results show that all three Golgi fractions have a peak that is similar in mobility on both crossed immunoelectrophoresis and SDS PAGE to antigen PM110 in the plasma membrane.

Since there was a possibility that a precursor to the three plasma membrane antigens might have a different mobility on crossed immunoelectrophoresis and a slightly different molecular weight, the major iodinated peaks from crossed immunoelectrophoresis were also eluted for a molecular weight determination. However, the major iodinated antigenic peaks other than that which corresponded in mobility to PM110 from GF₂ and GF₁ showed no polypeptides with similar mobilities to PM105 or PM160 of the N₂ᵤ fraction (Fig. 9).

SDS PAGE of the membrane fractions iodinated in the presence of Zwittergent 314 (Fig. 9C) revealed two major bands having the apparent molecular weights of antigens PM110 and PM160 in the plasma membrane fraction, N₂ᵤ. These bands could not be distinguished in any of the Golgi or endoplasmic reticulum fractions (Fig. 9C). Bands with mobilities similar to PM110 did not prove to be either PM110 or PM105 when fractions were run together. Hence, although PM110 is present in the Golgi fraction, it is present at a concentration ≥10-fold lower than in the plasma membrane fraction. PM105 and PM160 are present, if at all, in Golgi fractions in even lower relative concentrations.

Crossed Immunoelectrophoresis of the ¹²⁵I-Golgi Fractions Mixed with N₂ᵤ

A mixing experiment in which ¹²⁵I-Golgi fractions were mixed with plasma membrane as the carrier was done to obtain further information about the possible presence of antigens PM105 and PM160 in Golgi fractions. The antigen consisted of 48 μg of N₂ᵤ and iodinated GF₂, GF₁, or N₂ᵤ. The amount of ¹²⁵I added was four times higher in GF₂ and GF₁ than in N₂ᵤ. After crossed immunoelectrophoresis, the

antigens were eluted for SDS PAGE. Only the plasma membrane antigen with the mobility of PM110 was detected. Again, the antigens corresponding to PM105 and PM160 of the plasma membrane fraction were present at much lower concentrations in the Golgi fractions than was antigen PM110.

Antigens Present in Rat Serum

It has already been shown by tandem-crossed immunoelectrophoresis that rat serum does not contain the antigens PM105, PM110, and PM160, which are the major constituents of the plasma membrane fraction (Fig. 8). In addition, crossed immunoelectrophoresis of ¹²⁵I serum from rats followed by SDS PAGE of the major antigens in serum reacting with the antisera to plasma membrane fraction N₂ᵤ showed that the other antigens present in the Golgi fraction (shown in Fig. 9) were also probably serum constituents enroute to being secreted. One of the major antigens in GF₂, for example, had an apparent molecular weight of 69,000 and was probably serum albumin.

All of the previous studies suggest that the three glycopeptide antigens denoted PM105, PM110, and PM160 are present in a domain of the plasma membrane enriched in the N₂ᵤ fraction.

Identification of the Plasma Membrane Domain Containing the Glycoprotein Antigens PM105, PM110, and PM160

Rather than attempting to define the plasma membrane domain containing PM105, PM110, and PM160 by trying to characterize the N₂ᵤ fraction by a battery of marker enzyme assays or by morphological criteria, we chose to prepare antibodies to each of the antigens that predominate in the fraction. To do this, immunoprecipitin lines corresponding to PM160, PM110, and PM105 were cut from the crossed
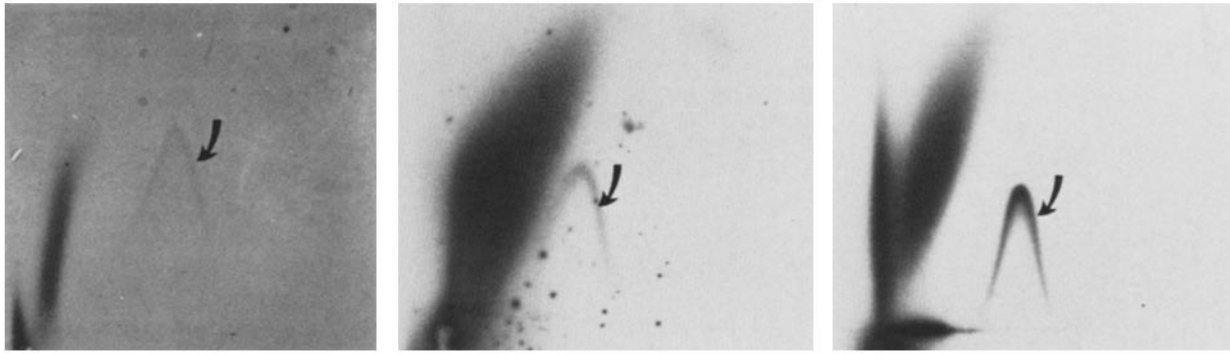
FIGURE 10 Analysis of antiserum produced in rabbits to the immune complex of PM110. N_{2u} membrane (30–100 μg unlabeled protein) was reacted with antiserum (4%) to this fraction as shown in Fig. 1. After crossed immune electrophoresis and washing of the gel, the immune complex of PM110, PM105, and PM160 were each cut out and boiled in 1% SDS. The boiled extracts then were loaded onto a 2.5 mm × 3.5 cm glass tube with dialysis tubing covering the end. The sample was subjected to electrophoresis in 0.025 M Tris-HCl 0.25 M glycine, pH 8.2 (4 mA/tube, 4 h). The material in the dialysis chamber was injected into rabbits, 5 mg per injection, three injections at 10-d intervals. Antiserum was obtained after 1 mo. The *left* side shows the specificity of the antiserum to PM110 to the N_{2u} fraction as assessed by Coomassie Blue stain. The middle shows the specificity of the N_{2u} fraction iodinated as described in the text and the right-hand side shows the specificity to total membrane extracts prepared from hepatocytes iodinated in situ via lactoperoxidase-catalyzed iodination. The arrows denote the one immune precipitin band for PM110 produced in each of the extracts by the specific immune serum. Similar results were obtained with each of the other specific antisera to PM105 and 160.

immunoelectrophoresis gels and were injected into rabbits. The rabbits responded by making distinct antibodies to each of the plasma membrane antigens. Fig. 10 shows a crossed immunoelectrophoresis analysis of the antibody produced to PM110. A single precipitin was found when either iodinated hepatocytes, iodinated liver N_{2u}, or the N_{2u} fraction itself was analyzed by the rabbit antisera. Similar results were obtained with the antisera directed to PM105 and PM160, although the antiserum to PM160 is of lower titer (data not shown). These antisera were sufficiently specific to be used for localization studies and were used to locate by indirect immunocytochemical methods the antigens in sections of rat liver fixed in glutaraldehyde-formaldehyde.

As shown in Fig. 11, B and C, the antisera to PM110 and the antiserum to PM105 (Fig. 11 E) stained a very characteristic domain of the hepatocyte plasma membrane. The specific antisera to PM160 stained the same domain. As shown in Fig. 12, which is a three-dimensional diagrammatic depiction of the liver, this domain is the bile canaliculus. This is the only region of the liver that binds the antibodies specific to the glycoproteins PM160, PM110, and PM105. As controls for the indirect immunochemical localizations of the bile canalicular antigens in Fig. 11, liver sections were also reacted with an antiserum specific for the hepatocyte-specific plasma membrane binding protein that recognizes galactose-terminated glycoproteins. As shown in Fig. 11 F, the latter antiserum did not give the characteristic bile canalicular pattern of staining of the antibodies specific for plasma membrane glycoproteins PM160, PM110, and PM105. Rather, it stained the sinusoidal surface of the hepatocyte, the domain where the receptor is localized. Further, there was no specific staining of any liver structure when preimmune serum was used as primary antibody or when any of the reaction components were omitted (Fig. 11, A and D). Finally, we used the high-titer antisera specific to PM110 and PM105 to localize these glycoproteins at the electron-microscopic level. For these studies we used the same liver tissues fixed and embedded in polyethylene glycol that were used for the light-microscopic localization. The second antibody, the IgG fraction of goat

anti-rabbit IgG, however, was tagged with colloidal gold. Although the preservation of morphology after polyethylene glycol embedding is not as good as with other more traditional fixation and embedding methods for electron microscopy, the result can be more readily compared with the light-microscopic localization of Fig. 11. As shown in Fig. 13, the indirect immunocytochemical methods using colloidal gold conjugated to the IgG fraction of goat anti-rabbit IgG localized the primary rabbit antibodies to PM110 and PM105 specifically and exclusively to the bile canaliculus of the rat hepatocyte.

DISCUSSION

The overall goal of this research was to identify in hepatocytes sets of proteins that make up the different domains of the plasma membrane and to compare the route of biogenesis and metabolism of these membrane domains. Toward this goal, we have isolated from homogenates of liver a membrane fraction that is heavily enriched in 5'-nucleotidase activity. We believed that this membrane originated from a domain of the liver different from the sinusoidal domain. We prepared antisera to this membrane fraction, denoted N_{2u}, and showed by crossed immunoelectrophoresis that three major antigens, denoted PM105, PM110, and PM160, are recognized in the membrane fraction by the immune serum. These antigens are externally oriented membrane proteins, as shown by their accessibility when hepatocytes in cell culture are subjected to lactoperoxidase-catalyzed iodination. As shown by metabolic labeling with [^{35}S]methionine, they are synthesized by the hepatocyte; they are not major secretory proteins in that they are not detected by crossed immunoelectrophoretic analyses of serum. The antigens PM105, PM110, and PM160 isolated from the immune precipitin complex after crossed immunoelectrophoresis had molecular weights of 105,000, 110,000, and 160,000, respectively.

These three polypeptides were unrelated to each other, as assessed by the pattern of peptide fragments released from each of them by limited proteolysis. Each of the membrane polypeptides binds when detergent extracts of membrane
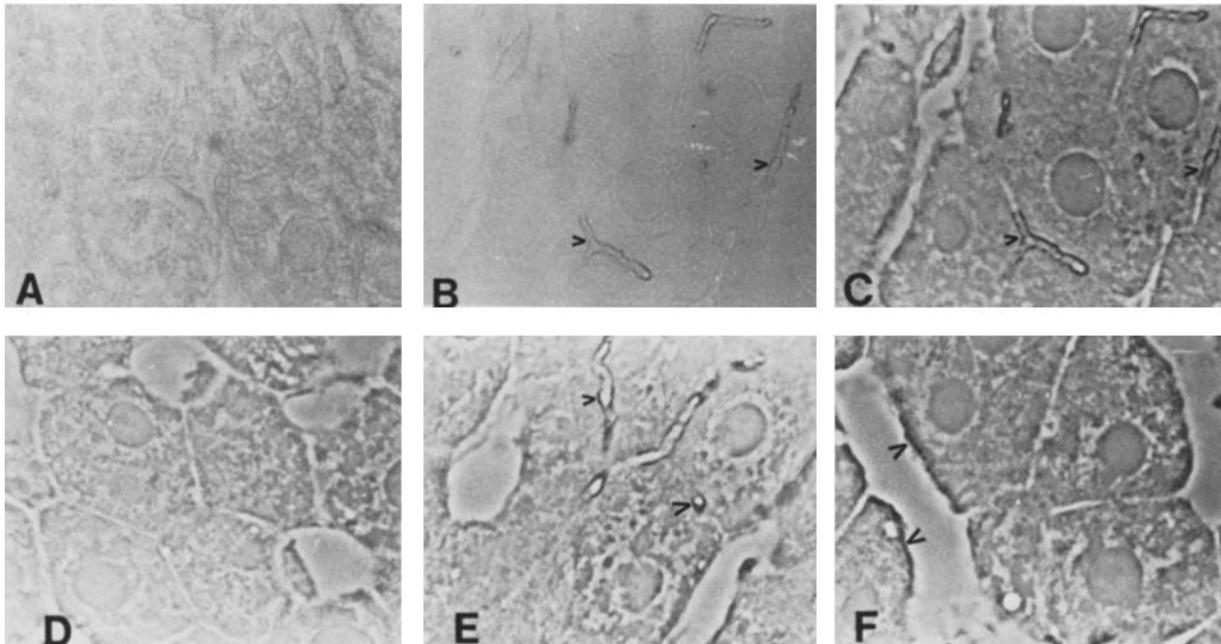
FIGURE 11 Light-microscopic immunocytochemical localization of plasma membrane glycoproteins. Adult rat liver was fixed in situ by perfusion for 30 min with 0.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The liver then was removed from the animal and cut into 2-3-mm^3 blocks. The fixed liver was washed in 0.1 M phosphate buffer at 4°C, dehydrated in ethanol and embedded in polyethylene glycol 4,000 before sectioning. The blocks of tissue embedded in polyethylene glycol were dipped into liquid nitrogen before cutting sections (4-10 μm) at room temperature. The sections were placed on a drop of water on carbon coated glass slides. The slides were warmed to 60°C to promote adhesion of the section to the glass. Sections then were rehydrated through a graded series of ethanol-water. The rehydrated sections were placed in 5 mm Tris-buffered saline, pH 7.6. Sections were incubated for 3 h at room temperature with the IgG fraction using either rabbit preimmune IgG (100 μg/ml) (A and D), rabbit antibody to PM110 100 μg/ml characterized in Fig. 10 (B and C), rabbit anti PM105, 100 μg/ml (E), rabbit antibody to the hepatocyte receptor for galactose terminated glycoproteins 100 μg/ml (F). Sections then were rinsed and washed for 30 min with Tris-buffered saline. Sections were then incubated with an IgG fraction of goat anti-rabbit IgG (1:30 dilution) for 30 min. Finally sections were washed and incubated with rabbit anti-horseradish peroxidase:peroxidase complex (1:100 dilution) for 30 min. The benzidine reaction product was localized in unstained sections using either bright-field (A and B) or phase-contrast microscopy (C, D, E, and F). There was no benzidine reaction product when preimmune IgG was used as the primary antibody (A and D) or if any of the other components were omitted from the indirect immune localization procedure. Primary antibodies to PM110 and 105 were localized exclusively to the bile canalicular domain of the plasma membrane (arrows in B, C, and E; see also Figs. 12 and 13). Primary antibody to PM160 showed exactly the same localization. Primary antibodies to the asialoglycoprotein receptor were localized by this procedure exclusively to the sinusoidal domain of the plasma membrane (arrow in F). × 1,250.
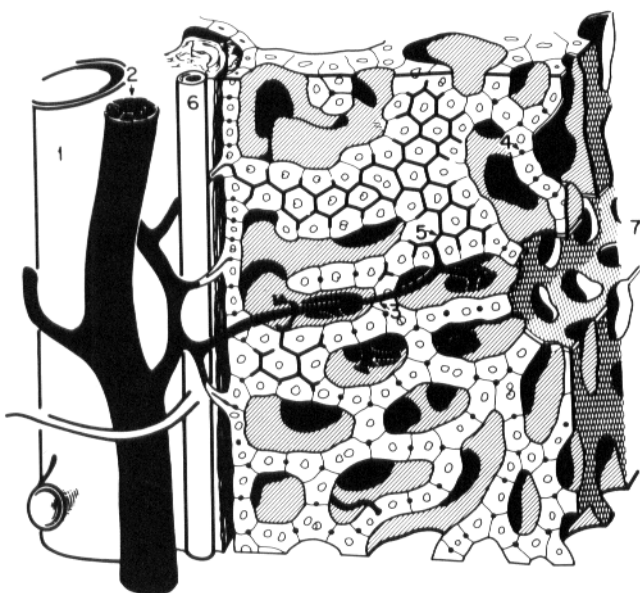
fractions are applied to columns of concanavalin A. The polypeptides are released from the lectin specifically by α-methyl mannoside, which suggests that they are membrane glycoproteins. The three glycoproteins are enriched about three- to fourfold in the N$_{2u}$ fraction relative to the P$_2$ fraction of membrane, as assessed by the degree of enrichment for iodide incorporated into three glycoproteins via lactoperoxidase-catalyzed iodination of primary cultures of hepatocytes. The three glycoproteins are also present in lower concentration in the N$_{2i}$ fraction of plasma membrane. The P$_2$ fraction of plasma membrane is enriched in membrane receptor glycoproteins specific for galactose-terminated glycoproteins and insulin (7), which suggests that this fraction is enriched in the

FIGURE 12 Schematic three-dimensional representation of the liver. Compare this representation to the two dimensional immunochemical localization of PM110 (and 105, 160) of Fig. 11 (redrawn from reference 30). 1, portal vein. 2, bile ducts. 3, intralobular cholangiole. 4, bile canalculi. 5, bile canalculi within liver plates. 6, hepatic artery. 7, central vein.
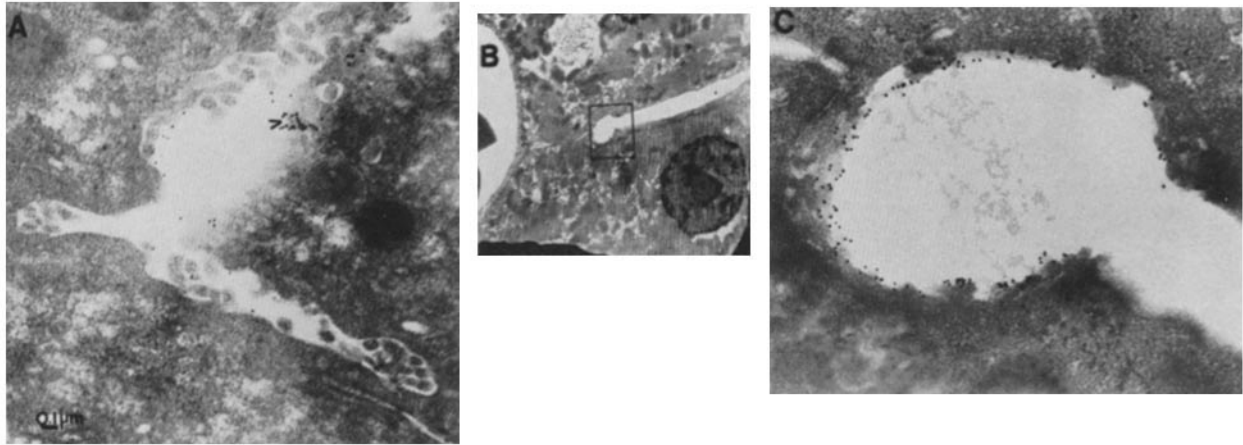
FIGURE 13 Electron-microscopic immunocytochemical localization of antibodies to bile canalicular-specific liver proteins. Sections prepared as described in Fig. 11 were treated with primary antibodies as rabbit IgG fractions directed against either PM110 or PM105 exactly as described in the legend to Fig. 11. After incubation and washing, the sections were incubated with gold-conjugated Ig fraction of goat anti-rabbit Ig for 30 min at room temperature. The sections then were rinsed and washed with Tris-buffered saline and water. The immunostained sections were post fixed with 2% OsO₄ for 15 min at 4°C. They then were rinsed twice with water and stained en bloc with uranyl acetate for 15 min at 4°C. The sections next were washed with water and the sections on slides were dehydrated in graded ethanol 70, 90, and 100% each for 10 min at 4°C followed by clearing in propylene oxide for 5 min, a mixture of propylene oxide: complete EPON 812 for 10 min and finally EPON 812 for 10 min. Open-tipped plastic capsules were positioned over the sections and filled with complete EPON, which was allowed to polymerize at 65°C for 48–72 h before sectioning. Capsules with sections embedded in their tips were snapped free. Sections of ~100 nm were cut and stained with uranyl acetate and lead citrate before viewing in a Siemans electron microscope. (A) The arrowhead indicates colloidal gold localization of PM110 to the bile canalicular domain. (B) A lower magnification of a rat hepatocyte; the outlined area is part of the bile canalicular, which is shown at higher magnification in C. Antibody to PM105 showed the same localization. Antibody to PM160 was of insufficient titer to be used for convincing electron microscope localization studies. (A) × 25,000; (B) × 2,100; (C) × 27,300.

sinusoidal domain of plasma membrane. Many of the proteins that are characteristic of the sinusoidal domain of the plasma membrane are in fact not unique to the plasma membrane. They are also found in relatively large concentrations on intracellular membranes, particularly membranes of the Golgi and/or GERL systems of the cell (2). In contrast, the three glycoprotein antigens characteristic of the N$_{2u}$ fraction of the plasma membrane are either absent or are present in very limited concentration in the intracellular membranes of the Golgi and endoplasmic reticulum systems of the hepatocyte. We isolated immune precipitates corresponding to PM160, PM110, and PM105 after crossed immunoelectrophoresis and used these complexes as immunogens in rabbits. The antisera obtained could distinguish and were specific for PM160, PM110, and PM105 in crude liver membrane fractions and membrane fractions prepared from iodinated hepatocytes in primary culture. These antisera were used to localize the corresponding antigens in sections of fixed liver. Each of the antibodies stained the same domain of the liver and this domain has the characteristic morphology of the bile canaliculus. Our goal in subsequent studies will be to use these antibodies and others specific to each of the proteins of the different plasma membrane domains to follow the biogenetic route by which the hepatocyte establishes a unique, differentiated domain at the plasma membrane. The methodology developed and used in this study can also be used to obtain immunospecific antibodies to other domains of the hepatocyte. Complicated methods of protein purification or tedious screening of hybridoma cells for production of useful monoclonal antibodies is not required. The monospecific antibodies can then also be of use in further purification of the complete membrane domain containing the antigen.

REFERENCES

1. Warren, R., H. Baumann, and D. Doyle. 1982. The cell surface and malignancy. In Membrane Abnormalities and Disease. M. Tao. editor. CRC Press, Inc., Boca Raton, FL. 51–96.
2. Doyle, D., J. Cook, and H. Baumann. 1982. Regulation of membrane protein concentration in hepatocytes. In The Liver: Biology and Pathobiology. I. Arias, H. Popper, H. Schachter, and D. Schafritz, editors. Academic Press, Inc., New York. 185–204.
3. Tweto, J., E. Friedman, and D. Doyle. 1978. Proteins of the hepatoma tissue culture cell plasma membrane. J. Supramol. Struct. 4:141–159.
4. Baumann, H., and D. Doyle. 1979. Localization of membrane glycoproteins by in situ neuraminidase treatment of rat hepatoma tissue culture cells and two-dimensional gel electrophoretic analysis of the modified proteins. J. Biol. Chem. 254:2542–2550.
5. Baumann, H., and D. Doyle. 1979. Effect of trypsin on the cell surface proteins of hepatoma tissue culture cells. J. Biol. Chem. 254:3935–3946.
6. Warren, R., and D. Doyle. 1981. Turnover of the surface proteins and the receptor for serum asialoglycoproteins in primary cultures of rat hepatocytes. J. Biol. Chem. 246:1346–1355.
7. Doyle, D., E. Hou, and R. Warren. 1979. Transfer of the hepatocyte receptor for serum asialoglycoproteins to the plasma membrane of a fibroblast. J. Biol. Chem. 254:6853–6856.
8. Aronson, N. N., and O. Touster. 1978. Isolation of rat liver plasma membrane fragments in isotonic sucrose. Methods Enzymol. 31:90–102.
9. Ehrenreich, J. H., J. J. M. Bergeron, P. Siekevitz, and G. E. Palade. 1973. Golgi fractions prepared from rat liver homogenates. J. Cell Biol. 59:45–72.
10. Baumann, H., and D. Doyle. 1980. Metabolic fate of cell surface glycoproteins during immunoglobulin-induced internalization. Cell. 21:897–907.
11. Bjerrum, D. J., and P. Lundahl. 1973. Detergent-containing gels for immunological studies of solubilized erythrocyte membrane components. Scand. J. Immunol. 2(Suppl. 1):139–143.
12. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680–685.
13. Chua, N.-H., and F. Blomberg. 1979. Immunochemical studies of thylaboid membrane polypeptides from spinach and Chamydomonas reinhardtii. J. Biol. Chem. 254:215–223.
14. Faulk, W. P., and G. M. Taylor. 1971. An immunocolloid method for the electron microscope. Immunochemistry. 8:1081–1083.

5. Geoghegan. W. D., and G. A. Ackerman. 1977. Adsorption of horseradish peroxidase, ovomucoid and anti-immunoglobulin to colloidal gold for the indirect detection of concanavalin A, wheat germ agglutinin and goat anti-human immunoglobulin G on cell surfaces at the electron microscope level: a new method, theory, and application. *J. Histochem. Cytochem.* 25:1187–1200.

6. Frens, G. 1973. Controlled nucleation for the regulation of the particle size in mono-disperse gold suspensions. *Nat. Physiol. Sci.* 241:20–22.

7. Seglen, P. O. 1976. Preparation of isolated rat liver cells. *Methods Cell Biol.* 13:29–83.

8. Laishes, B. A., and G. M. Williams. 1976. Conditions affecting primary cell cultures of functional adult rat hepatocytes. *In Vitro.* 12:821–832.

9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Bandall. 1951. Protein measure-ment with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.

0. Widnell, C. C., and J. C. Unkeless. 1968. Partial purification of a lipoprotein with 5' nucleotidase activity from membranes of rat liver cells. *Biochemistry* 61:1050–1057.

1. Ames, B. N., and D. T. Dubin. 1960. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. *J. Biol. Chem.* 235:769–775.

2. Chen, P. S., Jr., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phospho-rus. *Anal. Chem.* 28:1756–1758.

3. Sottocasa, G. L., B. Kuylenstierna, L. Ernster, and A. Bergstrand. 1967. An electron transport system associated with the outer membrane of liver mitochondria. *J. Cell Biol.* 32:415–437.

24. Swanson, M. A. 1955. Glucose-6-phosphatase from liver. *Methods Enzymol.* 2:541–543.

25. Hubscher, G., and G. R. West. 1965. Specific assays of some phosphatases in subcellular fractions of small intestinal mucosa. *Nature (Lond.).* 205:799–800.

26. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electro-phoresis. *J. Biol. Chem.* 252:1102–1106.

27. Touster, O., N. N. Aronson, Jr., J. T. Dulaney, and H. Hendrickson. 1970. Isolation of rat liver plasma membranes. Use of nucleotide pyrophosphatase and phosphodiesterase I as marker enzymes. *J. Cell Biol.* 47:604–618.

28. Howell, K. E., A. Ito, and G. E. Palade. 1978. Endoplasmic reticulum marker enzymes in Golgi fractions—what does this mean? *J. Cell Biol.* 79:581–589.

29. Sikstrom, R., J. Lanoix, and J. J. M. Bergeron. 1976. An enzymic analysis of a nuclear envelope fraction. *Biochim. Biophys. Acta.* 448:88–102.

30. Sherlock, S. 1975. Disease of the Liver and Biliary System. Fifth ed. Blackwell Scientific Publications, London.