Identification of Rat Hepatocyte Plasma Membrane Proteins Using Monoclonal Antibodies

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ABSTRACT We have localized and identified five rat hepatocyte plasma membrane proteins using hybridoma technology in combination with morphological and biochemical methods. Three different membrane preparations were used as immunogens: isolated hepatocytes, a preparation of plasma membrane sheets that contained all three recognizable surface domains of the intact hepatocyte (sinusoidal, lateral, and bile canalicular), and a glycoprotein subfraction of that plasma membrane preparation. We selected monoclonal IgGs that were hepatocyte specific and localized them using both immunofluorescence on 0.5-μm sections of frozen liver and immunoperoxidase at the ultrastructural level. One antigen (HA 4) was localized predominantly to the bile canalicular surface, whereas three (CE 9, HA 21, and HA 116) were localized predominantly to the lateral and sinusoidal surfaces. One antigen (HA 16) was present in all three domains. Only one antigen (HA 116) could be detected in intracellular structures both in the periphery of the cell and in the Golgi region. The antigens were all integral membrane proteins as judged by their stability to alkaline extraction and solubility in detergents. The apparent molecular weights of the antigens were established by immunoprecipitation and/or immunoblotting. In a related study (Bartles, J. R., L. T. Braiterman, and A. L. Hubbard, 1985, *J. Cell. Biol.*, 100:1126–1138), we present biochemical confirmation of the domain-specific localizations for two of the antigens, HA 4 and CE 9, and demonstrate their suitability as endogenous domain markers for monitoring the separation of bile canalicular and sinusoidal lateral membrane on sucrose density gradients.

Epithelial cells exhibit a striking polarity that reflects the different functions carried out at the different surfaces of the cell. The assumption that the functional differences among surface domains of such cells are matched by compositional differences has been generally confirmed (see references 8, 32, and 41). However, the extent of molecular restriction is still not known, because so few endogenous membrane molecules have been identified and definitively localized in epithelial cells. Furthermore, there is little information on the steady-state intracellular distributions of plasma membrane (PM)[1] molecules in this type of cell or on the cellular mechanisms of their surface expression.

The principal epithelial cell of the liver, the hepatocyte, has three functionally and morphologically distinct surface domains. The sinusoidal (*SF*) domain, specialized for exchange of metabolites with the blood, is characterized by irregular microvilli that extend into the space of Disse and by numerous coated pits. The lateral (*LS*) domain, which is contiguous to an LS domain of a neighboring hepatocyte, is specialized, at least in part, for cell attachments and cell-cell communication and thus is marked by junctional elements such as tight junctions, desmosomes, and gap junctions. The bile canalicular (*BC*) domain, which is separated from the LS domain by tight junctions, is specialized for bile secretion and is characterized by numerous microvilli.

We recently demonstrated by immunofluorescence and immunoelectron microscopy that leucine aminopeptidase, a membrane glycoprotein, was restricted to the BC domain of hepatocytes and thus could serve as a specific marker of this domain (30). We then used anti-leucine aminopeptidase antibody in an immunoadsorption approach to isolate mem-

[1] Abbreviations used in this paper: BC, bile canalicular; LS, lateral; Mabs, monoclonal antibodies; PBS, 0.15 M NaCl, 0.02 M NaP_i (pH 7.4); PBSa, PBS containing 0.02% (wt/vol) NaN_3; PFA, paraformaldehyde; PLP, 0.01 M Na-metaperiodate, 0.075 M lysine, 2% (wt/vol) paraformaldehyde; PM, plasma membrane; SF, sinusoidal; WGA, wheat germ agglutinin.

brane vesicles derived from the BC domain, and we partially characterized them (31). We have also reported that functional asialoglycoprotein receptors are enriched in the SF domain approximately 10-fold over their presence in the BC domain (16), a finding confirmed by other morphological approaches (25) (but see references 9 and 10). Thus it would appear from a few examples that the surface domains of this epithelial cell might be compositionally distinct.

To obtain more markers specific to the two major hepatocyte surface domains for use in domain isolation and biogenetic studies, we turned to hybridoma technology to generate antibodies to domain-specific antigens. We report here the identification of four such membrane proteins—one of which is localized predominantly to the BC domain and three to the SF and LS domains. A fifth protein appears to be present in all three domains. Portions of this work have been presented in abstract form (13).

MATERIALS AND METHODS

Materials

The following materials were obtained from the following sources: polyethylene glycol 4000 from E. Merck AG (Darmstadt, W. Germany); goat serum, lamb serum, rabbit typing sera for mouse IgG_1, IgG_{2A}, IgG_{2B}, IgG_3, IgM, and whole mouse Ig from Miles Laboratories Inc. (Elkhart, IN); goat anti-mouse $F(ab')_2$, rabbit anti-mouse and sheep anti-mouse IgGs (both rhodaminated), and rabbit anti-mouse IgG from Cappel Laboratories (Cochranville, PA); rhodaminated goat anti-rabbit IgG from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD); horseradish peroxidase-conjugated sheep anti-mouse Fab from Pasteur Productions (Marnes La Coquette, France); $Na^{125}I$ from Amersham Corp. (Arlington Heights, IL); Enzymobead radioiodination reagent from Bio-Rad Laboratories (Richmond CA); octyl-β-D-glucopyranoside from Calbiochem-Behring Corp. (San Diego, CA); agarose-bound wheat germ agglutinin (WGA) from Vector Laboratories, Inc. (Burlingame, CA); antipain from Peninsula Laboratories, Inc. (Belmont, CA); leupeptin from Vega Biochemicals (Tucson, AZ); and Immulon 1 Removawell strips from Dynatech Laboratories (Alexandria, VA). The sources of other reagents used in monoclonal antibody production and characterization are listed by Kiehart et al. (19). All other materials were from suppliers reported earlier (16, 30, 31) or were reagent grade. BALB/c mice were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA) or Harlan Sprague Dawley, Inc. (Indianapolis, IN); CD Sprague-Dawley rats were from Charles River Breeding Laboratories, and rat embryo skin fibroblasts (CRL 12-13) were from American Type Culture Collection (Rockville, MD).

Immunogen Preparation and Immunization

Three different hepatocyte membrane preparations were used as immunogens. In each case, BALB/c mice were immunized and boosted twice at 2-wk intervals. The first immunogen consisted of viable rat hepatocytes isolated by collagenase dissociation of livers as previously described (43). The preparations were 90–95% viable as judged by trypan blue dye exclusion. $1.5–2 \times 10^6$ cells were injected each time without adjuvant. The second immunogen was a preparation of isolated PM sheets (16) that had been extracted twice with 0.025 M Na_2CO_3 (pH 11.3) to remove filaments (14). The third immunogen was a glycoprotein-enriched subfraction of the PM sheets, which was isolated from 0.5% (vol/vol) Triton X-100 extracts of the alkaline-treated PM sheets by affinity chromatography on WGA-agarose. The 0.2 M *N*-acetylglucosamine eluate was then depleted of the highly immunogenic antigen HA 4 by incubation with anti-HA 4 monoclonal IgG-Sepharose 4B (23). Both the second and third immunogens were emulsified in complete Freund's adjuvant for the primary injection and in incomplete adjuvant for the booster injections (70–75 μg of protein/injection).

Production and Purification of Monoclonal Antibodies (Mabs)

3–4 d after the second boost, spleen cells were fused with mouse myeloma cells, P3X63Ag8U1 (P3U1, reference 42). The protocol described by Unkeless (35) was followed, except that erythrocytes were lysed, polyethylene glycol 4000 was used, and hypoxanthine-aminopterin-thymidine selection was performed according to the methods of Kiehart et al. (19). Hybridoma cell lines were maintained in 70% (vol/vol) Dulbecco's modified Eagle's medium, 10% (vol/vol) Medium NCTC 135, and 20% (vol/vol) fetal bovine serum supplemented as described (19). Culture supernates were screened for antibody 2–3 wk after the fusion (see PM BINDING ASSAY below). Hybridoma cells from wells whose supernates gave signals more than twice that of P3U1 cells were expanded and cloned once or twice (35). The culture supernates of the expanded clones were screened for anti-PM activity. The antibody isotypes were determined using the PM binding assay described below.

Pristane-primed BALB/c mice that had been irradiated with 400 rad (^{131}Cs, 128 rad/min) were used for ascites production. The ascites was heat-inactivated, clarified by centrifugation, and either stored at 4°C with 3 mM NaN_3 or dialyzed versus 5–10 mM KP_i (pH 8.0) and applied to a Whatman DE52 column (2 × 10 cm) (Whatman Chemical Separation Inc., Clifton, NJ). The bound IgG was eluted using a 180-ml gradient of 5–10 mM to 75 mM KP_i (pH 8.0) (40). Due to the low level of IgG in the P3U1-induced ascites, IgG could only be obtained by 50% ammonium sulfate precipitation (19).

Initial Screening

PM BINDING ASSAY: This solid-phase binding assay is based on that of Kiehart et al. (19). 2.5 μg of hepatocyte PM in 10 mM imidazole-HCl (pH 7.4) was dried onto the bottom of individual microtiter wells, which were subsequently rinsed in a buffer containing 0.1% (wt/vol) bovine serum albumin (BSA), 0.02% (vol/vol) Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, 0.02% (wt/vol) NaN_3 (pH 7.7) (TTX-BSA). Culture supernates or ascites, diluted in PBSa (0.15 M NaCl, 0.02 M NaP_i, 0.02% [wt/vol] NaN_3 [pH 7.4]) containing 1% (wt/vol) BSA, were applied to the wells, incubated 12–17 h at 4°C, rinsed with TTX-BSA and then with this same rinse buffer without Triton X-100. ^{125}I-Goat-anti-mouse $F(ab')_2$ in 1% (wt/vol) BSA-PBSa was applied for 1 h at 4°C and rinsed as before. The radioactivity in the wells was measured in a Beckman 400 gamma counter (Beckman Instruments, Inc., Palo Alto, CA).

DETERMINATION OF ANTIBODY ISOTYPE: After incubation of culture supernates in PM-coated microtiter wells as described above, one of six different rabbit anti-mouse Ig types (see *Materials* above) was applied to a well followed by ^{125}I-Protein A, each for 1 h at room temperature. Processing was similar to the PM binding assay described above.

Indirect Immunofluorescence Localization

LIVER: In early experiments, the fixation of livers in 4% (wt/vol) paraformaldehyde (PFA)-0.1 M NaP_i (pH 7.4), and the processing and immunostaining of 5-μm and 0.5-μm frozen sections were performed as described (30), with minor modifications. For screening purposes on 5-μm sections, 50 μl of undiluted culture supernate was used as the first antibody. An IgG fraction of the hybridoma ascites or P3U1 ascites diluted in 0.2% (wt/vol) gelatin-PBSa was used on the 0.5-μm sections. The second antibody was either goat, rabbit, or sheep anti-mouse, all rhodaminated. They were adsorbed 1–3 times against fixed-quenched liver homogenates (30) and diluted in PBSa containing 10% (vol/vol) goat, rabbit, or sheep whole serum for 5-μm sections and in 0.2% (wt/vol) gelatin-PBSa for 0.5-μm sections. More recently, we have found that perfusion with 0.01 M Na-metaperiodate, 0.075 M lysine, 2% (wt/vol) PFA (PLP) for 5 min in situ (27), followed by immersion in the same fixative for only 5 h at 4°C, increased the specific signal of all the antigens. In addition, 0.5-μm sections from these livers were not postfixed twice with 3% (wt/vol) PFA before antibody staining. We also adopted an enhancement labeling protocol in later experiments. After incubation with Mabs, the sections (0.5- or 5-μm) were first incubated with rabbit anti-mouse IgG (15–30 min) and then incubated with rhodaminated, affinity-purified goat anti-rabbit IgG (15 min). Both second and third antibodies were preadsorbed on liver homogenates and diluted as described above. Sections were examined using either a Leitz or Zeiss fluorescent microscope, and photographs were taken as described (30).

OTHER TISSUES: Rat small intestines were removed after fixation by cardiac perfusion for 5 min and further fixed by immersion in 4% (wt/vol) PFA for 3 h at 4°C. Rat kidneys and pancreas were removed and fixed by immersion for 4 h at 4°C. These tissues were frozen, and 5-μm sections prepared and stained as described above.

ISOLATED HEPATOCYTES: Isolated hepatocytes were fixed in PLP for 30 min at 4°C and quenched with 0.013 M $NaBH_4$ in 0.1 M Tris-HCl (pH 7.4) for 10 min at room temperature. Labeling with primary and secondary antibodies was performed for 30 min at 37°C in 10% (vol/vol) serum in PBS (0.15 M NaCl, 0.02 M NaPi [pH 7.4]), and the cells were examined in buffered glycerol (30).

RAT EMBRYO FIBROBLASTS: Rat embryo fibroblasts were maintained as recommended by the American Type Culture Collection. Subconfluent monolayers were washed with PBS and fixed in 2% (wt/vol) PFA-0.1 M NaP_i (pH 7.4) for 45 min at 4°C. The plates were quenched and labeled essentially as described for hepatocytes above.

Immunoperoxidase Localization

The immunoperoxidase labeling method recently described by Brown and Farquhar (4) was followed. Concentrations of monoclonal IgG ranging from 20 to 180 μg/ml were used, and the second antibody was a horseradish peroxidase conjugate of sheep-anti-mouse Fab.

Identification of PM Antigens

IODINATION OF PM VESICLES: PM sheets (2 mg/ml of protein in 0.25 M sucrose) were mixed gently with an equal volume of 0.05 M Na_2CO_3 (pH 11) and centrifuged at 1,500 *g* for 15 min. The pellet was resuspended in PBS containing 2% (wt/vol) glucose to a final protein concentration of 1 mg/ml and sonicated in an ice bath until sheets were no longer visible by phase-contrast microscopy (usually ten 10-s bursts). The preparation was then centrifuged at 1,500 *g* for 15 min, the supernate (containing PM vesicles) was removed, and ~300 μg of PM vesicle protein was iodinated using Enzymobeads and 2 mCi of carrier-free $Na^{125}I$, according to the manufacturer's directions. The reaction was terminated by sedimentation of the beads, and the supernate (containing ^{125}I-PM vesicles) was diluted with an equal volume of PBS containing protease inhibitors (200 U/ml of Trasylol, 2 μg/ml of antipain and leupeptin, 2 mM benzamidine and 2 mM EDTA) and dialyzed against PBS containing 1 μM di-isopropylfluorophosphate for 4–6 h at 0°C. The ^{125}I-PM vesicles were sedimented at 100,000 *g* for 45 min, resuspended in RIPA buffer (1% [vol/vol] Triton X-100, 1% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] SDS, 0.15 M NaCl, and 0.05 M Tris-HCl [pH 7.2]) (see reference 5) containing protease inhibitors (100 U/ml of Trasylol, 1 μg/ml of antipain and leupeptin, 1 mM benzamidine and 1 mM EDTA), incubated in this buffer for 12–16 h at 4°C, and the resulting extract cleared by centrifugation at 100,000 *g* for 45 min.

IMMUNOPRECIPITATION: Extracts of ^{125}I-PM vesicles in RIPA buffer (~5 μg of protein and ~10^7 cpm) were incubated with Mab-Sepharose in a total volume of 400 μl of RIPA buffer or Buffer A (20 mM octyl-β-D-glucopyranoside, 0.5% [vol/vol] Triton X-100, 0.3 M NaCl, 0.025 M NaP_i [pH 7.4]) containing 0.5% (wt/vol) BSA at 4°C with continuous shaking for 1–2 h. After sedimentation (10,000 *g*, 1 min, Eppendorf 5412 microfuge), the beads were washed once in 0.5% (wt/vol) BSA-Buffer A, twice in Buffer A minus octylglucoside, and once in 0.15 M NaCl, 10 mM Tris-HCl (pH 7.2). The immunoprecipitates were directly solubilized in SDS and separated by electrophoresis on SDS polyacrylamide gels; the gels were dried and exposed to x-ray film at −70°C with intensifying screens.

Other Methods

SDS PAGE: Samples were prepared for electrophoresis by precipitation in 10% (wt/vol) trichloroacetic acid in the presence of 0.17% (wt/vol) sodium deoxycholate, 0.125% (vol/vol) Emulphogene BC-720 and 0.02% (wt/vol) SDS, followed by extraction of the resulting pellet in 90% (vol/vol) acetone-0.1 N HCl at −20°C. The final precipitate was reduced with 20 mM dithiothreitol, solubilized in 3.6% (wt/vol) SDS, boiled for 3–5 min, and alkylated with 36 mM iodoacetamide. Nonreduced samples were prepared by solubilization in 2% (wt/vol) SDS and incubation at 50°C for 10 min. The samples were then electrophoresed on 7.5% polyacrylamide slab gels prepared by the method of Maizel (22) as previously described (14).

IMMUNOBLOTS: Polypeptides separated on SDS gels were electrophoretically transferred to nitrocellulose according to the method of Towbin et al. (34). Antigens were detected as previously described (30), using either a one-step (^{125}I-labeled monoclonal IgG) or two-step (ascites and then ^{125}I-goat anti-mouse $F[ab']_2$) method with two modifications. Blot strips were quenched with 2% (wt/vol) gelatin in wash buffer prior to incubation with antibodies and rinsed three or four times for 3–5 min each in wash buffer after the incubations (i.e., no urea was included in the washes).

ASSAYS: Protein was determined by the modified Lowry method of Markwell et al. (24) using BSA as a standard.

IODINATION: Monoclonal antibodies and goat anti-mouse $F(ab')_2$ were iodinated using chloramine T (11). Tyrosine (1 mM) was used to stop the reaction, and the final specific radioactivities ranged from 3–7 × 10^6 cpm/μg. Protein A and WGA were iodinated as described previously (1, 30) with similar specific radioactivities.

RESULTS

Selection of Hybridomas

The three immunogens described above yielded 47 positive clones out of a total of 106 wells exhibiting growth. The solid-phase PM binding assay was used for all immunogens, and those clones whose signals were greater than twofold over that of P3U1 were selected for the second screen. For the initial immunofluorescence experiments on 5-μm liver sections, we selected only clones producing IgGs, due to the ease of handling this isotype. Eleven such clones gave immunofluorescence labeling patterns that appeared to be specific for one domain of the hepatocyte surface. The culture supernates from five of these clones labeled the SF domain, and the other six labeled the BC domain. Based on these initial results, we next characterized the antigens recognized by these eleven Mabs in additional immunofluorescence, ultrastructural localization, and biochemical studies.

Immunofluorescence Studies

The putative BC antigens, as exemplified by HA 4, gave a very distinctive immunofluorescence labeling pattern on 0.5-μm sections (Fig. 1). Small circular profiles and the edges of long channels were labeled. These regions corresponded under phase-contrast optics to clear pockets between adjacent hepatocytes. The labeling pattern observed with HA 4 was identical for all the other putative BC antigens and was similar to that observed for the BC antigen, leucine aminopeptidase (30). Neither the SF domain of hepatocytes nor surfaces of any sinusoidal lining cells were labeled with any of the BC Mabs. No intracellular labeling was apparent, even in sections from lightly-fixed (PLP) livers.

The putative SF antigen, CE 9, was present on both the SF and LS surfaces of the hepatocytes (Fig. 2, *a* and *b*). Neither the BC domain nor cells lining the sinusoids were labeled. In addition, no intracellular labeling was observed even in lightly-fixed (PLP) livers. HA 21 gave essentially the same localization, but it was much weaker than that of CE 9 and was only observed on sections from PLP-fixed livers. HA 116, another SF antigen, appeared to be localized predominantly to the SF domain of hepatocytes, not to the LS domain. In addition, intracellular labeling was clearly evident when livers fixed by the PLP method were examined (Fig. 2, *e* and *f*). The intracellular structures were present in phase-lucent zones around bile canaliculi and adjacent to nuclei, where Golgi apparatus, lysosomes, and endosomes are located. Anti-HA 107 gave an identical pattern to that of anti-HA 116. The immunofluorescence localization of HA 16 was the most variable of the SF Mabs and was detected only on 0.5-μm sections from PLP-fixed livers (Fig. 2, *c* and *d*). In five livers examined using anti-HA 16, the SF and LS domains were labeled. In three of those livers, labeling of the BC domain was also observed. Sinusoidal lining cells also appeared to be labeled with anti-HA 16 Mab (Fig. 2*c*) as were other cells in portal triad regions (data not shown).

When livers were dissociated with collagenase and isolated hepatocytes were fixed and labeled, positive specific labeling was observed with each Mab (data not shown). Thus, all the epitopes appeared to be externally oriented, since no permeabilization was needed to obtain specific labeling.

We also examined by immunofluorescence other rat epithelia to determine whether our BC antigens were present on analogous surfaces in these cells. Two BC Mabs (anti-HA 2 and anti-HA 4) reacted with the brush border (apical surface) of epithelial cells in the small intestine but not with either pancreas or kidney epithelia. The signal-to-noise ratios observed in these same epithelia using the Mabs directed against putative SF antigens were too low to allow any conclusions to be drawn. Finally, anti-HA 16 but not anti-HA 4 was found to label rat embryo skin fibroblasts (data not shown).

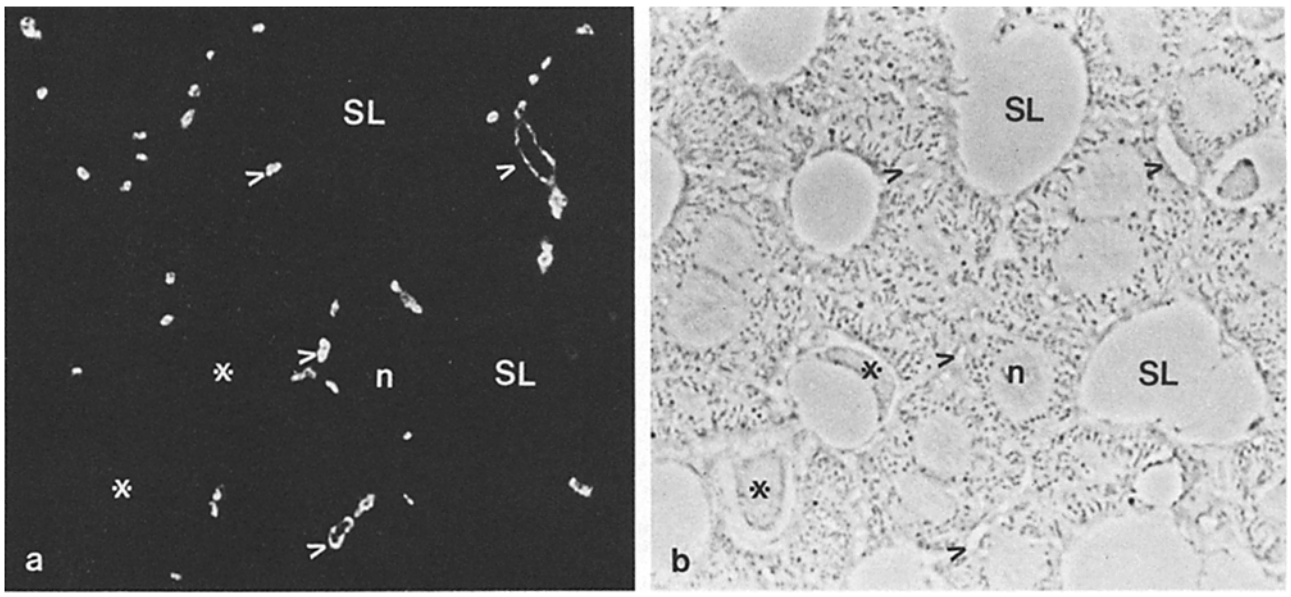

FIGURE 1 Immunofluorescence localization of HA 4, a BC antigen. 0.5-μm sections from 4% (wt/vol) PFA-fixed liver were labeled by sequential incubation with anti-HA 4 ascites, rabbit anti-mouse IgG, and then rhodaminated goat anti-rabbit IgG; the sections were then viewed with fluorescence (*a*) and phase-contrast (*b*) optics. Labeling is restricted to small circular profiles and the edges of channels between adjacent hepatocytes (>). These are bile canaliculi. There is no positive labeling of sinusoidal lining cells (*), hepatocyte membrane facing the sinusoidal lumen (*SL*), or intracellular structures. *n*, Nucleus. × 1,000.

Ultrastructural Localization of the Hepatocyte Antigens

The patterns observed with our Mabs at the light microscopic level using immunofluorescence were confirmed at the ultrastructural level using immunoperoxidase. The BC antigen, HA 4 (Fig. 3*a*), was almost entirely restricted to the BC domain and was randomly distributed throughout this domain. Very light reaction product was occasionally evident along the LS and SF membranes, but the labeling intensity was much lower than that at the BC membrane. There was virtually no intracellular HA 4, even when the cryostat sections were incubated with fivefold higher concentrations of anti-HA 4 Mab. The apical surfaces of bile duct epithelial cells were not labeled (data not shown).

A surface distribution reciprocal to that of HA 4 was observed for CE 9 (Fig. 3, *b* and *c*). That is, this antigen appeared entirely restricted to the SF and LS domains and was uniformly distributed within each. There was no evidence of CE 9 either along the BC membrane or in intracellular sites. However, CE 9 did appear to be present in the basolateral membrane of bile duct epithelia (data not shown).

The labeling intensity of HA 16 using immunoperoxidase was weaker than that of either of the above antigens (Fig. 3*d*) and was variable from region to region. However, in a majority of the cells examined, the antigen appeared to be present in all three surface domains—BC, SF and LS. There was no concentration of antigen within any particular region of a given domain. Small vesicles near bile canaliculi were occasionally labeled.

Another SF antigen, HA 116, was present along the SF membrane (Fig. 3*e*) and appeared to be concentrated in pits (Fig. 4), whose size and location at the base of microvilli along the SF domain correspond to coated pits (37). At the electron microscopic level, using immunoperoxidase, the antigen could be detected along the LS membrane. However, it was absent from the BC domain. In accordance with the immunofluorescence results, HA 116 had several intracellular locations which could be identified at the electron microscopic level (Fig. 4). HA 116 was found in the peripheral cytoplasm in small vesicles and tubular structures, and in the Golgi region in larger vesicles and vacuoles. It was absent from bonafide stacks of Golgi but seemed to be preferentially on one side of the stacks, in more dilated structures.

Finally, HA 21 was detected along the SF membrane with immunoperoxidase, but the signal was sufficiently low to preclude comments about other ultrastructural localizations of the antigen.

Identification and Characterization of the Hepatocyte Domain-specific Antigens

IDENTIFICATION BY IMMUNOBLOTTING: Each of the Mabs was tested for its reactivity with PM polypeptides that had been separated by SDS PAGE and then transferred onto nitrocellulose. Seven of the Mabs recognized denatured PM polypeptides; four did not. The BC antigen recognized by all six of the BC Mabs was a molecule of M_r 113,000 ± 4,000 (Fig. 5). This antigen (which we call HA 4, since it is the IgG from this Mab-producing clone that we have routinely used) consisted of a broad band centered at M_r 110,000, but spanning ~15,000 M_r units, and a narrow band of slightly slower electrophoretic mobility. The CE 9 antigen, which by morphological techniques was entirely restricted to the SF and LS domains, exhibited an M_r of ~39,000. The M_r assignment of CE 9 is tentative, since it reacted with antibody only when the solubilized PMs were not reduced before electrophoresis. None of the antigens recognized on immunoblots appeared to correspond to major Coomassie Blue-staining polypeptides on gels (Fig. 5) or blots.

IMMUNOPRECIPITATION: The identification of the four remaining PM antigens recognized by our Mabs was

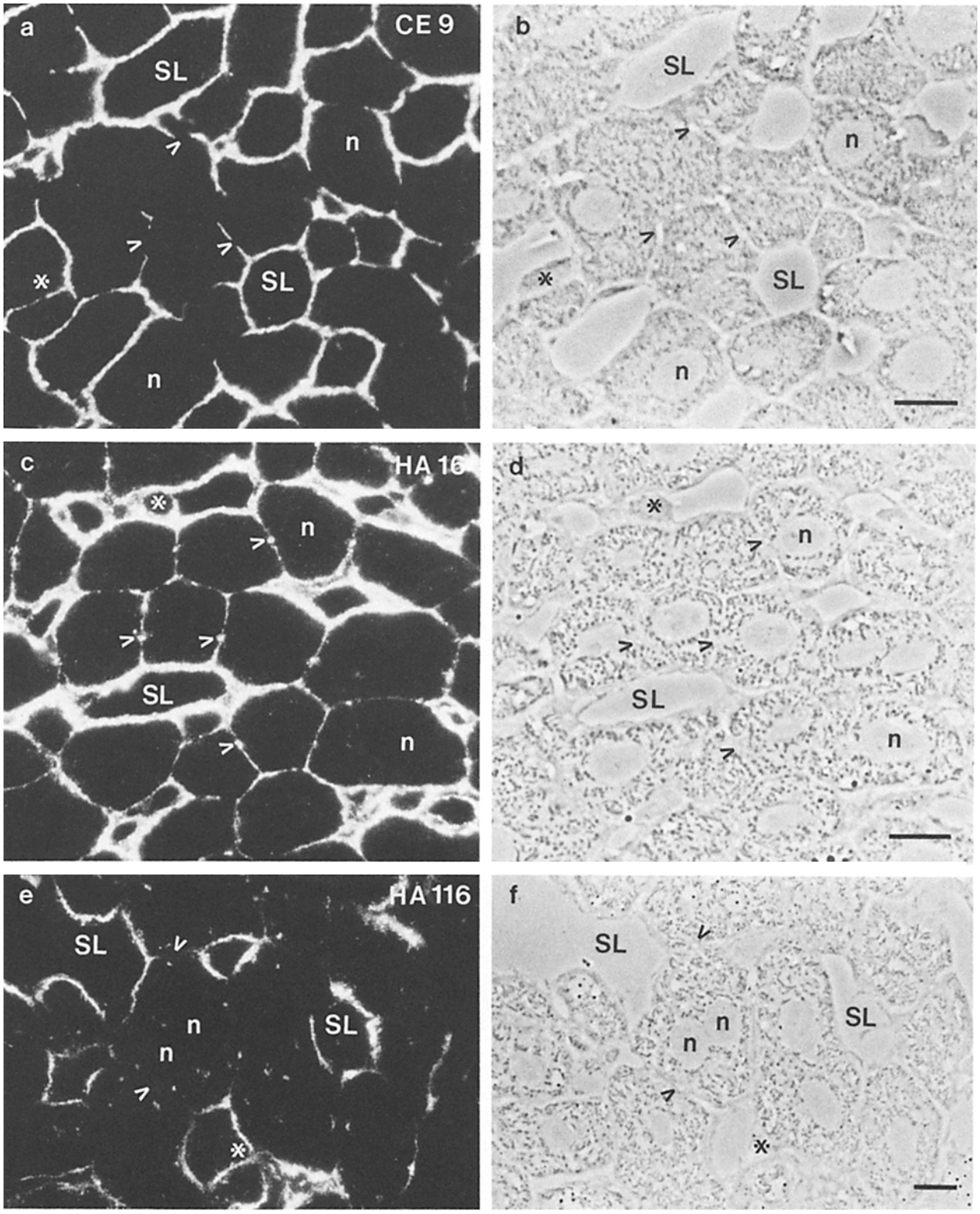

FIGURE 2 Immunofluorescence localization of three putative SF antigens. 0.5-μm sections from livers fixed in 4% (wt/vol) PFA (CE 9) or PLP (HA 16 and HA 116) were labeled as described in Fig. 1 and viewed with fluorescence (*left column*) and phase-contrast (*right column*) optics. (*a and b*) CE 9 appears to be located exclusively along the SF and LS domains of hepatocytes. It is absent from the BC domain (>), sinusoidal lining cells (*), and intracellular structures. *n*, Nucleus; *SL*, sinusoidal lumen. (*c and d*) HA 16 is present in all surface domains of the hepatocyte. It is also associated with cells (*) lining the sinusoidal lumen. Positively labeled structures are occasionally seen in the cytoplasm of hepatocytes. >, Bile canaliculi. (*e and f*) HA 116 exhibits a punctate appearance along the SF domain of hepatocytes. It appears to be absent from both the *BC* (>) and LS domains, but is present in intracellular structures near canaliculi. Bars, 10 μm.

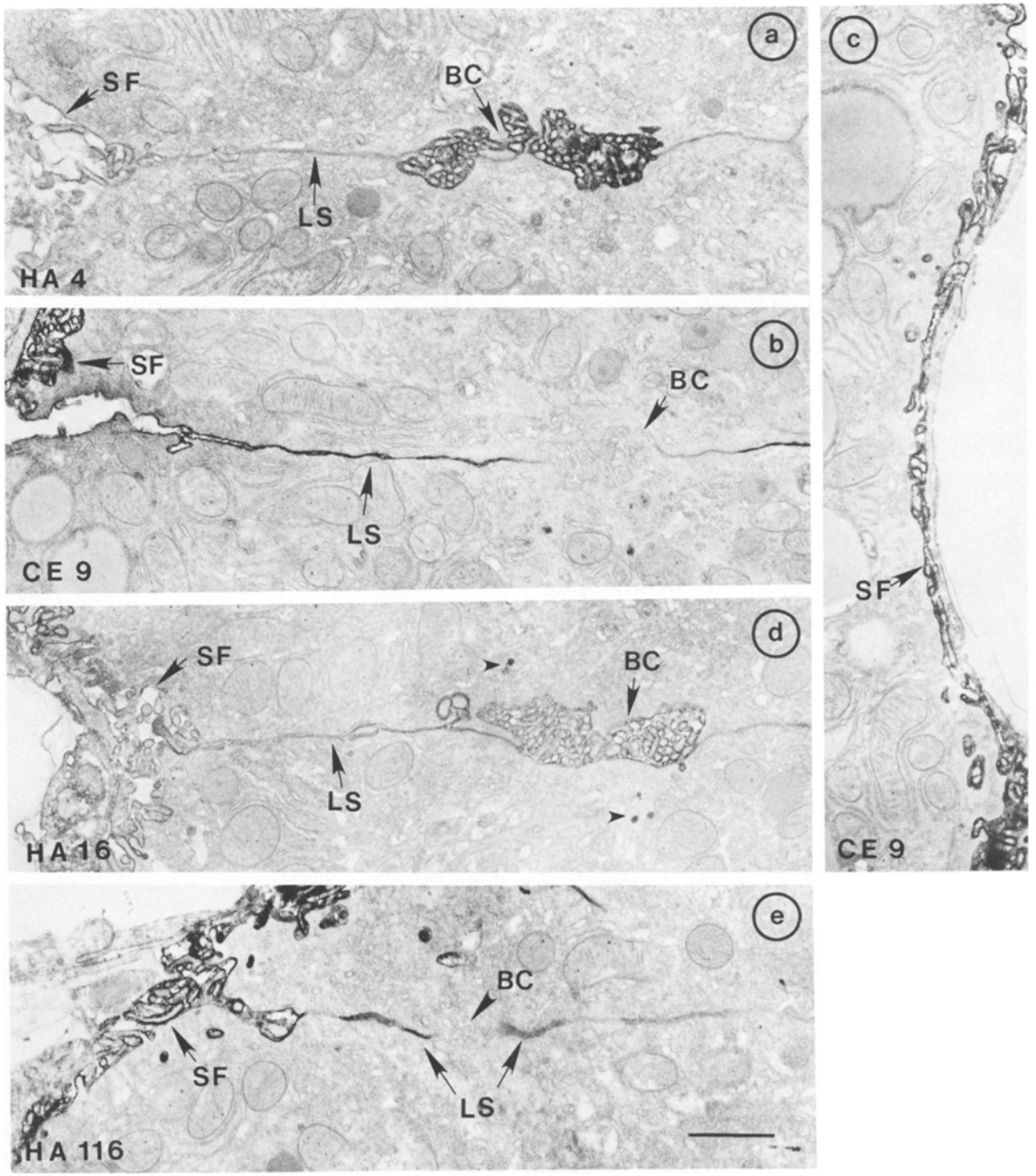

FIGURE 3 Immunoperoxidase localization of four hepatocyte PM antigens in situ. Livers were fixed, frozen, sectioned, and incubated with first and second antibodies as described in Materials and Methods. The second antibody, sheep anti-mouse Fab conjugated to horseradish peroxidase, was then visualized using diaminobenzidene, and the tissue was processed for electron microscopy. The localizations observed with immunofluorescence (Figs. 1 and 2) were confirmed at the ultrastructural level. (*a*) HA 4 is highly concentrated in the BC membrane, with very low (but detectable) levels along the *LS* and *SF* membranes, which may be due to diffusion of the strong reaction product in the BC. (*b and c*) CE 9 is present in both the *LS* and *SF* membranes but appears to be totally absent from the *BC*. (*d*) HA 16 is found in all domains (*SF*, *LS*, and *BC*) with perhaps a slight concentration in the *BC* membrane. (*e*) HA 116 is distributed at the cell surface in both the *LS* and *SF* domains and is absent from the *BC* domains. However, it is also present in intracellular structures (see Fig. 4). Lysosomes near the *BC* domain (e.g., small arrowheads in *d*) often contain lipid inclusions that stain with OsO_4, but do not represent specific reaction product. Bar, 1 μm. × 14,000.

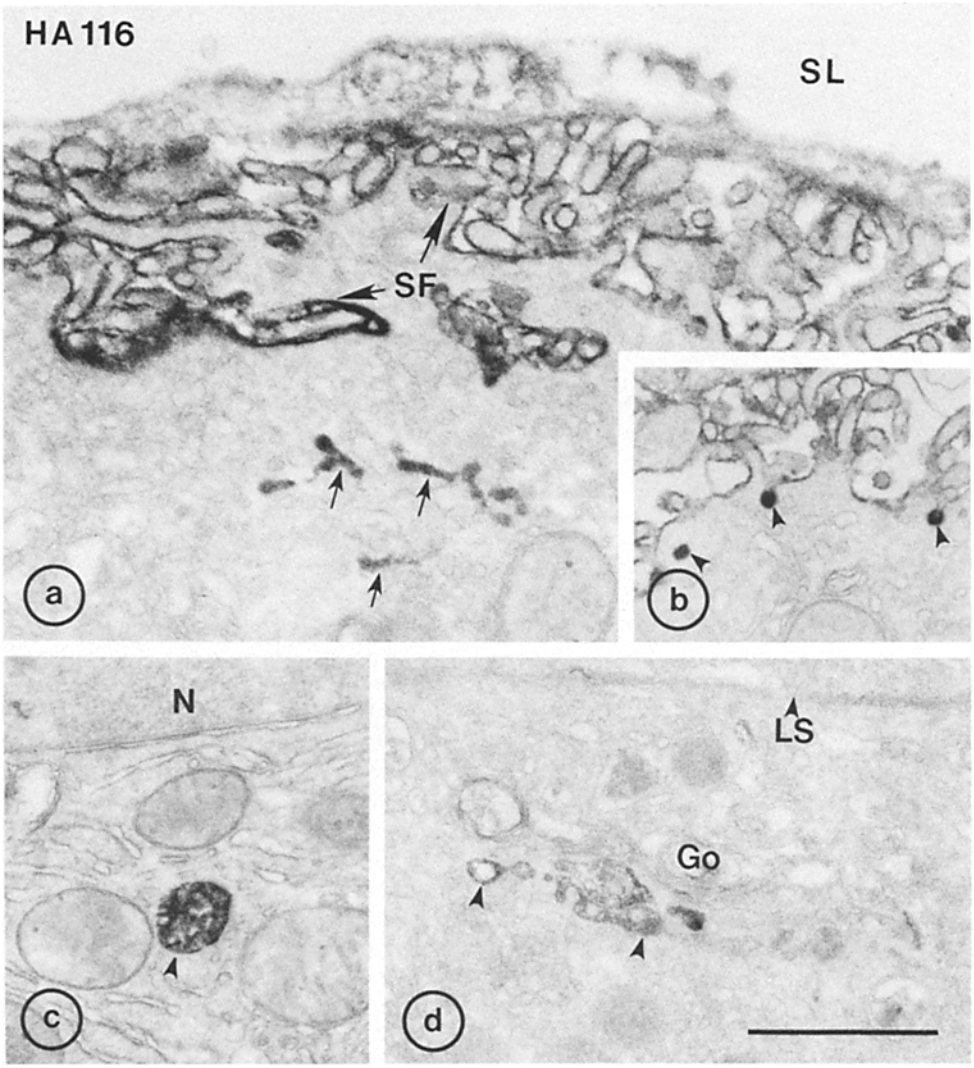

FIGURE 4 Immunoperoxidase localization of the SF antigen, HA 116. Rat livers were processed as described in Fig. 3. (*a*) Dense reaction product can be seen all along the *SF* domain and also in small tubules and vesicles in the peripheral cytoplasm. (*b*) Small pits (arrowheads) of ~1,000 Å diameter at the base of the *SF* membrane appear to contain more reaction product than adjacent *SF* membranes. (*c and d*) Reaction product can also be seen in structures in the interior of the cell (arrowheads). (*c*) Vesicles larger than those seen in the periphery appear to contain HA 116. The reaction product fills the lumen. *N*, Nucleus. (*d*) Reaction product is also found quite frequently in the region of the Golgi complex (*Go*). *LS*, lateral surface. In the absence of a second marker (e.g., thiamine pyrophosphatase, galactosyl transferase, an endocytosed ligand) it is impossible to identify the HA 116–positive compartment. Bar, 1 μm. × 25,000.

accomplished by their immunoprecipitation from detergent-solubilized preparations of alkaline-extracted and iodinated PM (Fig. 6). We found that all of the antigens were recovered predominantly in the sedimentable fraction after alkaline treatment, which suggests that they were integral membrane molecules (14). The BC antigen, HA 4, which we detected on immunoblots, was also readily identified by this method and exhibited the same appearance—that is, a broad band at M_r ~ 110,000 with a trailing shoulder (Fig. 6*A*). HA 16, the antigen localized to all three domains, was a doublet with bands at M_r's 90,000 ± 4,000 and 76,000 ± 4,000. These two ^{125}I-labeled bands were detected in a ~1.5:1 ratio in six separate PM preparations. HA 21, an SF antigen, was a single band with an apparent M_r of 85,000 ± 4,000. Because the electrophoretic mobilities of the major bands of HA 21 and 16 were very similar, we exposed the same ^{125}I-PM extract sample sequentially to anti-HA 16-Sepharose, then anti-HA 21-Sepharose (or vice versa). We found that the antigens were distinct. That is, immunoprecipitation of HA 21 did not alter the amount of HA 16 subsequently immunoprecipitated from the same sample. CE 9, a third SF antigen that we had identified on immunoblots of nonreduced samples, was difficult to detect after reduction because it migrated at M_r ~ 43,000, where nonspecific precipitates were found (see control, Fig. 6*A*). Anti-HA 116 and anti-HA 107 immunoprecipitates were identical and consisted of three ^{125}I-polypeptide bands migrating at M_r's 43,000, 52,000 and 64,000 after reduction (Fig. 6*C*). The middle band was the most prominent. All three bands were immunoprecipitated specifically, because excess soluble Mab during incubation effectively competed with Mab-Sepharose for all three bands (data not shown).

We found the mobilities of the immunoprecipitated antigens on SDS gels to be variously affected by omission of the

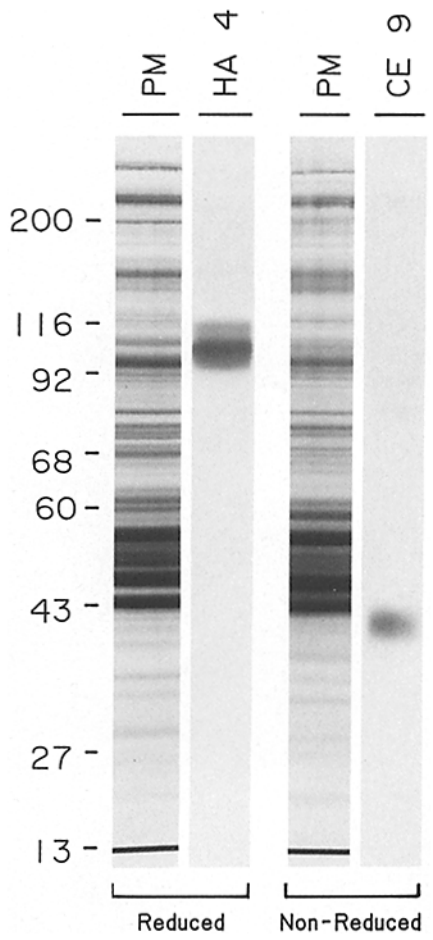

FIGURE 5 Identification of two PM antigens by immunoblot analysis. Isolated PM sheets were directly solubilized in SDS (with or without reduction-alkylation); the polypeptides were separated by SDS PAGE; one edge was cut and stained with Coomassie Blue (*left lanes*) and the remainder electrophoretically transferred to nitrocellulose. A strip from the reduced sample was incubated with ^{125}I-anti-HA 4 IgG and one from the nonreduced sample with anti-CE 9 IgG followed by ^{125}I-goat anti-mouse F(ab')$_2$. Numbers at left indicate approximate $M_r \times 10^{-3}$ based on comparison of the PM profiles with known standards.

reduction-alkylation steps (Fig. 6*B*). In general, all antigens electrophoresed more rapidly in the nonreduced state, but HA 4, HA 16, and HA 21 exhibited large M_r shifts in the reduced versus nonreduced states (~20,000), whereas CE 9 and HA 116 were less affected (~4,000).

DISCUSSION

In this report we have identified and localized five hepatocyte PM proteins using hybridoma technology in combination with morphological and biochemical methods. Table I summarizes our findings, several of which were unexpected, in light of current views concerning the surface and intracellular distributions of PM proteins.

Distribution of PM Proteins at the Hepatocyte Surface

Due to the distinct functions known to be carried out at the apical and basolateral surfaces of epithelial cells, it was expected that the molecular compositions of the two major surface domains would also be distinct. Results from early histochemical and cytochemical studies (see references 3 and 36), as well as those from more recent immunofluorescence work (see reference 21) tended to support this view. However, in the last two years, more detailed morphological examinations of the surface distributions of several membrane molecules (e.g., 5'-nucleotidase [reference 26], and the asialoglycoprotein receptor [references 9 and 10]) have been performed at the ultrastructural level. The results of many of these studies suggest that molecular restriction may not be complete.

By morphological criteria, most molecules we identified (i.e., CE 9, HA 4, HA 116, and HA 21) appear to be excluded from at least one surface domain. We can confidently assign an SF + LS location to the M_r 39,000 protein, CE 9. By immunofluorescence as well as the more sensitive immunoperoxidase method, we found no evidence for CE 9 in the BC domain. HA 21 and HA 116 also appear to be restricted to the SF and LS surface domains. The presence of these three antigens in both the SF and LS domains suggests that these two surfaces can be considered as one: the basolateral domain. Aside from the obvious surface specializations that form the lateral junctional complexes, the LS domain is in physical continuity with the SF domain and is most likely a functional equivalent. Conversely, HA 4 is highly concentrated in the BC domain, but we cannot exclude the possibility that it may be present to a much lesser extent in the other two, given the results using immunoperoxidase (see Fig. 3*a*).

The surface distribution of HA 16 was the biggest surprise, since it was found by morphological methods in all three domains. However, there are common morphologic features among the domains which could be matched by common membrane molecules. For example, both the BC and SF membranes have microvilli with abundant cytoplasmic actin filaments (see reference 7). It is possible that the same type of membrane molecule associates with such cytoplasmic assemblies in the different domains. However, these putative molecules have not yet been identified.

We found only one antigen, HA 116, to be concentrated in coated pits in the SF domain of hepatocytes. However, the antigen was also present elsewhere along the SF membrane. This distribution was very similar to that found by us for asialoglycoprotein binding sites (38). By peptide mapping and immunological cross-reaction, we have recently confirmed that the HA 116 antigen is indeed the asialoglycoprotein receptor.[2] The other SF antigens appeared to be more uniformly present both in this specialized region as well as along microvillar membrane. The antigen, HA 4, was also uniform in its distribution within the BC domain. However, potential artifacts due to diffusion of reaction product cannot be excluded at present. Therefore, we are also using particulate (gold) tracers to localize the antigens on isolated PM sheets to determine whether or not they are present in microdomains of the type recently described by Kerjaschki et al. (18) in kidney.

Our concern that one approach to protein localization (whether morphological or biochemical) could yield spurious results has prompted us to extend our morphological findings to biochemical analyses of PM subfractions. As described in the following article by Bartles et al. (2), we have already obtained confirmation of the domain-specific localization of

[2] Bartles, J. R., L. T. Braiterman, and A. L. Hubbard, manuscript in preparation.

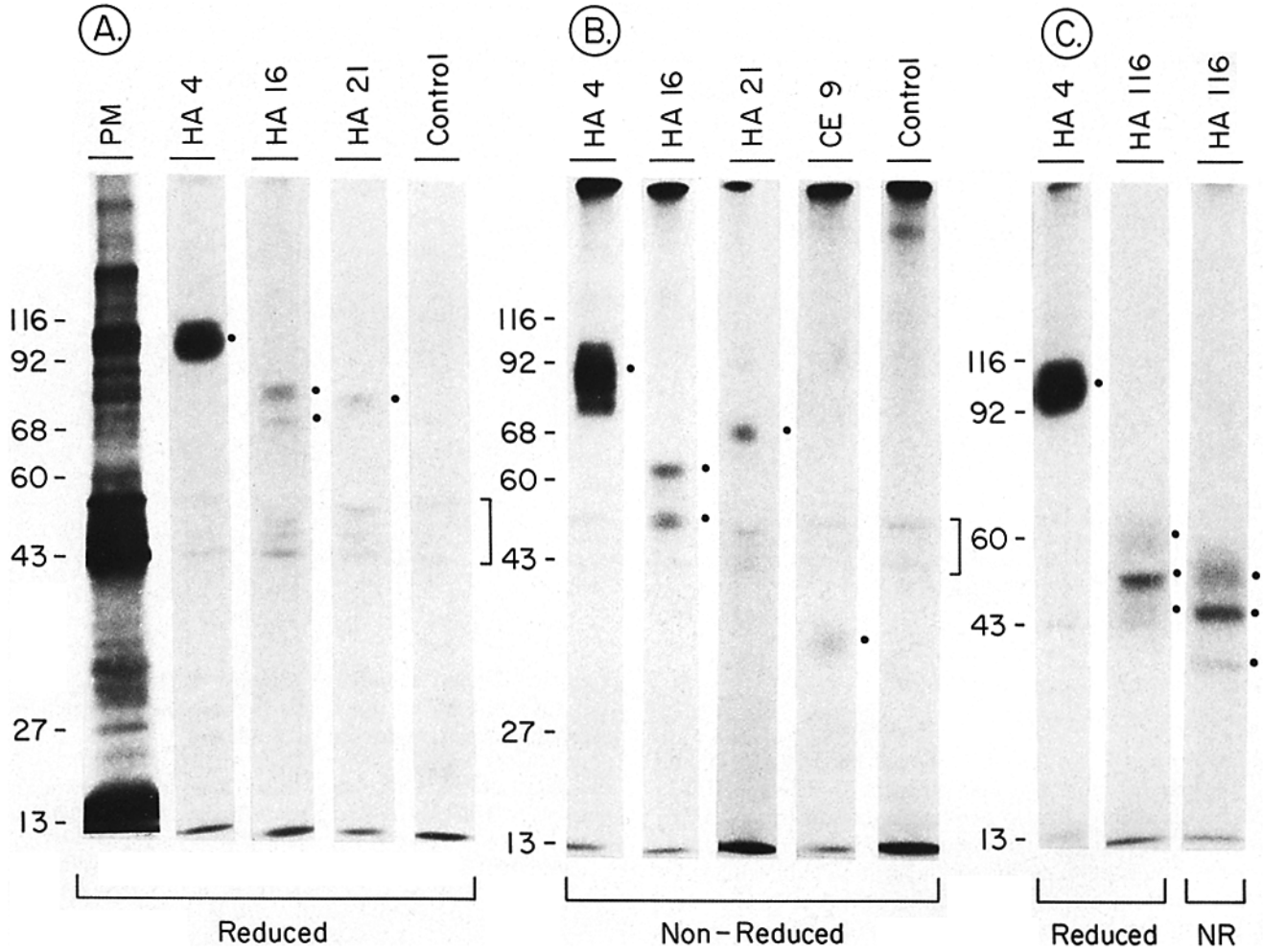

FIGURE 6 Identification of the PM antigens by immunoprecipitation and SDS PAGE. Detergent extracts of ^{125}I-PM were incubated with the indicated Mab-Sepharose; extracts were then rinsed, and the bound material was solubilized and resolved by SDS PAGE as described in Materials and Methods. Gels were dried, exposed to x-ray film, and the autoradiograms were developed. Labels at the top of each lane indicate the Mab-Sepharose used and the solid circles at the right of each lane indicate the ^{125}I-bands specifically immunoprecipitated. PM, Initial ^{125}I-PM extract at ~1% the amount used for the immunoprecipitations. Control, Protein A-Sepharose incubated with ^{125}I-PM. (*A*) All samples reduced before SDS PAGE. (*B*) All samples solubilized in SDS without reduction. Note the less efficient solubilization. (C) HA 116 either reduced or not reduced (*NR*) before electrophoresis. Numbers at left in each panel correspond to position of markers of known M_r ($\times 10^{-3}$). Brackets between M_rs of 43,000 and 60,000 in *A* and *B* indicate regions where nonspecific ^{125}I-contaminants migrated. Examination of the PM lane in *A* reveals the presence of large amounts of radioactivity in this region (residual filament proteins, see reference 14).

CE 9 and HA 4 and the multi-domain localization for HA 16.

The Intracellular Distribution of PM Proteins

The morphological finding that only one membrane protein, HA 116, could be detected inside the hepatocyte was somewhat unexpected. Recent reports have emphasized the presence of plasma membrane proteins inside cells (e.g., 5′-nucleotidase [references 33 and 39], and leucine aminopeptidase [reference 21]) and suggested that these pools comprise recycling molecules. Our morphological analysis of the steady-state distributions of HA 4 and CE 9 suggests that, if these two membrane proteins recycle, the cycling must either be fast or infrequent; otherwise, we would have expected more intracellular antigen. We are currently assessing the intracellular pools by biochemical means. The small amounts (~10%) we do detect in internal membrane compartments by immunoblotting (unpublished data) could represent the biosynthetic pools of these two membrane molecules. Of course, since we are using Mabs, we cannot rule out the possibility that some intracellular form of these antigens is present but not detectable with our reagents. For this reason, we are currently preparing polyclonal antibodies to each antigen.

Our morphological findings for HA 116, the asialoglycoprotein receptor,[2] are consistent with our own and others' results of subcellular fractionation. That is, there is a latent pool of receptor. We find HA 116 in dilated structures near stacks of Golgi apparatus and in structures in the peripheral cytoplasm that are morphologically similar to peripheral endosomes in these cells (37). The internal structures are not morphologically similar to internal endosomes (37), but fixation conditions used in the immunoperoxidase method do not preserve ultrastructural detail as well as those used in conventional peroxidase cytochemistry. Nonetheless, the intracellular distribution we observed for HA 116 is consistent with that reported by Geuze et al. (10) for the asialoglycoprotein receptor.

TABLE I

Characteristics of the Hepatocyte PM Antigens

Antigen	Mabs	Immunogen*	M_r‡	Distribution			
				Surface			
				BC	SF	LS	Intracellular
HA 4	anti-HA 4, HA 2; HA 112, HA 109; BC 5, EB 9	1, 2	113,000	+	−	−	−
HA 16	HA 16	1	90,000; 76,000	+	+	+	?§
HA 21	HA 21	1	85,000	−	+	+	?
CE 9	CE 9	3	39,000	−	+	+	−
HA 116	HA 116, HA 107	2	64,000; 52,000‖; 43,000	−	+	+	+

* 1, isolated hepatocytes; 2, alkaline-extracted PM; 3, glycoprotein subfraction of PM.
‡ Deduced by SDS PAGE. All M_r's were determined under reducing conditions, except that of CE 9.
§ Labeling at the hepatocyte surface was too weak to make any conclusions regarding the level of intracellular labeling.
‖ Major band (see Fig. 6).

Biochemical Characteristics of the Membrane Proteins

Two of our Mabs (anti-HA 16 and anti-HA 116) immunoprecipitated more than one polypeptide. The anti-HA 16 Mab immunoprecipitated two bands that differed in M_r by ~14,000 on SDS gels. The two polypeptides were not associated by disulfide bonds, since solubilization in the absence of reduction still yielded two bands after SDS PAGE. Therefore, it appears that these two molecules have at least one epitope in common. We have recently found the tryptic and chymotryptic peptide maps of these two bands to be virtually identical.[2] Three bands were immunoprecipitated by the anti-HA 116-Sepharose; the rat asialoglycoprotein receptor has already been shown to consist of three related polypeptides (12).

The BC antigen, HA 4, proved to be particularly immunogenic, generating many antibodies when either whole cells or isolated PM were used as immunogens (Table I). We have found that this molecule binds the lectin WGA in large amounts,[2] suggesting that it may be heavily sialylated (1). A number of rat liver PM proteins have been reported to have mobilities on SDS PAGE and domain localizations similar to those of HA 4 (6, 20, 28). However, with the exception of HA 116 (the asialoglycoprotein receptor),[2] none of the antigens we described have as yet been directly correlated with known hepatocyte plasma membrane proteins.

Methodology

The hybridoma approach proved to be quite useful in this study, since it allowed us to use heterogeneous immunogens and then a sensitive screening procedure to select the most interesting antigens for further study. A similar approach has been reported for another epithelial cell, the Madin-Darby canine kidney cell line (29), and for an unpolarized cell, the mouse fibroblast (17). To our knowledge, this is the first report of the generation of mouse monoclonal antibodies to rat hepatocyte membrane antigens. The availability of well-characterized subcellular fractions from liver now allows us to study the synthesis, processing, and sorting to the respective domains of these membrane proteins. Such a study is not currently possible in other cells because the corresponding subcellular fractions are not yet available.

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