Isolation of Rat Hepatocyte Plasma Membranes. II. Identification of Membrane-associated Cytoskeletal Proteins

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ABSTRACT Rat liver plasma membranes were isolated as presented in the preceding paper (Hubbard, A. L., D. A. Wall, and A. Ma., 1983, *J. Cell Biol.*, 96: 217–229) and found to contain many filaments associated both with desmosomes along the lateral surface and with the cytoplasmic aspects of membranes comprising each of the three domains (lateral [LS], bile canalicular [BC] and sinusoidal [SF]). Exposure of the plasma membranes to alkaline media (up to pH 11) resulted in loss of recognizable filaments without loss of domain morphology or membrane enzyme activities. Electrophoretic analysis of solubilized components from control and alkaline-extracted plasma membranes revealed that three major polypeptides present at 43, 52, and 56 kdaltons in the control had been released by alkaline treatment (pH 11) and could be quantitatively recovered in the supernate. The 43-kdalton component was identified as cytoplasmic actin by comparison of its tryptic ^{125}I-peptide map to those of muscle (α) and brush border (β, γ) actins. The 52- and 56-kdalton polypeptides were identified as tonofilament components by their solubility properties and their ability to reassemble into 9.5-nm filaments from monomers present in an alkaline extract.

In the preceding paper (1), we presented a method that yields rat liver plasma membrane (PM)[1] sheets containing each of the three domains in substantial amount. An important part of our initial characterization of this PM has been to identify molecular constituents of the preparation. In this regard we have succeeded in identifying three components of the liver cytoskeleton that are associated with the isolated plasma membrane. The first two are polypeptides that compose the intermediate filaments (tonofilaments) associated with desmosomes present in the lateral surface domain. The third is actin. A preliminary report of this work has been presented (2).

MATERIALS AND METHODS

Materials

Methylene bis acrylamide and glycine were purchased from Eastman Organic Chemicals Div., Eastman Kodak Co. (Rochester, NY); leupeptin from Vega-Fox Biochemicals Div., Newbery Energy Corp. (Tucson, AZ); Trasylol from Mobay Chem. Corp. (New York, NY); urea (ultrapure) and the molecular weight markers, bovine serum albumin, chymotrypsinogen, and cytochrome c, from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, NY); benzamidine, phosphorylase a, and 2-mercaptoethanol (2-ME) from Sigma Chemical Co. (St. Louis, MO); SDS from British Drug House (Poole, England); acrylamide from Bio-Rad Laboratories (Richmond, CA); ampholines from LKB (Bromma, Sweden); cellulose gel plates from Mallinckrodt Inc. (St. Louis, MO); L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone-trypsin (TPCK-trypsin) and catalase from Worthington Biochemical Corp. (Freehold, NJ); and antipain from Peninsula Laboratories, Inc., (San Carlos, CA). Other chemicals were purchased from the sources listed in the preceding paper (1) or were of reagent grade.

General

The preparation of liver PM sheets, enzyme assays, chemical determinations, and general microscopic procedures have been described (1).

Morphology

The glutaraldehyde fixation and processing described in the procedures of the preceding paper (1) were followed. However, when the effect of a particular extraction procedure on plasma membrane morphology was to be evaluated (e.g., high pH, see below), the PM suspension was treated, centrifuged, and the pellet then fixed without resuspension for 30–45 min by overlaying ice-cold 2% glutaraldehyde in 0.1 M Na cacodylate, pH 7.4. This protocol avoided possible changes in morphology caused by resuspension of fragile structures. The resulting pellet was processed as described (1).

Samples were negatively stained as follows: Formvar carbon-coated copper grids were inverted over a drop of sample (0.1–1 mg/ml) for 30–60 s, blotted with filter paper, stained for 30–60 s with a drop of 2% uranyl acetate, blotted, and examined in the EM.

[1] The abbreviations used in this article, in addition to those given in the preceding paper (1) are: 2-mercaptoethanol (1-ME), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), isoelectric focusing (IEF), bromphenol blue (BB), dithiothreitol (DTT).

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 JANUARY 1983 230-239

Analytical Methods

SDS PAGE: Linear gradients of polyacrylamide were prepared according to Laemmli (3) as previously described (4). Slab gels of dimensions 25 × 20 × 0.1 cm were used. Samples in volumes >25 μl were first concentrated by precipitation in deoxycholate-trichloroacetic acid (5), and the precipitates were solubilized in 40 mM Tris-PO_4, pH 6.9, 5 mM EDTA, 20 mM dithiothreitol, 4% SDS, and 0.005% bromophenol blue (BϕB). After boiling the samples for 5 min, iodoacetamide (to 40 mM) and sucrose (to 0.3 M) were added and the total sample volume was applied to a gel. Electrophoresis, staining with Coomassie Blue, and destaining were carried out as previously described (4, 6). Gels were scanned at 650 nm, using a spectrophotometer equipped with a linear transport (Gilford Instrument Laboratories Inc., Oberlin, OH). The percent of total Coomassie Blue-staining material present in a particular peak was determined by weighing a cut-out trace of the total gel scan and of the peak of interest and taking the ratio of their weights.

TWO-DIMENSIONAL ANALYSIS IN POLYACRYLAMIDE GELS: Samples were first subjected to iso-electric focusing (IEF) and then SDS PAGE. The IEF procedure of Ames and Nikaido (7) was followed with minor modifications. The samples were applied in 50-μg aliquots to 3.8% polyacrylamide tube gels (8 cm long × 4 mm diameter), and the gels were focused at 10°C in an IEF apparatus (Medical Research Associates, Clearwater, FL). After IEF, the gels were removed and equilibrated (7) for 30 min to 6 h at room temperature for second-dimension analysis in SDS PAGE. Gels were stored at −70°C for up to three months before SDS PAGE.

Equilibrated gels were embedded in molten 0.5% agarose on top of SDS polyacrylamide 1-mm slabs consisting of 3.75% spacer (6-cm long) and 10% resolving gels (16.5 cm). Following electrophoresis, gels were processed through 10% TCA and a solution of 25% 2-propanol: 10% acetic acid: 65% H_2O to remove the detergent and Ampholines (8) before being stained with 0.2% Coomassie Blue R as for SDS PAGE.

PEPTIDE MAP ANALYSIS: Polypeptides previously separated on one-dimensional (SDS PAGE) or two-dimensional (IEF then SDS PAGE) gels were excised, iodinated, and digested with TPCK-trypsin as described by Elder et al. (9). The iodinated peptides (50,000–500,000 cpm) were separated on 10 × 10 cm cellulose plates (9) and the plates autoradiographed at −70°C using Kodak X-OMAT film and Cronex Lightning Plus intensifying screens.

Isolation of Cytoskeletal Proteins from Sources Other than PM

Desmin, the major component of intermediate filaments from chicken gizzard, was isolated according to Franke et al. (10). *Vimentin* was isolated from human skin fibroblasts following the method of Osborn and Weber (11). *Muscle actin (α)* was prepared from an acetone powder of rabbit skeletal muscle (12). Several preparations were provided by J. Cooper. *Intestinal brush border actin (β, γ)* was prepared from chickens (13) and provided by Dr. S. Craig. *Tubulin* from hog brain (14) was provided by Dr. D. Murphy.

Selective Extraction of the Plasma Membrane

ALKALINE EXTRACTION: Aliquots of PM in 0.25 M sucrose were mixed with sufficient 1 N NaOH to achieve a pH of 8, 9, 10, or 11. Alternatively, 50 mM $NaHCO_3/CO_3$, at a specified pH (pH 8–11), was mixed with an equal volume of PM (1–2 mg/ml). After 5–60 min on ice, the suspension was centrifuged at 106,500 g_{av} for 30 or 60 min (40,000 rpm, SW 50.1 or 50 Ti rotors, Beckman Instruments, Inc., Palo Alto, CA), and the supernate and pellet were separated and saved for subsequent analysis.

DETERGENT EXTRACTION: 4–5 volumes of 1–2% Triton X-100 in 0.15 M NaCl, 20 mM PO_4, pH 7.4, and 5 mM EDTA (15) were added to 1 volume of PM (1–2 mg/ml) in 0.25 M sucrose. The suspension was centrifuged at 106,500 g_{av} for 60 min and the supernate and pellet were analyzed.

Reassembly of Filaments from Components Extracted from the PM

Freshly isolated PM (3–6 mg/ml) was extracted at pH 9 or 11 as described above. After centrifugation at 106,500 g_{av} for 30–60 min (50 Ti rotor, Beckman Instruments, Inc.), the supernate was dialyzed overnight at 4°C against 100 volumes of 50 mM Tris-HCl, pH 7.6, and 50 mM 2-ME. Modifications of this basic procedure are described in Results. After an aliquot of the dialyzed material was removed for morphologic examination by negative staining, the remainder was centrifuged at 106,500 g_{av} for 60–120 min (50 Ti rotor) and the supernate and pellet were analyzed.

RESULTS

In the preceding paper, we characterized in detail the biochemistry and morphology of our highly enriched hepatocyte PM preparation (1). One striking morphologic feature of the PM sheets was the abundance of filaments, some associated with the desmosomes and others forming filamentous networks along the cytoplasmic aspect of the SF membranes and the basal portion of the BC membrane (Figs. 2 and 3 in the preceding paper and Fig. 1 *a* and *b*, this paper). In addition, numerous small vesicles, some with ribosomes attached, were often associated with the filaments. In this section we first describe conditions that disassemble the PM-associated filaments and demonstrate that the integrity of the PM sheets is maintained. We then identify the three major components of the filaments.

Selective Extraction of the PM Filaments

Likely candidates for the filaments in our PM preparation were actin filaments and/or tonofilaments, the latter being a class of intermediate filaments associated with desmosomes in epithelial cells. Both have been localized to hepatocytes *in situ* (e.g. 16, 17, 18). Therefore, we chose a limited number of conditions reported to extract either one or both types of filaments and examined their effect on the morphology and biochemistry of the PM. The following conditions reported to depolymerize F actin were not successful in removing the filaments: (*a*) 0.6 M KI, 1 mM ATP, 1 mM DTT, 20 mM Tris-HCl, pH 7.2 (see reference 20); or (*b*) 2 mM Tris-HCl, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, pH 7.4 (12). Of the conditions tested, only extremes of pH released significant amounts of protein from the PM and/or showed selective extraction of PM proteins. However, incubation of the PM at acid pH's led to aggregation of membranes and partial inactivation of several enzymes; therefore, only the alkaline treatment was examined in greater detail.

Separate aliquots of PM were exposed to alkaline media (pH 8–11) and the resulting supernates and pellets analyzed for the presence of protein and several enzyme activities. The extremes (control and pH 11) are presented in Table I, where it can be seen that 40% of the total protein was released by pH-11 treatment. However, >95% of each of three plasma membrane enzymes remained sedimentable. Furthermore, when the alkaline-treated PM suspension was centrifuged at lower *g* forces (e. g. 1,500 *g* 10 min), a similar distribution of these markers (data not shown), plus that of the ASGP receptor (Table I), was observed. This result suggested that no extensive vesiculation of either BC or SF domain membrane had occurred. The selective extraction of non-enzymatic protein increased the specific activities of APDE I, 5′-nucleotidase, and the ASGP receptor by 1.5-fold over untreated PM (Table I), raising the final relative enrichment of these enzymes in pH 11-extracted PM to ~60-fold over the starting homogenate. (See the preceding paper [1] for documentation of the relative enrichments in these enzyme activities from homogenate to PM.) There was no selective release of the ER marker; therefore, alkaline extraction did not reduce the relative contamination of this organelle.

A striking difference was observed when the morphology of the pellet from PM extracted at pH 11 was compared to that from control PM; the filaments associated with desmosomes, as well as the network present along the cytoplasmic surface of the SF membrane, had been lost to a substantial extent by the

TABLE I

Effect of pH and Detergent on the Distribution of Protein and Enzyme Activities in the PM Fraction *

Treatment	Protein	Alkaline phosphodiesterase I	5′-Nucleotidase	Alkaline phosphatase	ASGP receptor‡	NADH-Cytochrome c reductase
			Per cent in Pellet			
None (H_2O)	92.5	99.5	99	98.5	85	91
pH 11	60	99.2	99	96.2§	81	67
Tx-100	35	2	92	ND	ND	ND

* PM was incubated with detergent or at pH 11, as described in Materials and Methods, the suspension centrifuged at 100,000 g_{av} for 60 min, and the supernates and resuspended pellets assayed for the indicated activities. The values represent the per cent of total recovered activity found in the pellet. (Recoveries >85%).

‡ After the treatment indicated, the PM suspension was centrifuged at 1,500 *g* for 10 min and the resuspended pellet analyzed. The binding activity present in the pellets is expressed as percent of the initial activity in untreated PM.

§ 55% of the initial alkaline phosphatase activity was inactivated by exposure to alkaline conditions.

alkaline treatment (Fig. 1). However, the domain morphology was retained; that is, the BC and LS were easily identified in alkaline-treated PM, as were tight junctions (Fig. 1 *b* and *d*) and gap junctions (not shown). Remnants of desmosomes were evident due to the presence of amorphous material on the cytoplasmic surfaces of adjacent LS membrane. The SF domain appeared more vesiculated but was still recognizable; however, coated membrane regions were no longer evident (not shown). Finally, small vesicles remained associated with the LS membrane.

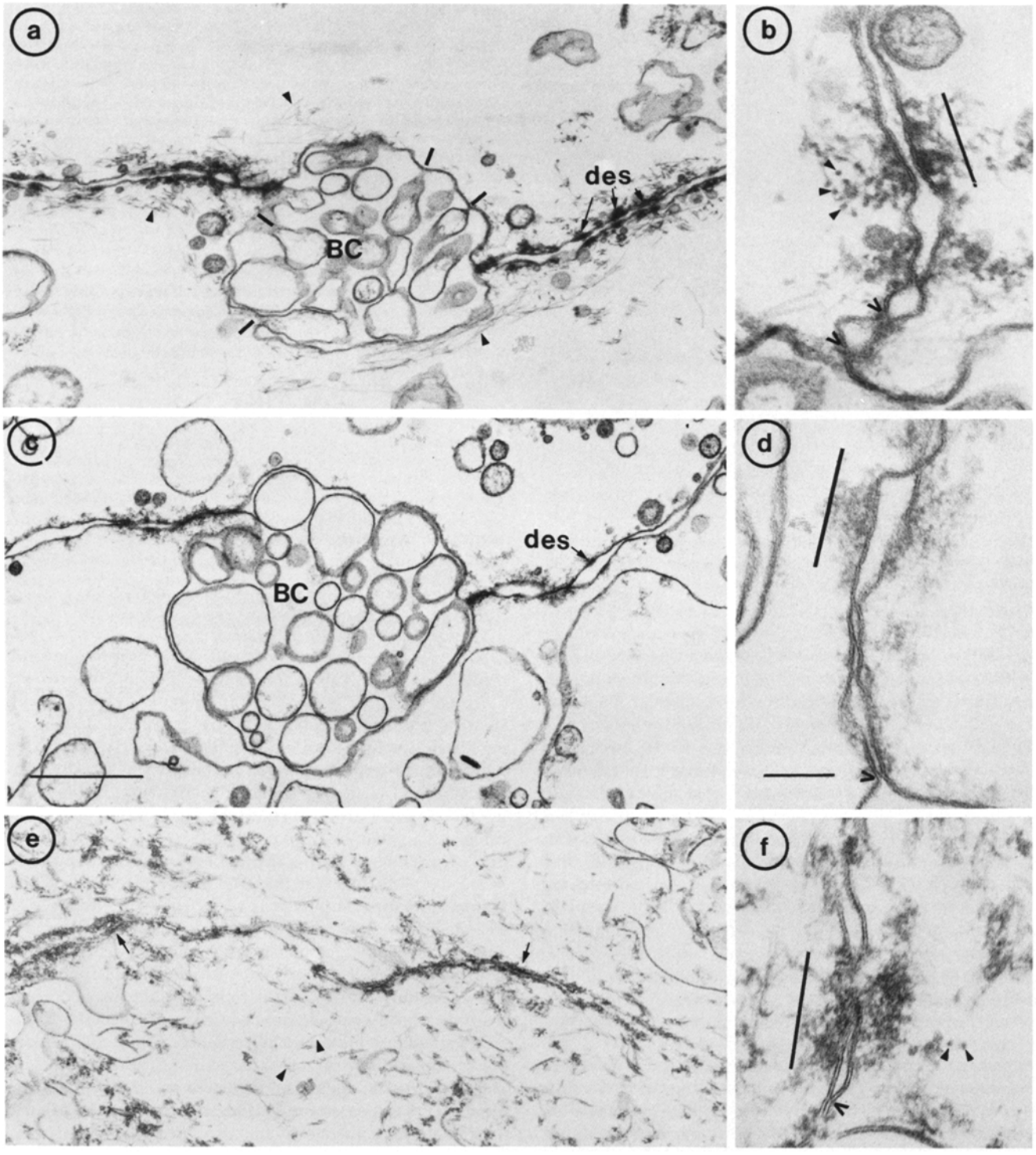
Having established a treatment that successfully removed the filaments, at least morphologically, we next analyzed by SDS PAGE the supernates and pellets derived from PM aliquots exposed to alkaline conditions (pH 8–11). The results are presented in Fig. 2, where it can be seen that several major PM components were selectively and progressively released by the alkaline treatment. The three major bands that were released had apparent molecular weights of 56 kdaltons, 52 kdaltons, and 43 kdaltons. Together they comprised 20–30% of the total Coomassie Blue-staining material of control PM and only ~5% of the pellet of pH 11-treated PM (lane *4*, Fig. 2). The 52- and 56-kdalton components appeared to be released in parallel with one another, but not with the 43-kdalton component. In addition, the amount of 43-kdalton varied in different PM preparations relative to the other two, whereas the 52- and 56-kdalton polypeptides were routinely present in approximately equimolar amounts in >50 separate PM preparations.

When the PM was exposed to 1% or 2% Triton X-100, under conditions that reportedly *do not* disassemble either actin or intermediate filaments but do solubilize integral membrane components (e.g. reference 15), a picture complementary to that found with alkaline treatment was obtained. That is, 98% of alkaline phosphodiesterase I activity was released in the presence of 1% TX-100, whereas 65% of the protein was solubilized (Table I). 5′-nucleotidase remained sedimentable but could be released if deoxycholate was added (data not shown). Morphologically, the pellet contained filaments, often in parallel arrays reminiscent of their distribution along the lateral surface in untreated PM (Fig. 1 *e* and *f*), but did not exhibit continuous membrane profiles or recognizable membrane domains. SDS PAGE analysis revealed that the three major polypeptides extracted by high pH were present in the pellet after detergent treatment (Fig. 3).

Comparison of the Three Major Polypeptides with Known Filament Proteins

We used two-dimensional gel analysis (IEF then SDS PAGE) to compare the three major polypeptide components of the PM to several well-characterized filament proteins having similar solubilities and apparent molecular weights. These included vimentin and desmin, two intermediate filament proteins found predominantly (but not exclusively) in mesenchymal and muscle cells, respectively (e.g. references 11, 19), tubulin, and actin. The results of this comparison are presented in Fig. 4. First, we found that Component 1 (56 kdaltons) had

FIGURE 1 Electron micrographs of control, alkaline-extracted and detergent-extracted PM. PM was incubated in 0.25 M sucrose (*a*, and control), 25 mM Na_2CO_3, pH 11 (*c* and *d*) or 1% Triton X-100 (*e* and *f*) as described in Materials and Methods. The suspensions were centrifuged and the pellets processed for EM visualization. (*a*) Untreated PM. The bile canalicular (*BC*) domain often appears to be composed of vesicles. However, in this section, several vesicle profiles (marked by bars) can be seen in various degrees of continuity with the encircling membrane. These images indicate that all of the membrane within the BC is continuous. Filaments (arrowheads) are evident all along the LS and the basal portion of the BC. Dense plaques are adherent to the cytoplasmic surface of the LS (*des*). (*b*) At higher magnification in control PM, the filaments can be seen in cross-section (arrowheads) and in close association with the dense plaques (line). Open arrowheads mark membrane fusion points, comprising the tight junctions in liver, which are immediately subjacent to the bile canaliculus. (*c*) In alkaline-extracted PM, the BC is intact although more vesiculated in appearance. Recognizable filaments are absent, but a fibrillar mat is visible along the LS. Parallel densities can be seen on the two membranes composing the LS (*des*). Associated vesicles still remain. (*d*) At higher magnification in alkaline-extracted PM, a region of the LS similar to that shown in *b* lacks recognizable filaments but does contain amorphous material associated with the membrane. Tight junctions can still be seen (open arrowheads), although they appear somewhat altered. (*e*) Pellet of detergent-extracted PM. The picture complementary to that observed in *c* is seen here. Membrane is largely absent, although short segments (open arrowheads) can be seen. Dense plaques are present that occasionally have membrane profiles attached (arrows). Short filaments are also apparent (arrowheads). (*f*) At high magnification in detergent-extracted PM, a region very similar to that shown in *b* has recognizable filaments (arrowheads) and remnants of trilaminar structure reminiscent of a membrane (open arrowheads). *a*, *c*, and *e*, × 37,500. Bar, 0.5 μm. *b*, *d*, and *f*, × 117,000. Bar, 0.1 μm).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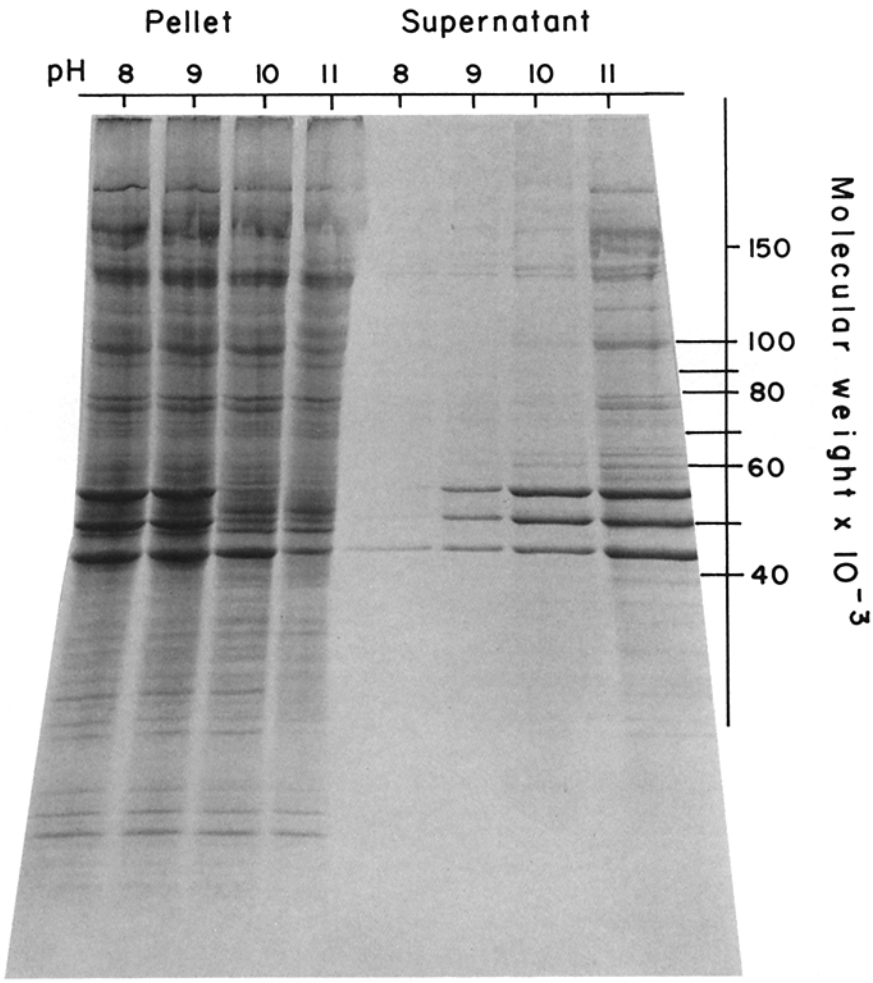

FIGURE 2 SDS PAGE of PM exposed to a pH range of 8–11. Aliquots of PM (200 μg) were first incubated in alkaline media (pH 8–11) and then the sedimentable and nonsedimentable material separated by centrifugation at 100,000 g_{av} for 30 min. The entire supernate was removed, precipitated with 10% TCA, then it and the entire pellet were prepared for SDS PAGE (see Materials and Methods). The resolving gel was a gradient of 5–15%. After electrophoresis, the gel was stained with Coomassie Blue. The molecular weight scale was determined from the electrophoretic mobilities of the following standards: phosphorylase a (94 kdaltons), bovine serum albumin (68 kdaltons), catalase (60 kdaltons), muscle actin (43 kdaltons), chymotrypsinogen (26 kdaltons), and cytochrome c (12 kdaltons).

an apparent pI of ~6.4, Component 2 (52 kdaltons) a pI of ~5.4, and Component 3 (43 kdaltons) a pI of ~5.4. Although desmin (Fig. 4 *a–c*) had an electrophoretic mobility similar to that of Component 1 (56 kdaltons), the former was more acidic. Vimentin was both larger *and* more acidic than Component 1 (Fig. 4 *d-f*). In both the desmin and vimentin preparations, a 43-kdalton component, presumed to be actin (11, 12), was found to co-migrate with Component 3 (43-kdaltons). When muscle actin (the α isozyme) was isolated and its behavior in this two-dimensional gel system compared to those of PM Components 1–3, it was also found to comigrate with the 43-kdalton spot (Fig. 4 *g–i*). The isozymes of actin were *not* resolved by the pH range used here. That is, the cytoplasmic (β, γ) and muscle (α) actins comigrated with the 43-kdalton spot. Finally, neither of the larger two components could be identified as the tubulins (Fig. 4 *j* and *k*).

Identification of Component 3 as Cytoplasmic Actin

Although the results of two-dimensional analysis suggested strongly that Component 3 was an actin, the solubility properties of this component did not correspond to those of other filamentous actins (see previous section). Furthermore, desmosomal filaments from intestinal brush border have been reported to contain a 40-kdalton component (20), suggesting that Component 3 might be a constituent of desmosomes in liver PM. Therefore, we used peptide maps to determine that the 43-kdalton polypeptide was actin. Bands from SDS PAGE gels were iodinated, incubated with TPCK-trypsin, and the peptides separated. The results are shown in Fig. 5. The pattern obtained for the 43-kdalton band from PM was virtually identical to that of brush border actin (Fig. 5 *a* and *b*), confirming that PM actin was of the β, γ type. However, there were significant differences between the patterns of muscle actin (α) and PM 43-kdaltons (compare Fig. 5 *b* to *c*). Although four major ^{125}I-spots were common to the two actins, muscle actin exhibited six spots *not* shared by either PM 43-kdaltons or brush border actin. Conversely, there were three ^{125}I-peptides in the liver PM 43-kdalton preparation and brush border actin that were *not* present in muscle actin. These differences were reproducible in four different PM and muscle actin preparations and four different iodinations.

To determine whether all of the 43 kdalton-material in our PM was actin, we compared ^{125}I-tryptic peptide maps of spots from two-dimensional gels or bands from one-dimensional gels corresponding to the 43-kdalton position from total PM with the supernate from pH 11-extracted PM. The patterns were virtually identical, indicating that only one polypeptide, PM actin, comprised the stained band migrating at 43 kdaltons, whether or not it was membrane-associated.

Characterization of the 52- and 56-kdalton Polypeptides

Having identified PM actin, we next turned to the two larger polypeptides. Two questions were asked: (*a*) were they unique or related polypeptides? and (*b*) could one or both be reassem-

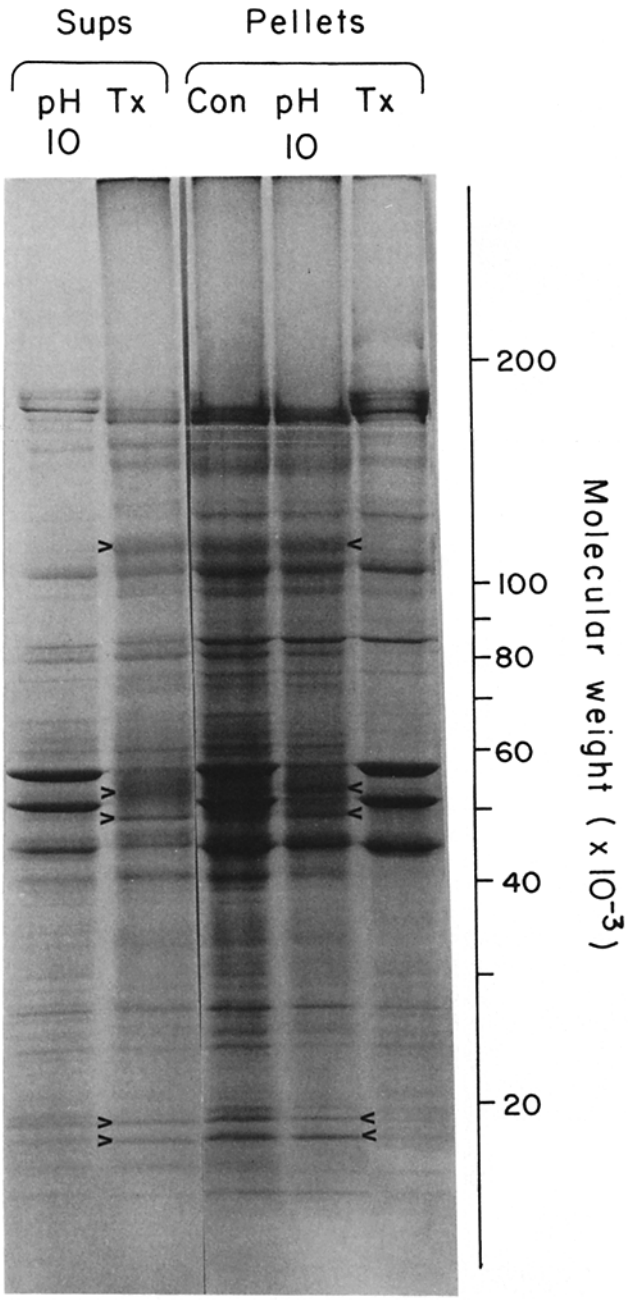

FIGURE 3 SDS PAGE of PM. Effect of Triton X-100 and pH. Aliquots of PM (200 μg) were extracted with NaOH, pH 10, or 1% Triton X-100 (*Tx*), sedimented and the pellets and supernates (*Sups*) prepared for SDS PAGE as described in Materials and Methods. After electrophoresis the gel was stained with Coomassie Blue. *Con*, untreated PM. Several bands (open arrowheads) exhibit the solubility characteristics of integral membrane components. However, the three major bands between 40 and 60 kdaltons in control exhibit the solubility properties of cytoskeletal elements and correlate with the distribution of filaments. That is, they are absent from the pellet of alkaline-treated PM and present in the pellet of detergent-extracted PM (see Fig. 1 for corresponding morphology of pellets from PM so treated).

bled into filamentous structures, under conditions known to promote assembly of intermediate filaments, which would then identify them as *bona fide* desmosomal filament proteins?

PEPTIDE MAP ANALYSIS: The peptide maps shown in Fig. 6 illustrate that the 52- and 56-kdalton bands from SDS PAGE represented distinct polypeptides. Furthermore, no significant change in the ratio of these polypeptides was seen by SDS PAGE when PM was isolated in the presence of protease inhibitors or when freshly-isolated PM was incubated at 37°C for up to 3 h. These results suggested that the two polypeptides were not proteolytic fragments generated *in vitro*.

REASSEMBLY: We next asked whether filaments could be reassembled from the 52- and/or 56-kdalton components or any molecules in the supernate from alkaline-treated PM. As stated above, reassembly constitutes a criterion for the identification of particular molecules as intermediate filament components (21).

Supernates from alkaline-extracted PM (pH 9 or pH 11) were first examined by negative staining and found to be devoid of filaments. Gel filtration of these supernates on Sephadex G-150 (at pH 11) revealed that >90% of the protein was included in the bed volume and that most (>75%) migrated just behind an albumin marker (data not shown). After dialysis overnight at 4°C against 50 mM Tris-HCl, pH 7.6, and 50 mM 2-ME, a solution that promotes intermediate filament assembly (22), filaments could be visualized (Fig. 7). They were long, had diameters of ~9.5 nm and stained central cores. Aggregates were also evident in the negatively stained preparations but when the dialyzed supernate was centrifuged, the pellet was processed, and sectioned material examined (Fig. 7 *c*), appeared to be composed primarily of filaments. Particles of a nonfilamentous nature were also apparent.

We found the inclusion of protease inhibitors to be a variable requirement in the disassembly-reassembly protocol. If PM was extracted at pH 9 rather than pH 11, the protease inhibitors antipain (1 μg/ml), leupeptin (1 μg/ml), benzamidine (1 mM), and Trasylol (100 units/ml) were necessary throughout the procedure. Otherwise, no filaments were found in the dialyzed extract and evidence of proteolysis during extraction and dialysis was apparent by SDS PAGE. When we measured the distribution of protein through the disassembly-reassembly procedure, there were variable losses during dialysis, making quantitation difficult. However, in six separate experiments where recoveries at the dialysis step were >75%, ~15% of the initial PM, protein was found in the final pellet.

SDS PAGE ANALYSIS OF REASSEMBLED FILAMENT PREPARATION: When filaments were observed morphologically in the dialyzed extracts, the final pellets always contained the 56-kdalton and 52-kdalton polypeptides as major components (Fig. 8) plus variable amounts of actin. Although other bands were also certainly present in the final pellet (Fig. 8), none were enriched to the extent that the 56- and 52-kdalton bands were. Supernates of the dialyzed extracts (*S II*'s in Fig. 8) contained the bulk of the original polypeptides.

WHAT HAPPENS TO PM ACTIN DURING REASSEMBLY? From Fig. 8 and our morphological finding of only one size population of filaments, it appeared that PM actin was not a major component of the reassembled filaments. However, a 43-kdalton band was present in the final pellets by SDS PAGE analysis, albeit in reduced amounts. Therefore, we asked whether PM filaments would reassemble under conditions (low ionic strength) known to depolymerize filamentous muscle actin and to maintain actins in the globular form (12). As a control, muscle G actin was carried in parallel with PM through the entire procedure, from alkaline extraction to SDS PAGE analysis. Protease inhibitors were present throughout and dialysis of the alkaline supernates was done against a solution composed of 5 mM Tris-HCl, pH 7.2, 0.2 mM $CaCl_2$,

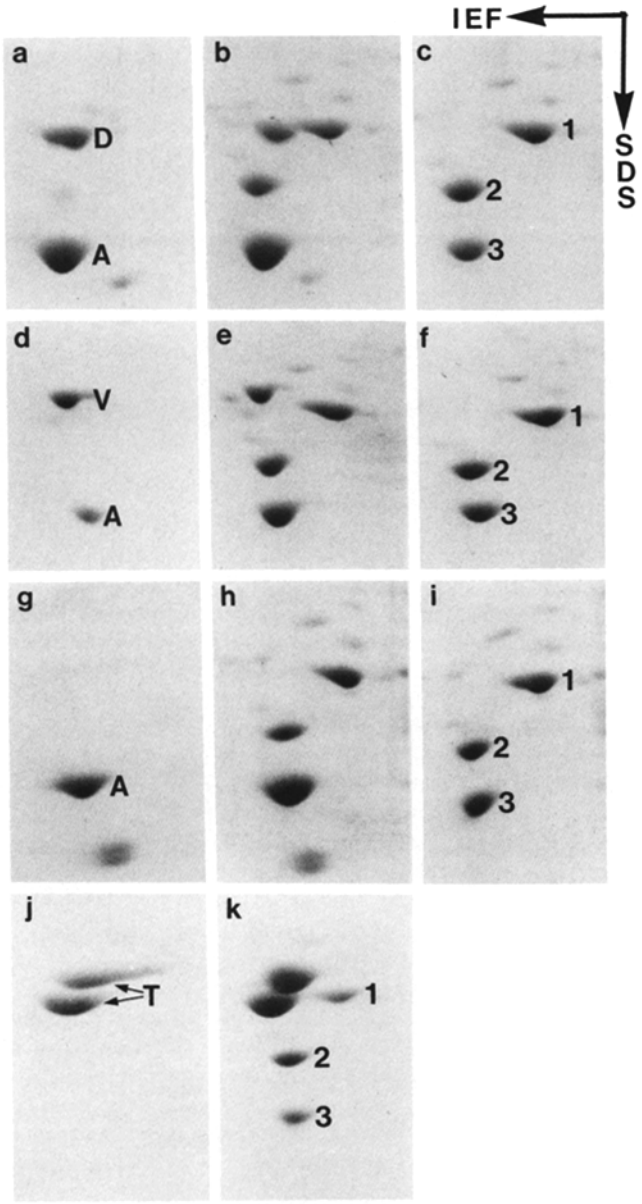

FIGURE 4 Comparison by two-dimensional analysis of the three major PM polypeptides to cytoskeletal proteins. PM and four different cytoskeletal proteins were solubilized in SDS-NP40 and focused both separately and as mixtures in polyacrylamide gels. IEF was from right to left (basic to acidic pH). As described in the text, three tube gels were cast onto the top of a single 10% slab SDS polyacrylamide gel and electrophoresed (top to bottom). Gels were stained with Coomassie Blue. Each row represents a separate experiment and slab gel. Only the regions of each gel containing the relevant polypeptides are pictured here. The three major PM polypeptides are denoted as *1* (56 kdaltons), *2* (52 kdaltons) and *3* (43 kdaltons). PM alone is in the right hand panel of each row (*c*, *f*, and *i*), the cytoskeletal protein alone in the left hand panels (*a*, *d*, *g*, and *j*), and mixtures in the center panels (*b*, *e*, *h*, and *k*). (*a*, *b*, and *c*) PM was compared to gizzard desmin (*D*). Actin (*A*) was also present in the desmin preparation. (*a*) Desmin alone; (*b*) desmin-PM mixture; (*c*) PM alone. In *d*, *e*, and *f*, PM was compared to fibroblast vimentin (*V*). (*d*) vimentin alone; (*e*) vimentin-PM mixture; (*f*) PM alone. In *g*, *h*, and *i*, PM was compared to muscle actin. (*g*) Actin alone; (*h*) actin-PM mixture; (*i*) PM alone. In *j* and *k*, PM was compared to brain tubulin (*T*). (*j*) Tubulin alone; (*k*) PM-tubulin mixture.

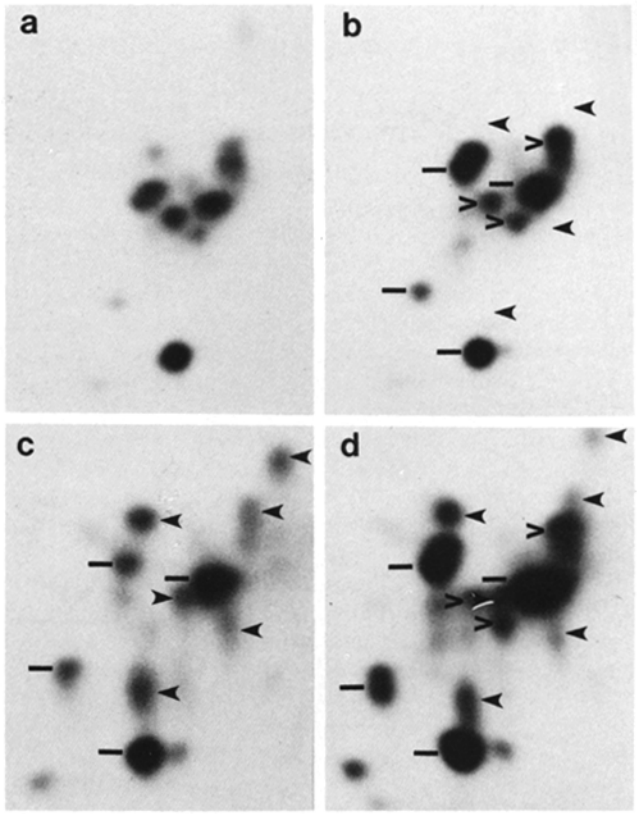

FIGURE 5 Tryptic peptide maps of the 43-kdalton component of liver PM and authentic actins. Bands from SDS PAGE gels were excised, iodinated, digested, and separated as described in Materials and Methods. Only the central regions of the autoradiograms are presented here. *a*, brush border actin; *b*, PM 43 kdaltons, *c*, muscle actin, *d*, PM 43 kdaltons and muscle actin mixed 2:1 by radioactivity. Bars indicate peptides found in both PM 43 kdaltons and muscle actin (*b* and *c*). Open arrowheads indicate spots found only in 43 kdaltons (*b* only) and solid arrowheads those found only in muscle actin (*c*).

0.2 mM ATP, and 50 mM 2-ME. On three separate occasions we found filaments in the dialyzed actin extract. SDS-PAGE analysis revealed the 56-kdalton and 52-kdalton bands to be the major components in the final pellet (*P II*). These results suggested that the reassembled filaments were composed predominantly of the 56- and 52-kdalton polypeptides and not of PM actin.

DISCUSSION

In this study we have described the removal of filaments that are associated with the isolated hepatocyte plasma membrane and the characterization of their major component proteins.

Alkaline Extraction of the PM

A procedure has recently been published (23) that utilizes alkaline media (0.1 M Na carbonate, pH 11.3) to isolate liver endomembrane from a subcellular fraction (e.g., peroxisomes [24], Golgi [25]). We have extended this alkaline extraction procedure to the liver PM and found that it yields a membrane sheet preparation in which associated filaments are largely lost, but the major membrane domains and at least four intrinsic membrane enzyme activities are retained. As described in the preceding paper, the enrichment of enzyme markers in the starting PM is 30- to 50-fold relative to the filtered homogenate; after alkaline extraction, this value increases to ~40- to 70-fold. Such enrichments are among the highest reported for a preparation of membrane sheets.

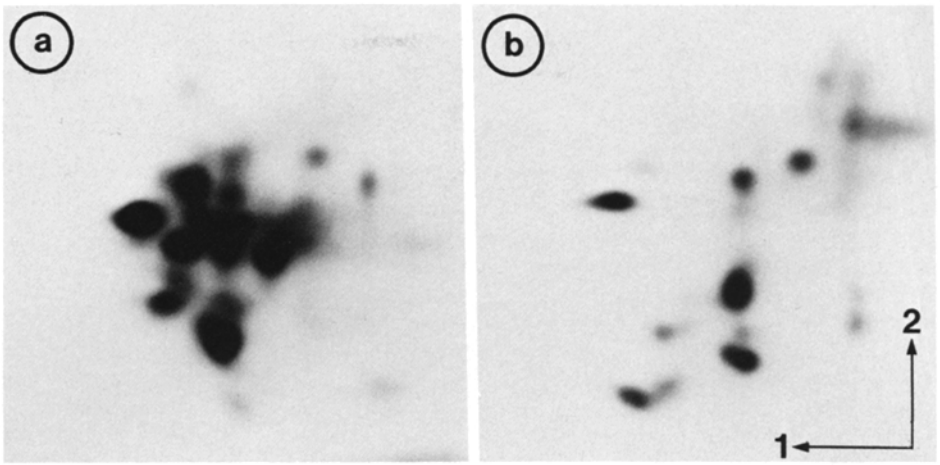

FIGURE 6 Tryptic peptide maps of the 52-kdalton (*a*) and 56-kdalton (*b*) polypeptides in PM. Bands from one-dimensional gels of total PM corresponding to two of the major PM polypeptides were excised, iodinated, digested, and separated as described in Materials and Methods. Only the central regions of the autoradiograms are presented. Electrophoresis (first) was from right to left and chromatography (second) from bottom to top.

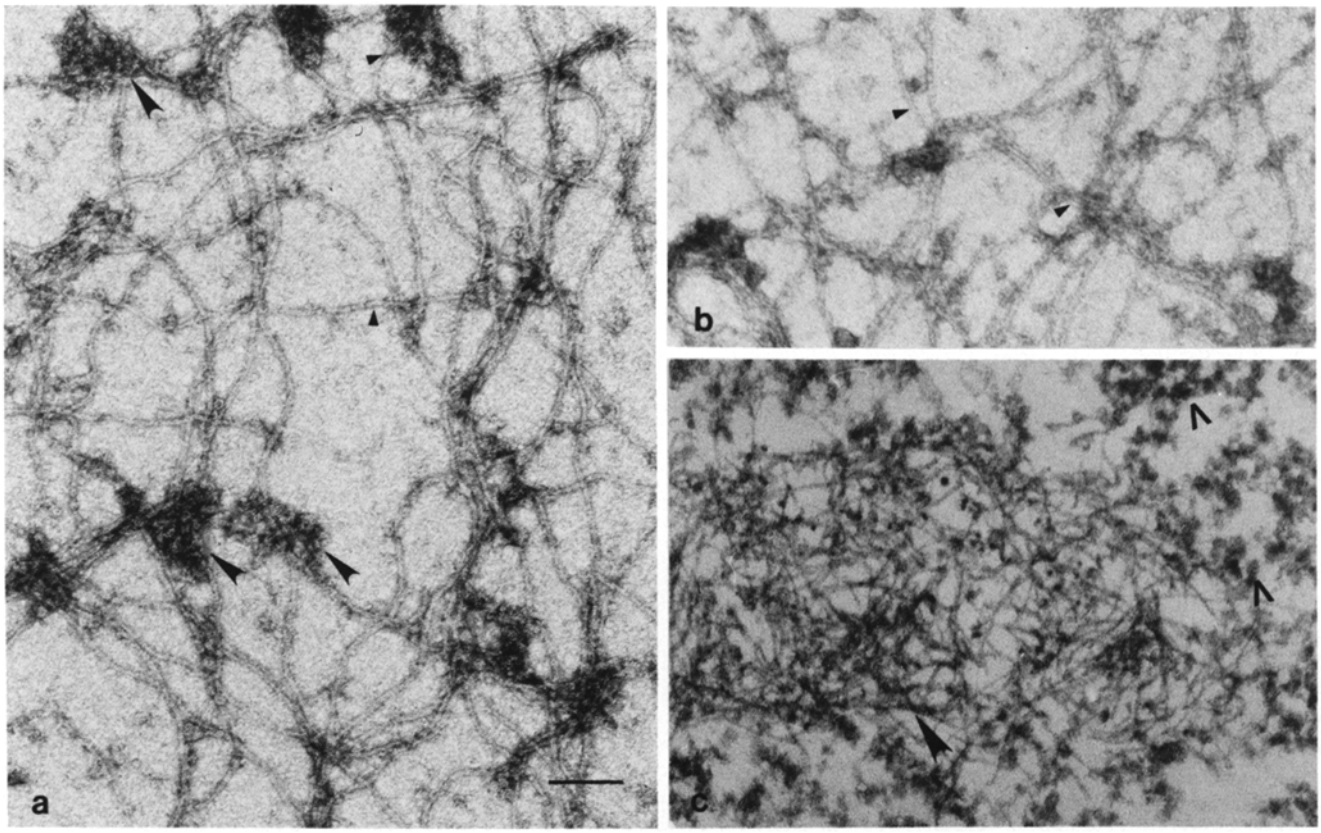

FIGURE 7 Electron micrographs of dialyzed alkaline extracts of PM. (*a*) Negative stain appearance of a preparation after extraction of PM at pH 9 (CO_3) and overnight dialysis of the supernate against 50 mM Tris-HCl, pH 7.6 and 50 mM 2-ME. Filaments (small arrowheads) and aggregates (large arrowheads) are abundant. (*b*) Negative-stain image of a preparation after extraction of the PM at pH 11 (CO_3) and processing as described above. (*c*) Thin-section appearance of the pellet from a pH 11 extract dialyzed against Tris only (no 2-ME). (The supernate contained no filaments when examined by negative staining.) The aggregates seen by negative stain in (*a*) and (*b*) appear to be composed predominantly of filaments which tend to be somewhat shorter when assembled in the absence of a reducing agent (21). Open arrowheads mark particles seen in pellets but not apparent in negatively-stained preparations. Bar, 0.1 μm. *a*, *b*, and *c*: × 95,200.

Actin in the PM

We have identified the cytoplasmic form(s) of actin (β, γ) as a major component that is removed from the PM during alkaline extraction. Our identification is based on the identity of a 43-kdalton polypeptide in PM with a known cytoplasmic actin (from brush border) in two analytic systems. All of the 43-kdalton material is actin, indicating that PM actin is ~5% of the total PM protein. The exact location and form of the actin in our PM is at present not clear. That is, no filaments exhibited heavy meromyosin binding our preparation (A. Hubbard, unpublished observation), although others have observed

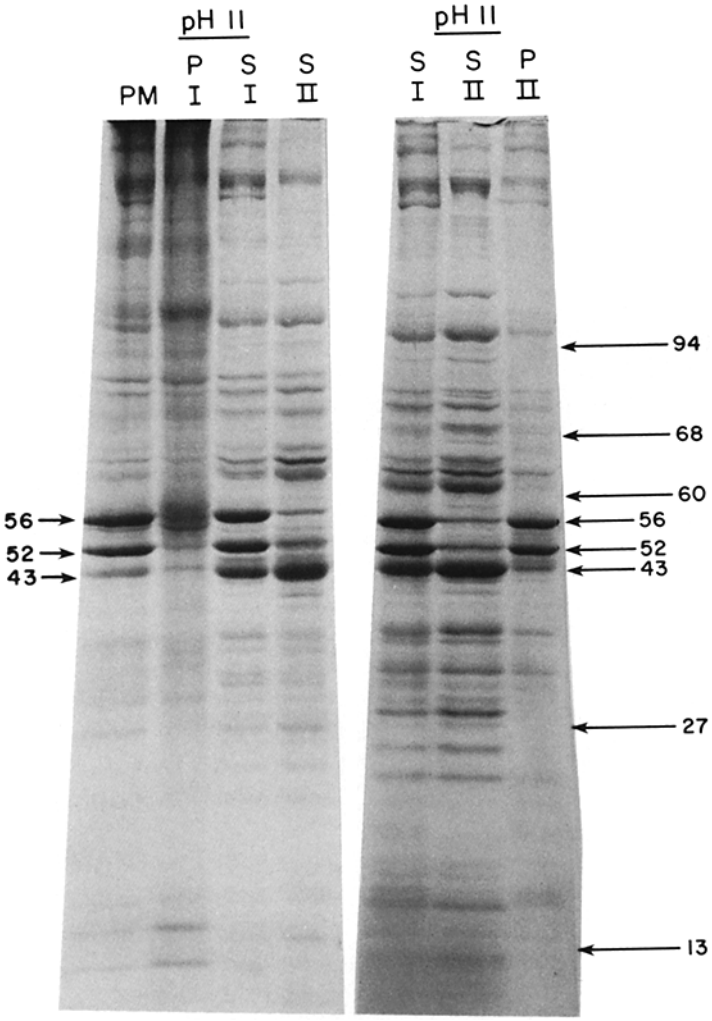

FIGURE 8 SDS PAGE analysis of fractions obtained during disassembly and re-assembly of PM filaments. Solubilized aliquots (150 μg) taken after PM extraction, supernate dialysis, and centrifugation, were electrophoresed and the gels then stained. *PM*, starting PM; *PI* and *SI*, pellet and supernate after alkaline extraction of PM and centrifugation at 100,000 g_{av} for 60 min; *SII* and *PII*, supernate and pellet of dialyzed extract (*SI*) after centrifugation at 100,000 g_{av} for 60 min. The predominant bands in PII migrate at 56 kdaltons and 52 kdaltons. The 43-kdalton component remains in the supernate (*SII*). The positions of the molecular weight standards are indicated on the right.

decorated filaments in intact liver extracted for prolonged periods in glycerol (26). Exposure of PM to either 0.6 M KI or a low ionic strength buffer, both reported to depolymerize filamentous actin in other systems, had little effect on releasing the PM actin. Furthermore, DNase I was not inhibited by actin in the PM until the latter was exposed to alkaline conditions, which also released actin from the membrane (A. Hubbard, unpublished observations). Thus, the possibility that the PM actin is associated with other molecules in the PM preparation, membrane and/or cytoskeletal components, remains to be determined. Recently, Bennett et al. (27) reported the presence of an analogue to brain spectrin in liver PM prepared as we have described.

Desmosomes and Tonofilaments

Although structurally similar in all cells so far studied, intermediate filaments can be grouped into five immunologically distinct classes (see reference 21 for review). Most epithelial cells of vertebrates contain proteins (cytokeratins) related to epidermal prekeratins which are components of a class of intermediate filaments (tonofilaments) associated with desmosomes in the epidermis. Franke et al. (20) have recently identified the proteins of tonofilaments associated with desmosomes present in isolated brush borders of rat intestinal epithelial cells. These proteins appear to differ immunologically from most cytokeratins in epidermal cells. The present study is the first to identify and characterize the desmosomal filaments in isolated rat liver plasma membrane. Although desmosomes were clearly recognizable in the micrographs of isolated PM preparations from other studies, by SDS PAGE analysis few tonofilaments appeared to be associated with them (e.g. 28–32). Franke et al. (33) found that components of intermediate filaments remaining after detergent extraction of rat hepatocytes *in situ* were similar to those found in intestinal epithelial brush borders. Two major components in the detergent-resistant fraction from total liver (A and D, reference 33) probably correspond to the 56- and 52-kdalton intermediate filament components we have identified in our isolated PM. Two points from the Franke study are relevant to our work: (*a*) antibody to D apparently did not cross-react with A, reinforcing our conclusion from the tryptic peptide map analysis that 56- and 52-kdaltons are distinct polypeptides; (*b*) immunofluorescent localization of component D revealed a filamentous organization in PTK cells, suggesting, by analogy, that our 52-kdaltons is a component of the filament and not any substructure (i.e., the cytoplasmic plaque). This view reinforces our conclusion from data on reassembly that both 52- and 56-kdaltons are part of the tonofilament itself. However, no localization of the latter component in this or any other system has yet revealed its organization.

Finally, the nature of the cytoplasmic plaque in the desmosomes of our PM preparation remains unknown. On the basis of selective extraction in metrizamide, Gorbsky et al. (34)

suggested that the plaque in bovine muzzle was composed of polypeptides much larger (~200 kdaltons) than the prekeratin polypeptides comprising the tonofilaments of the bovine epidermal system. Although polypeptides of this size can be detected by SDS PAGE in our PM and an alkaline extract of PM, their abundance does not match that of the desmosomal filament proteins (e.g. Figs. 2 and 8). Therefore, it is not presently known whether this component of hepatocyte desmosomes will be biochemically or immunologically similar to those of other epithelial cells.

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