Isolation of Cell-to-Cell Adherens Junctions from Rat Liver

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Abstract. A new isolation procedure for cell-to-cell adherens junctions has been developed using rat liver. From the bile canaliculi-enriched fraction obtained by homogenization of the liver and sucrose gradient centrifugation, the fraction rich in adherens junction was recovered by detergent treatment followed by sucrose gradient centrifugation. Light and electron microscopy revealed that this final fraction was mainly composed of the belt-like adherens junctions with their associated short actin filaments. Biochemical and immunological analyses have shown that vinculin is highly enriched in this fraction. Considering that vinculin is known to be localized in the cell-to-cell adherens junctions, we can conclude that we have succeeded in isolating the cell-to-cell adherens junctions. Furthermore, the constituents of the undercoat (dense layer underlying the membrane) of adherens junctions were selectively extracted from the fraction rich in junctions. Upon SDS electrophoresis of this extract, 10 polypeptides including vinculin, α-actinin, and actin were dominant. The results obtained are discussed with special reference to the molecular organization of the undercoats of cell-to-cell adherens junctions.

It is of central importance in cell biology to understand the cell adhesion mechanism and its regulation in molecular terms. In mammalian cells, the intercellular junctions are categorized into four types: tight junctions, adherens junctions, desmosomes, and gap junctions (7). Among them, adherens junctions and desmosomes are characterized by a prominent "undercoat" (dense layer underlying the plasma membrane; 18), through which actin and intermediate-sized filaments, respectively, are associated with the plasma membrane. Therefore, these two types of junctions offer advantageous models to analyze how the undercoat and cytoskeleton are involved in the cell-to-cell adhesion mechanism.

Desmosomes now can be isolated from epidermal cells (33). In desmosomes isolated from cow muzzle epidermis, the major constitutents were identified mainly on SDS-PAGE (14, 32, 34). Some of these proteins (desmoplakin I,II,III, desmocalmin, etc.) were shown to localize in the undercoat (desmosomal plaque) using immunological methods (4, 8, 15, 23). Polyclonal and monoclonal antibodies against these proteins have made it possible to analyze the dynamic behavior of these proteins induced by cell-to-cell adhesion (16, 19, 41).

Adherens junctions are the other cytoskeleton-associated junctions, which are thought to play crucial roles in the morphogenesis of organs and also in carcinogenesis (10). According to Geiger's group, the adherens junction should be subclassified into cell-to-cell and cell-to-substrate junctions (12). As a model of the cell-to-substrate adherens junction, the membrane fraction prepared from smooth muscle cells has been used, leading us to conclude that some unique proteins such as vinculin and talin localize in the cell-to-substrate junction (1, 2, 9, 10, 11). Talin was detected only in the cell-to-substrate junctions, while vinculin was localized in cell-to-cell junctions as well as in cell-to-substrate junctions. Some efforts have been made to isolate the cell-to-cell adherens junction, mainly from cardiac muscle cells (3, 20, 40). However, compared to the desmosome fractions from cow muzzle epidermis, the purity of the cell-to-cell adherens junction fraction from cardiac muscle seemed to be rather low. For example, on electrophoresis of this fraction, vinculin did not appear to dominate. Therefore, to analyze the molecular organization of the cell-to-cell adherens junction systematically, it is first necessary to develop a new procedure for the isolation of this type of junction. In recent years, we have attempted to isolate the cell-to-cell adherens junction. Our criteria for the isolation of the cell-to-cell adherens junction is as follows. (*a*) In the fraction obtained, the adherens junction should be highly enriched at the electron microscopic level. The prominent actin bundles should not be contained in this fraction. (*b*) On electrophoresis of the fraction, vinculin should be clearly identified as one of the major constituents. On the basis of these criteria, we have searched for the "nonmuscle" organ appropriate for the isolation of the cell-to-cell adherens junctions.

In the present study, a new procedure was developed for the isolation of the cell-to-cell adherens junction from rat liver. This isolation procedure is basically the selective detergent extraction of bile canaliculi, prepared by a previously published method (35). The bile canaliculi are enriched in adherens junctions and in plasma membrane components not involved in cell-to-cell adhesion. The nonionic detergent NP-40 then was used to selectively solubilize the membrane components that are not stabilized by strong hydrophilic in-

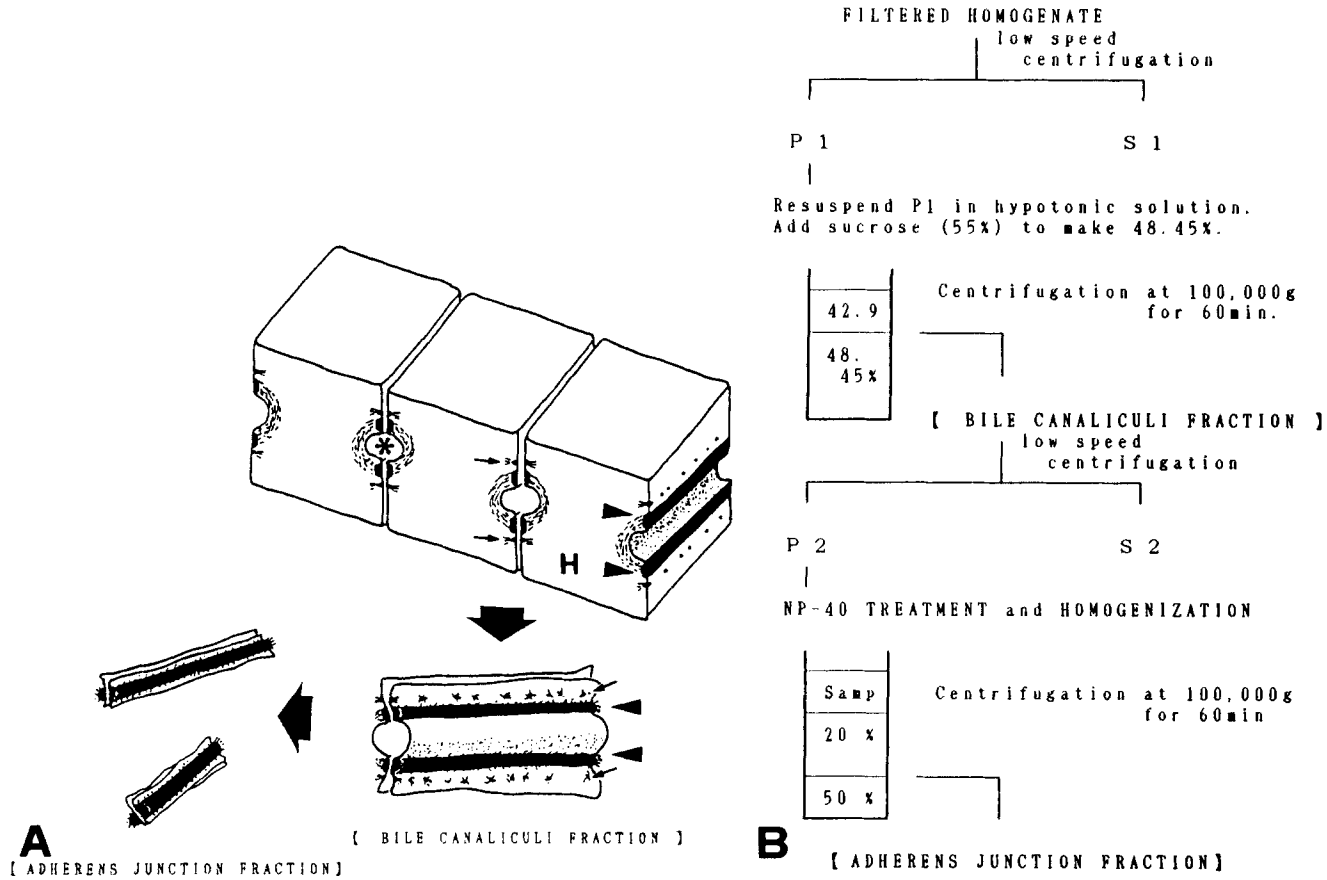

Figure 1. A scheme for the isolation of adherens junctions from rat liver. (*A*) The hepatic cells (*H*) are arranged to form laminae and the apposed sides of neighboring cells form a bile canaliculus (*) between them. The isolation procedure includes two steps: isolation of bile canaliculi and isolation of the belts of adherens junction. *Arrowhead*, adherens junctions; *arrows*, desmosomes. (*B*) Specific details are given in Materials and Methods.

teractions, leaving behind the proteins engaged in cell-to-cell and membrane-to-cytoskeleton attachments. Similar strategies have been used many times to isolate other specialized domains stabilized by strong hydrophilic interactions; for example, erythrocyte membrane skeleton (30, 42), gap junctions (13), desmosomes (6, 14, 34), zonula occludens (37), and nonjunctional areas of cell–cell contact (17, 28). In this study, we further succeeded in selectively extracting the major constituents of the cytoskeletal structures underlying the plasma membrane of the cell-to-cell adherens junction. We believe our isolation procedure will show a new way to analyze the molecular organization of the cell-to-cell adherens junction in nonmuscle cells.

Materials and Methods

Isolation of Adherens Junctions

For each isolation experiment, three 8-wk-old Wister rats were used. After decapitation, the livers were carefully taken out and soaked in an ice-chilled physiological saline solution. All subsequent procedures were carried out at 4°C. As the first step, the fraction rich in bile canaliculi was obtained according to a modification of the method described by Song et al. (35). The liver was minced with razor blades and treated with "hypotonic solution" consisting of 1 mM $NaHCO_3$ and 2 μg/ml leupeptin (pH 7.5) for 30 min. The swollen samples were homogenized in 2 vol of hypotonic solution by the use of a loose-fitting homogenizer (Dounce, Vineland, NJ). The homogenate was diluted with hypotonic solution up to 400 ml, filtered twice through four layers of gauze, and centrifuged at 1,500 *g* for 10 min. The pellets were resuspended in hypotonic solution, diluted up to 13.1 ml, and mixed with 96.9 ml of 55% (wt/vol) sucrose solution to make a 48.45% sucrose solution. This was divided into six tubes and carefully layered with 42.9% sucrose solution. After centrifugation for 60 min at 100,000 *g*, the bile canaliculi were recovered at 42.9:48.5% interface.

The fraction rich in bile canaliculi was diluted with 10 vol of hypotonic solution and centrifuged for 30 min at 4,000 *g*. The pellets obtained were resuspended in NP-40 solution consisting of 100 mM KCl, 1 mM $MgCl_2$, 0.1% (vol/vol) NP-40, 10 mM Hepes (pH 7.5). The sample was passed twice through a 23-gauge needle and stirred in NP-40 solution for 20 min. Then it was fractionated by centrifugation at 100,000 *g* for 60 min on a discontinuous gradient consisting of 20% (wt/vol) and 50% (wt/vol) sucrose. Adherens junctions were recovered at the 20:50% interface.

Selective Extraction of Undercoat of Adherens Junctions

The fraction rich in adherens junction was diluted with an equal volume of "extraction" solution and dialyzed against extraction solution for 12 h. Extraction solution is composed of 1 mM EGTA, 0.5 mM PMSF, and 2 mM Tris-HCl (pH 9.2). The sample was centrifuged at 100,000 *g* for 60 min and the supernatant was used as the "extract" from adherens junctions.

Gel Electrophoresis

One-dimensional SDS-PAGE was based on the method of Laemmli (21) and stained with Coomassie Brilliant Blue R-250 or with a silver staining kit (Wako Pure Chemical Industries, Tokyo, Japan). For two-dimensional gel electrophoresis, the isoelectric focussing was followed by SDS-PAGE, ac-

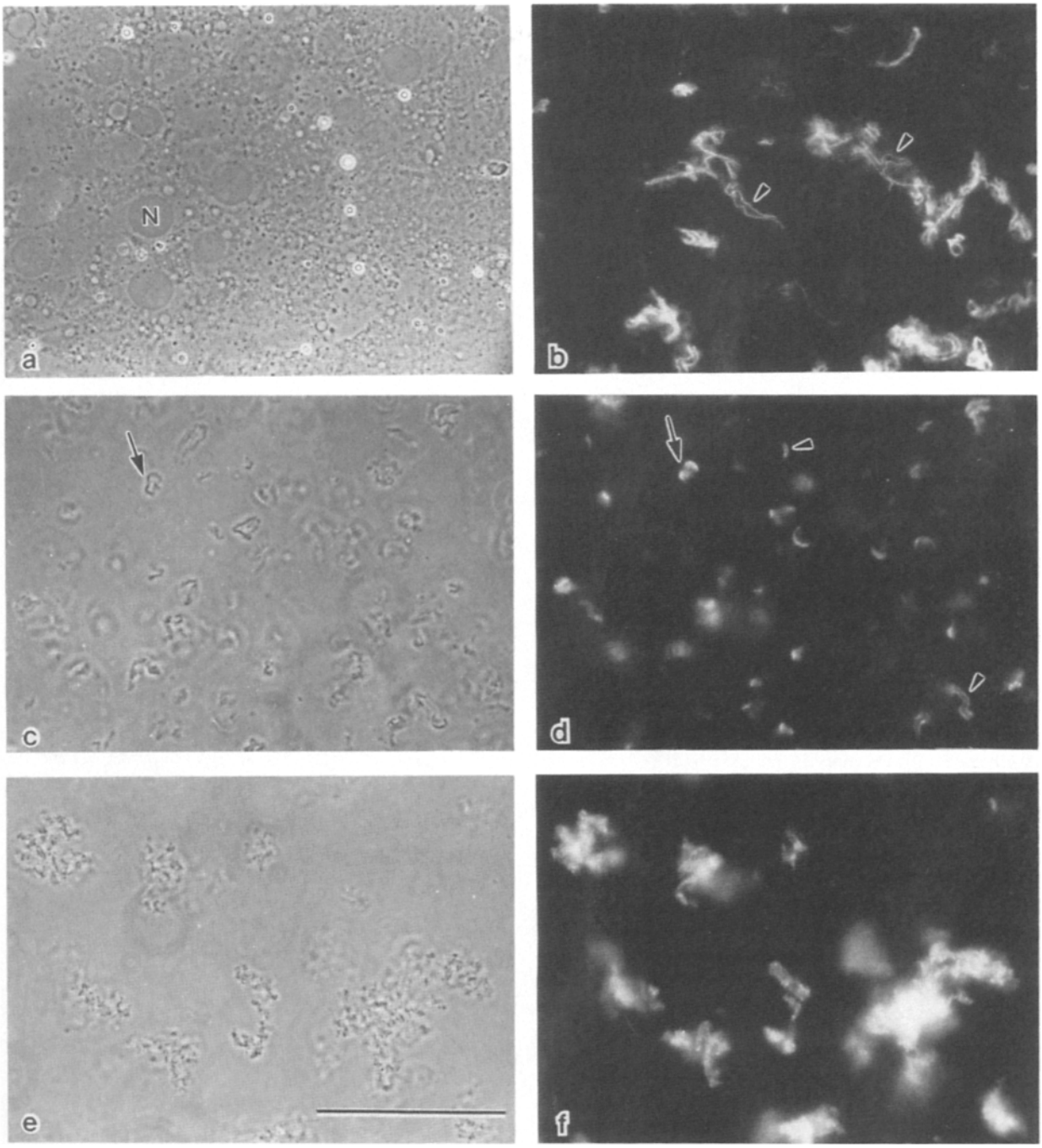

Figure 2. Isolation of adherens junctions from rat liver. (*a*, *c*, and *e*) Phase-contrast micrographs. (*b*, *d*, and *f*) Actin pattern visualized by incubation with rhodamine-phalloidin. (*a* and *b*) The homogenate of liver with hypotonic solution. Among cell debris and nuclei (*N*), many bile canaliculi (*arrowheads*) can be clearly identified, which are specifically stained with rhodamine-phalloidin. (*c* and *d*) The fraction rich in bile canaliculi. This fraction is characterized by a large number of membrane fragments (*arrows*), which contained one or two bile canaliculi (*arrowheads*). (*e* and *f*) The fraction rich in belt-like adherens junctions. Note the aggregated thin strands, all of which are intensively stained with rhodamine-phalloidin. Bar, 100 μm.

cording to the method of O'Farrell (24), with the slight modification that the sample was applied on the acidic sides in the isoelectric focussing. As molecular markers, chick gizzard vinculin (9), chick gizzard α-actinin, and chick gizzard talin (1) were kindly provided by Drs. A. Asano and T. Ohtaki (Sapporo Medical College).

Thin-Section Electron Microscopy

Samples were processed as previously described (38), using 0.5% tannic acid, 2.5% glutaraldehyde, 0.1 M sodium cacodylate buffer (pH 7.4) as a fixative.

Immunological Method

After electrophoresis of the extract from the adherens junctions obtained from 60 rats, the bands corresponding to a molecular mass of 130 kD were removed with a razor blade. Such bands were pooled and the 130-kD polypeptide was eluted electrophoretically. The eluted protein was precipitated

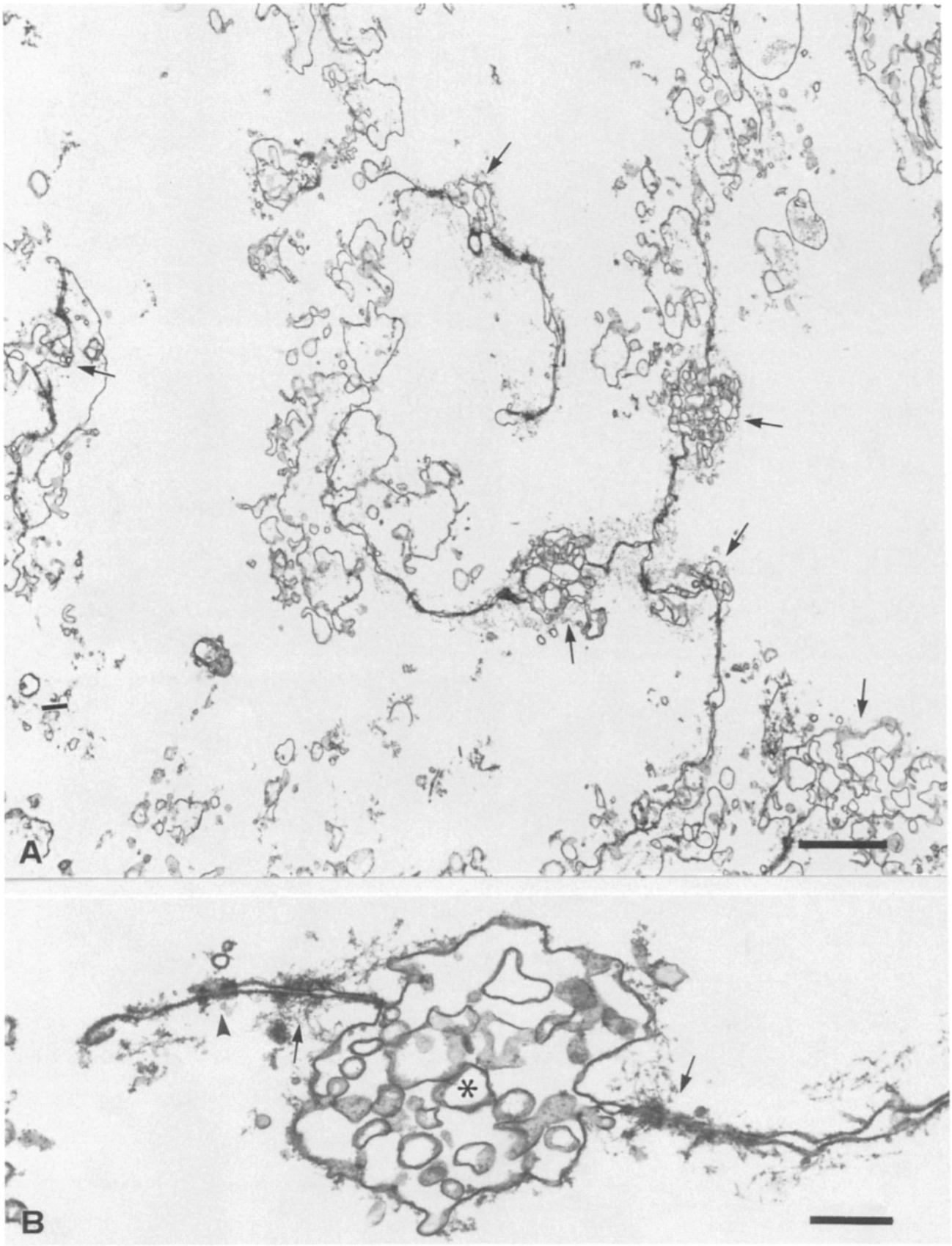

Figure 3. Thin-section electron microscopy of the fraction rich in bile canaliculi. At low magnification (*A*), the enrichment of the preparation was apparent. *Arrows*, isolated bile canaliculi. At higher magnification (*B*), the isolated bile canaliculus (*) is associated with adherens junctions (*arrows*) and desmosome (*arrowhead*). Note that small amounts of actin filaments are seen to grow out of adherens junctions. Bars: (*A*) 2 μm; (*B*) 0.5 μm.

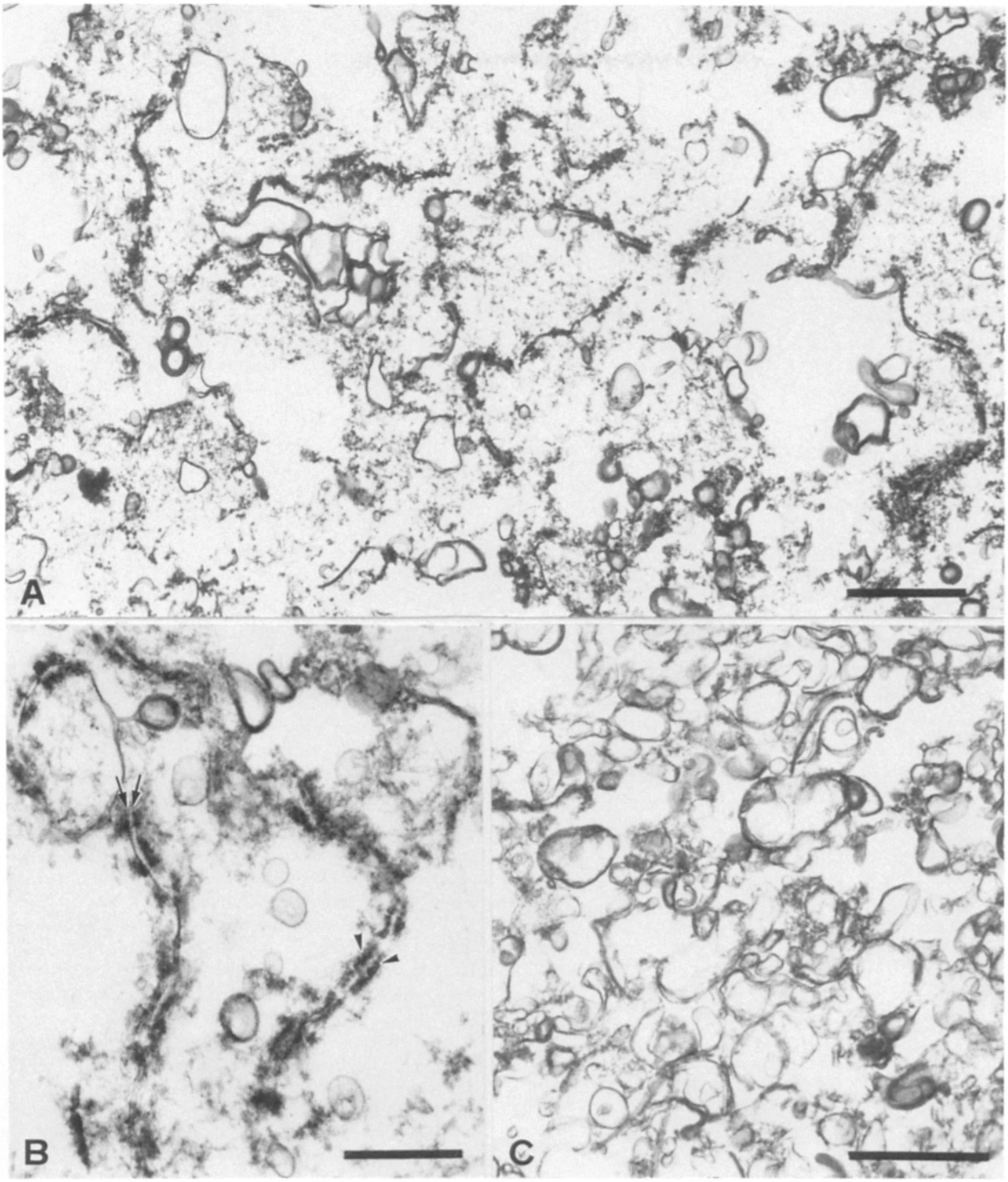

Figure 4. Thin section electron microscopy of the fraction rich in adherens junctions (*A* and *B*) and of the pellet of adherens junctions after extraction of the proteins of undercoats (*C*). The adherens junctions with short actin filaments are recovered at the 20/50% interface (*A*) of sucrose density gradient after treatment of bile canaliculi fraction (see Fig. 3) with NP-40. At higher magnification (*B*), the isolated adherens junctions are seen to be composed of partially extracted, apposed membranes (*arrows*), dense layers (undercoat) underlying membranes (*arrowheads*), and short actin filaments. After treatment of this fraction with low salt solution, almost all undercoat and actin filaments are removed, leaving vesiculated membranes (*C*). Bars: (*A* and *C*) 1 μm; (*B*) 0.5 μm.

by adding acetone, and the pellet was resuspended in 1 ml of distilled water. The sample was emulsified using Freund's complete adjuvant. A breed rabbit was injected subcutaneously with ~100 μg of 130-kD polypeptide. The same amount of antigen without adjuvant was reinjected subcutaneously on day 21, and this procedure was repeated at day 28. At day 31, the rabbit was bled by ear puncture, and serum was prepared. IgG was purified from the serum using ammonium sulfate precipitation and DEAE-cellulose column chromatography (38).

Immune blotting was performed by one-dimensional electrophoresis, followed by electrophoretic transfer to nitrocellulose sheets (39). Nitrocellu-

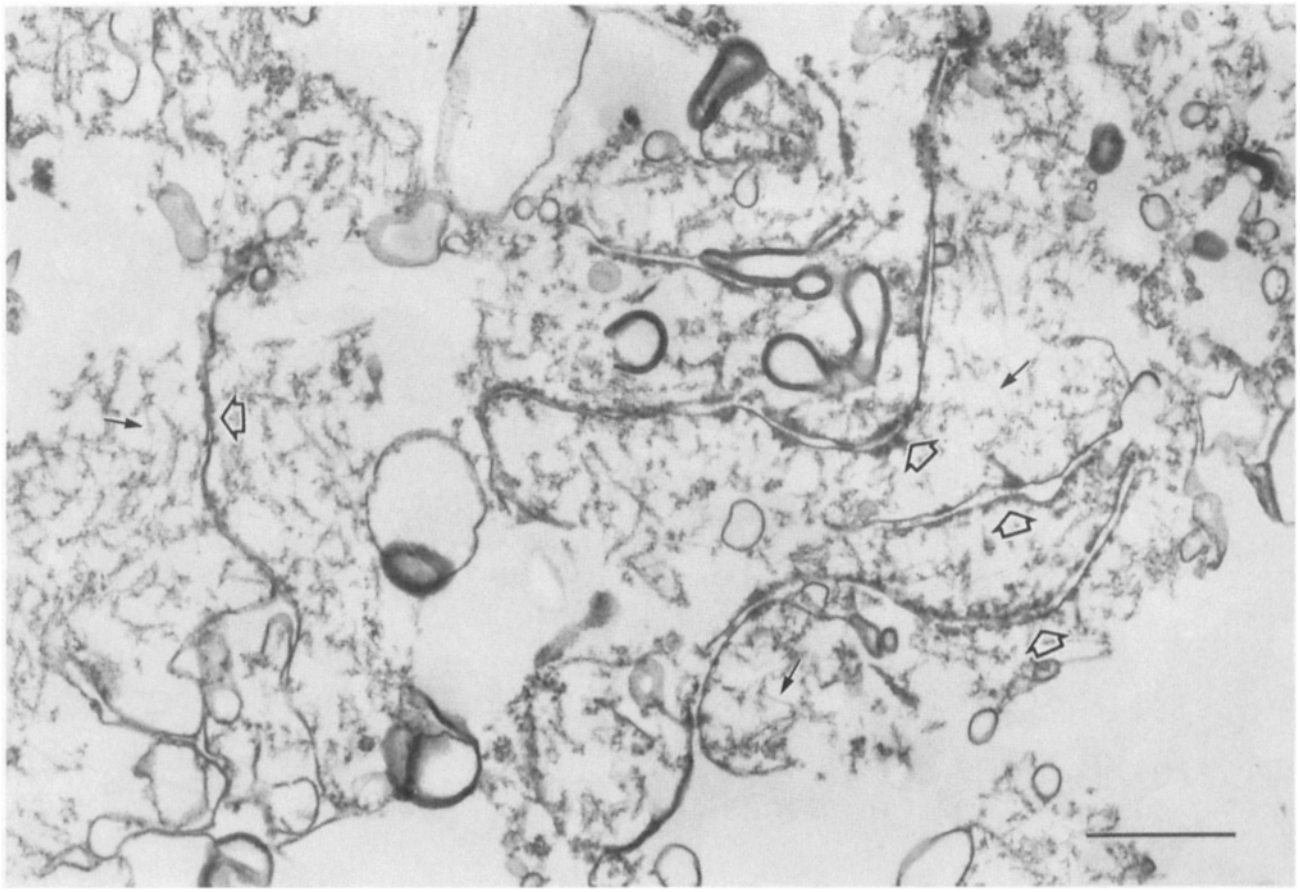

Figure 5. Thin section electron microscopy of the fraction rich in adherens junctions treated with heavy meromyosin. Note that almost all filamentous structures seen in this fraction are decorated with heavy meromyosin, forming typical arrowhead structures. *Open arrows*, isolated adherens junctions; *small arrows*, undecorated filaments. Bar 0.5 μm.

lose sheets were treated with antibody followed by horseradish peroxidase-labeled goat anti-rabbit IgG (Bio-Rad Laboratories, Cambridge, MA) and the localization of peroxidase was detected by the reaction using diaminobenzidine in the presence of Ni and Co ions (5).

For indirect immunofluorescence microscopy of cultured cells, the rat 3Y1 cells were fixed with 1% formaldehyde in PBS for 15 min at room temperature, treated with 0.2% Triton X-100 in PBS for 15 min, and washed three times with PBS. After being soaked in PBS containing 1% BSA for 10 min, the sample was treated with the first antibody diluted with PBS containing 1% BSA for 1 h in a moist chamber. It was then washed with PBS containing 1% BSA three times, followed by incubation with the second antibody (FITC-labeled goat anti-rabbit IgG) for 30 min. In some samples, rhodamine-labeled phalloidin was added in the second antibody solution to detect actin filaments. After incubation, the sample was washed with PBS three times and examined with an Olympus Vanox-S microscope. For indirect immunofluorescence microscopy of frozen sections, samples were processed as previously described (38).

Anti-desmoplakin I/II (guinea pig polyclonal antibody) was provided from Dr. A. Kusumi (University of Tokyo), and anti-cytokeratin (mouse monoclonal antibody, PKK1; specific to cytokeratin type 8,18,19) from Labsystems Inc. (Finland). Anti-chick gizzard vinculin (mouse monoclonal antibody) was produced in our laboratory by Mr. T. Yagi.

Results

Morphological Evaluation of the Isolation Procedure

In the liver, hepatic cells are arranged to form laminae, one to two cells thick, and the apposed sides of neighboring cells form a bile canaliculus between them. The principle and essential steps of our newly developed procedure for the isolation of adherens junctions from rat liver are summarized in Fig. 1. As the first step, the fraction rich in bile canaliculi ("B.C. fraction") was isolated according to a modification of the method developed by Song et al. (35). In the second step, the fraction rich in the long belts of adherens junctions ("A.J. fraction") was recovered from the B.C. fraction by treatment with NP-40.

At first, we evaluated this isolation procedure using rhodamine-phalloidin at the light microscopic level (Fig. 2). Three fractions (liver homogenate, B.C. fraction, and A.J. fraction) were examined by phase-contrast and fluorescence microscopy after rhodamine-phalloidin treatment. In liver homogenate, among various types of structures were clearly identified the bile canaliculi, which were specifically stained with rhodamine-phalloidin. The B.C. fraction was characterized by a large number of membrane fragments in the phase-contrast image, and fluorescence microscopy revealed that each membrane fragment contained one or more bile canaliculi. In sharp contrast with B.C. fraction, the A.J. fraction was composed of long thin strands, all of which were intensively stained with rhodamine-phalloidin. These thin strands were occasionally aggregated. Taken together with the diagram in Fig. 1, these results seemed to show that the membrane fragments in B.C. fraction and the thin strands in A.J. fraction might correspond to the isolated bile canaliculi

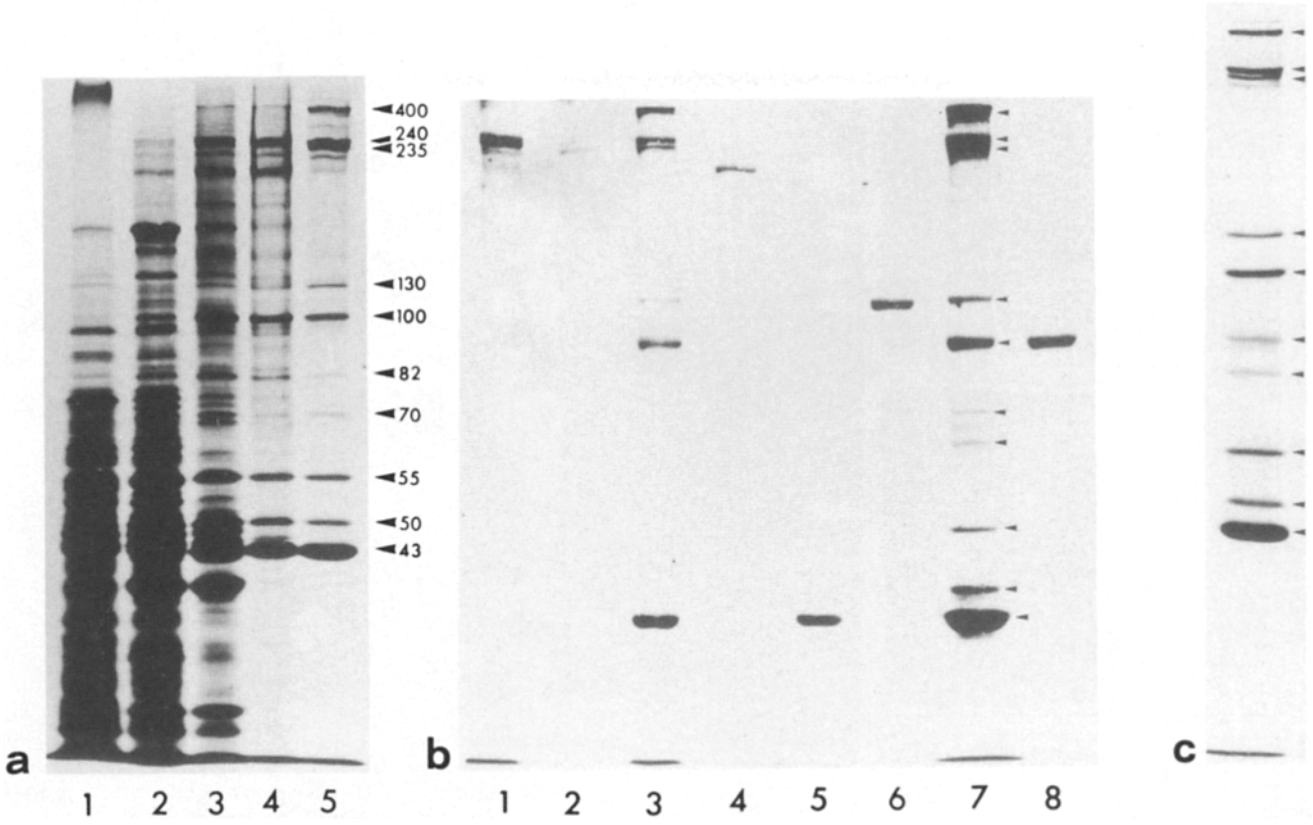

Figure 6. (*a*) Changes in electrophoretic banding patterns during isolation procedures of adherens junction. (Lane *1*) Rat whole liver; (lane *2*) pellet after homogenization of liver with hypotonic solution and centrifugation; (lane *3*) fraction rich in bile canaliculi; (lane *4*) fraction rich in adherens junction; (lane *5*) low salt extract from adherens junction (A.J. extract). (*b*) Comparison of molecular masses between low salt extract of adherens junction (lanes *3* and *7*), rat brain spectrin (fodrin; lane *1*), chick talin (lane 2), rabbit myosin (lane *4*), rabbit actin (lane *5*), chick vinculin (lane *6*), and chick α-actinin (lane *8*). (*c*) Typical banding pattern of the A.J. extract. In this extract, 10 major polypeptides (*arrowheads*) are reproducibly identified. Judging from *b*, the molecular masses of these polypeptides are estimated as 400, 240, 235, 130, 100, 82, 70, 55, 50, and 43 kD, from the top.

and the belts of adherens junctions, respectively. To confirm this interpretation, each fraction was examined by thin-section electron microscopy.

As shown in Fig. 3, in the B.C. fraction numerous bile canaliculi were clearly identified. The isolated bile canaliculi consisted of the plasma membrane forming the canaliculi wall, the junctional complex (tight junction, adherens junction with actin filaments, and desmosome with intermediate-sized filaments), and the apposed plasma membranes. Gap junctions were occasionally found. In the A.J. fraction, almost all plasma membranes disappeared, with the exception of the membranes with well-developed undercoats; a small number of membrane vesicles without undercoats were also observed to be scattered in this fraction (Fig. 4). The major components of this fraction were the belts of adherens junctions, which were composed of the apposed plasma membrane, two rows of electron-dense undercoats, and the associated actin filaments. When this fraction was treated with heavy meromyosin prepared from rabbit skeletal muscle, almost all filamentous structures observed in this fraction were decorated, forming typical arrowhead structures; close inspection revealed that only a small number of undecorated filaments, probably 10-nm filaments, were scattered to be intermingled with a large number of decorated actin filaments (Fig. 5). These observations indicate that contamination of the desmosome is, at least morphologically, negligible.

Biochemical and Immunological Evaluation of the Isolation Procedure

Using the condition in which vinculin was extracted from the membrane fraction of the chick gizzard, we have treated the A.J. fraction to extract the protein of the undercoat (1, 9). As shown in Fig. 4 *c*, this treatment removed almost all the electron-dense undercoat, leaving the vesiculated smooth membranes without underlying structures. We tentatively call this extract the "A.J. extract."

First, the electrophoretic banding pattern of each fraction was compared (Fig. 6 *a*). The most characteristic feature of the changes in the banding pattern during isolation of adherens junction was that high molecular mass proteins were enriched in the B.C. fraction and further in the A.J. fraction. The banding pattern of the A.J. fraction was similar to that of the A.J. extract. Interestingly, most of the major polypeptides of the A.J. extract were not predominant in the starting material, the whole liver. 10 major polypeptides were reproducibly identified in the A.J. extract (Fig. 6 *c*). When purified rat fodrin, chick talin, rabbit myosin, chick vincu-

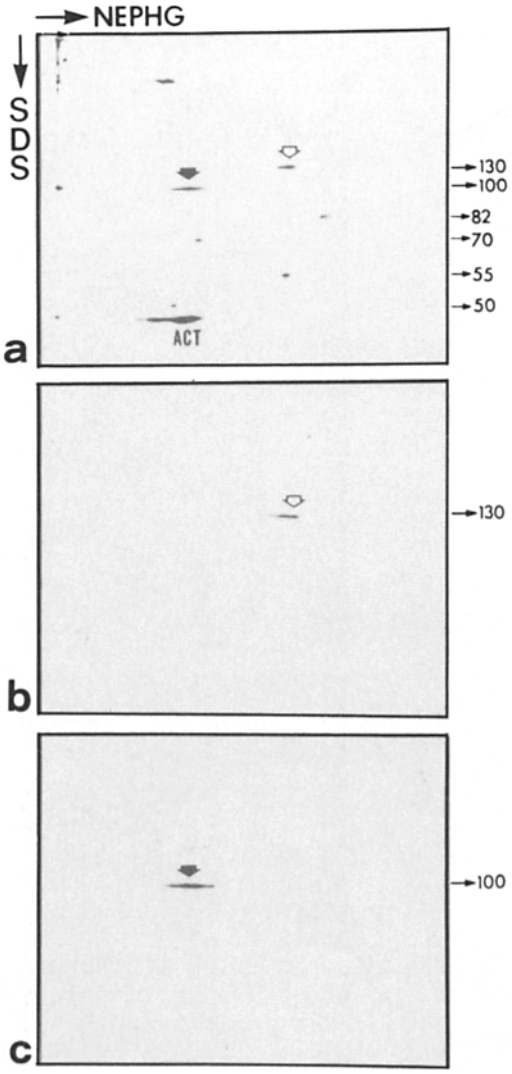

Figure 7. Two-dimensional electrophoretic profiles of the A.J. extract (*a*), chick vinculin (*b*), and chick α-actinin (*c*). The samples are applied on the acidic sides in the isoelectric focusing. The gels are stained by the silver staining method. *Closed arrows*, α-actinin; *open arrows*, vinculin; *ACT*, actin. Molecular mass of each spot is shown in the right.

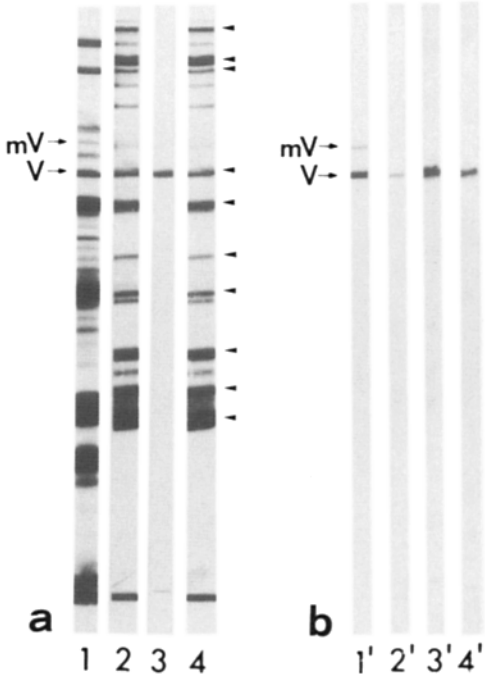

Figure 8. One-dimensional gel electrophoresis (*a*) and accompanying immunoblot (*b*). (*a* and *b*) Lane *1*, vinculin-enriched fraction obtained from chick gizzard. Lanes *2* and *4*, A.J. extract. Lane *3*, 130-kD protein that is electrophoretically isolated from A.J. extract. *V*, vinculin; *mV*, metavinculin. Lanes *1'* and *2'*, monoclonal antibody against chick gizzard vinculin. Lanes *3'* and *4'*, polyclonal antibody against 130-kD protein of A.J. extract. *Arrowheads*, 10 major polypeptides identified in A.J. extract.

lin, chick α-actinin, and rabbit actin were subjected to electrophoresis together with the A.J. extract, the molecular masses of these 10 polypeptides were estimated as approximately 400, 240, 235, 130, 100, 82, 70, 55, 50, and 43 kD (Fig. 6 *b*). The mobility of 130, 100, and 43 kD was identical to that of vinculin, α-actinin, and actin, respectively. Fig. 7 shows the two-dimensional electrophoretic profile of the A.J. extract, vinculin, and α-actinin, indicating that 130- and 100-kD polypeptides in the A.J. extract showed the same isoelectric point as purified vinculin and α-actinin, respectively.

To biochemically evaluate our newly developed procedure for the isolation of cell-to-cell adherens junction, a key question is whether vinculin is enriched in the A.J. extract. As shown in Figs. 6 *b* and 7, one of the major polypeptides of the A.J. extract with a molecular mass of 130 kD showed the same electrophoretic behavior as that of authentic chick gizzard vinculin. Some of the monoclonal antibodies against chick vinculin weakly reacted with this 130-kD polypeptide (Fig. 8). Furthermore, we have purified this 130-kD polypeptide electrophoretically, and raised a polyclonal antibody against this polypeptide. By immune blotting procedure, this polyclonal antibody specifically stained the chick gizzard vinculin as well as the 130-kD polypeptide (Fig. 8). Next, using this antibody, we have tested the localization of the 130-kD protein in rat-cultured fibroblasts and in rat liver cells and cardiac muscle cells (Fig. 9). In the cultured fibroblasts, under indirect immunofluorescence microscopy, the 130-kD protein-specific fluorescence was highly concentrated at the tips of the stress fibers, the so-called focal contacts (Fig. 9 *b*). In the frozen section of the rat liver, characteristic staining of the junctional complex region was revealed (Fig. 9 *c*). In the longitudinal-section view of each bile canaliculus, a pair of parallel continuous lines were visible; a pair of intensively-stained dots were observed in cross-section. In the rat heart, the intercalated discs including adherens junctions were intensively stained, and, in addition, the punctate staining was clearly detected along the side of each muscle cell, showing a typical "costamere" pattern (27; Fig. 9 *d*). Taking these findings together, we can conclude that rat vinculin was highly enriched in the A.J. fraction we have obtained here.

Next, to test whether desmosomal proteins were enriched in the A.J. fraction and the A.J. extract, an immune blot analysis using monoclonal antibody specific for desmoplakin I/II was employed (Fig. 10 *a*). In this experiment, the liver homogenate, B.C. fraction, A.J. fraction, and A.J. extract were subjected to electrophoresis, so that the amount of vinculin

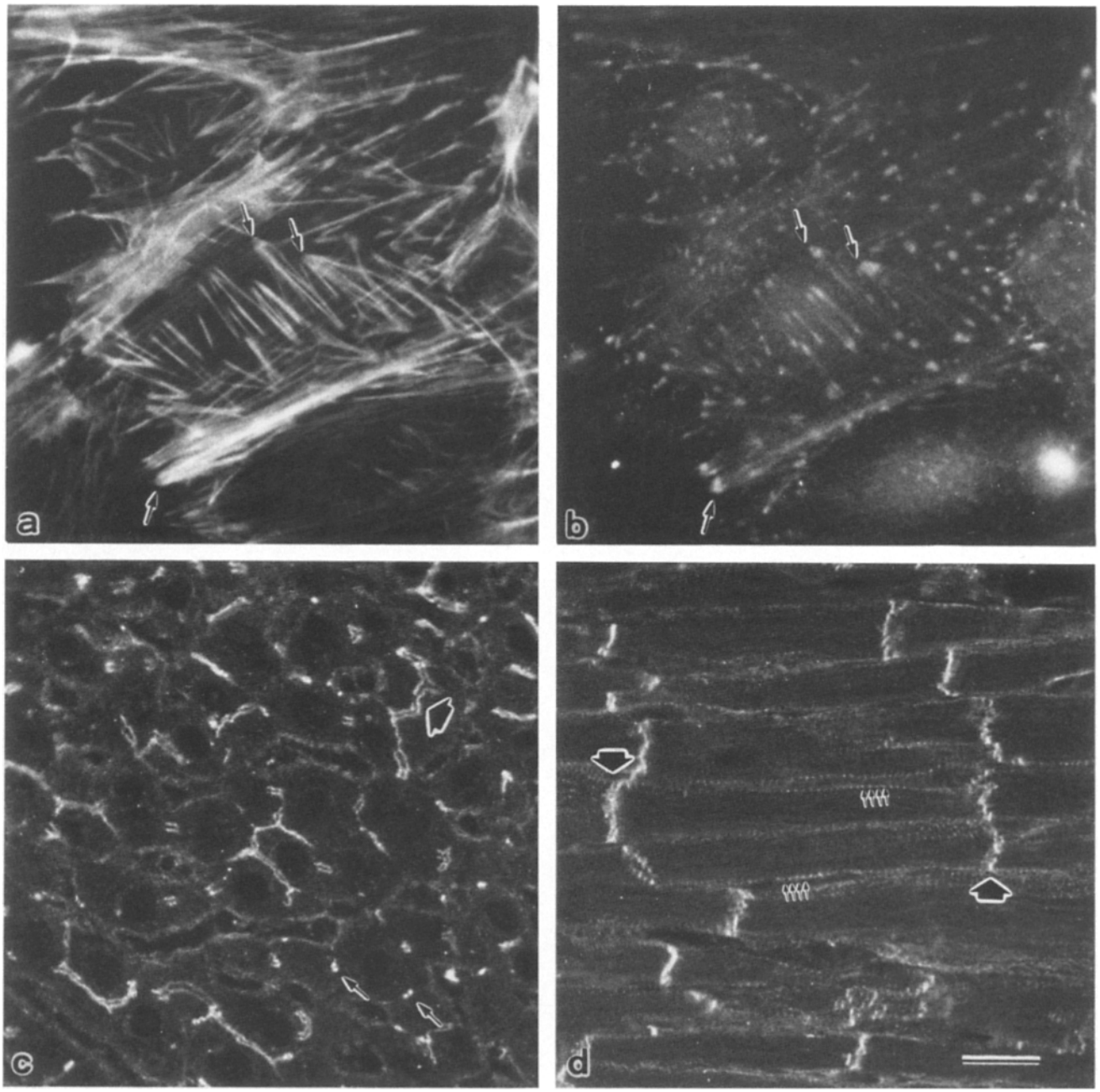

Figure 9. Localization of 130-kD protein identified in A.J. extract. (*a* and *b*) Double staining of rat cultured fibroblasts (3Y1 cells) with rhodamine-phalloidin (*a*) and anti-130-kD protein antibody (*b*). The 130-kD protein-specific fluorescence is highly concentrated at the tips of each stress fiber (*arrows*). (*c* and *d*) Indirect immunofluorescence image with anti-130-kD protein antibody of the frozen section of rat liver (*c*) and heart (*d*). In the liver, intensive staining at the junctional complex along bile canaliculi can be detected. *Large arrows*, longitudinal section view of bile canaliculus; *small arrows*, cross section view of bile canaliculus. In the cardiac muscle (*d*), the intercalated discs including adherens junctions are intensively stained (*large arrows*). Note the punctate staining pattern along the side of each cardiac cell (*small arrows*). Bar, 20 μm.

in each fraction was almost constant; the amount of vinculin was estimated by the immune blot with the polyclonal antibody to the 130-kD protein. Then, these fractions that were subjected to electrophoresis were analyzed by the immune blot method to determine the amount of desmoplakin I/II in each fraction. As a result, both in the liver homogenate and B.C. fraction, clear staining of two bands at 240 (desmoplakin I) and 200 kD (desmoplakin II) was observed, while in the A.J. fraction a very small amount of desmoplakin was detected, indicating that the ratio of adherens junction/desmosome increased remarkably during the isolation procedure. No desmoplakins were detected in the A.J. extract. These results indicate that only a small amount of desmosomes are found biochemically in the A.J. fraction; the desmosomal plaque proteins are not extracted under the condition used in this study.

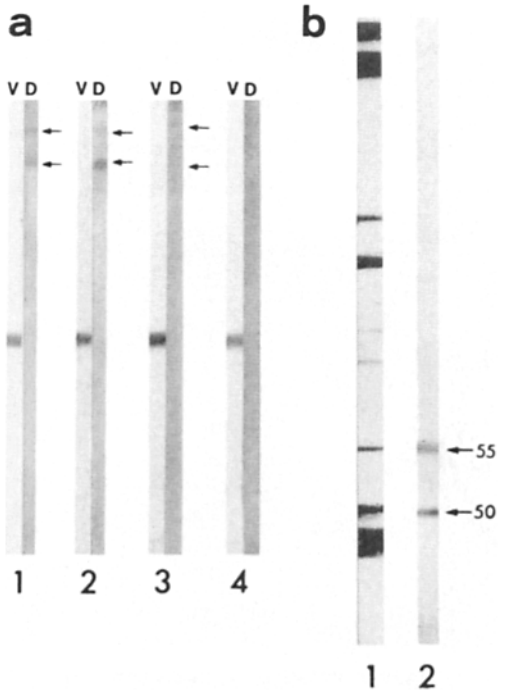

Figure 10. Immune blot analyses of the occurrence of desmoplakins (*a*) and cytokeratins (*b*) in the fraction rich in adherens junctions. (*a*) The liver homogenate (lane *1*), B.C. fraction (lane *2*), A.J. fraction (lane *3*), and A.J. extract (lane *4*) were subjected to electrophoresis so that the amount of vinculin in each fraction was almost constant; the amount of vinculin was estimated by the immune blot with the polyclonal antibody to the 130-kD protein (*V*). Then, these fractions that were subjected to electrophoresis were analyzed by the immune blot method to determine the amount of desmoplakin I/II (*D*) in each fraction. Both in the liver homogenate (lane *1*) and B.C. fraction (lane *2*), clear staining of two bands (*arrows*) was observed, while in the A.J. fraction (lane *3*) a very small amount of desmoplakin was detected. No desmoplakins were detected in the A.J. extract (lane *4*). (*b*) One-dimensional electrophoresis of A.J. extract (lane *1*) and accompanying immunoblot with anti-cytokeratin antibody (lane *2*). This antibody has recognized 55- (*55*) and 50-kD polypeptides (*50*).

Finally, to study the possibility that the 55 and 50-kD polypeptide in the A.J. fraction and the A.J. extract may be identical to the hepatocyte cytokeratins (type 18 and 8, respectively), we have performed the immune blot analysis with the monoclonal antibodies specific to these cytokeratins (Fig. 10 *b*). As a result, interestingly, this monoclonal antibody has recognized the 55 and 50-kD polypeptides. Judging from the results that in electron microscopy the intermediate-sized filaments were observed to be very small in number in the A.J. fraction (see Fig. 5) and that the amount of desmoplakins in the A.J. fraction was very small, it may be reasonable to speculate that nonfilamentous form of cytokeratin or very short cytokeratin filaments are associated with the adherens junctions, nonspecifically or specifically. A similar situation is known in the case of the isolated desmosomes; actin can be detected as one of the major components of the isolated desmosomes. Further studies are required to know the exact meaning to the occurrence of the cytokeratins in the A.J. fraction and the A.J. extract.

Discussion

So far, the structural aspects of the adherens junctions have been intensively studied, mainly using intestinal or retinal pigmented epithelial cells and cardiac muscle cells (7, 10, 25, 26, 36). However, in both types of cells, well-developed bundles of actin filaments ("circumferential microfilament bundles" and "myofibrils," respectively) were tightly associated with the adherens junctions, so that it was very difficult to isolate the adherens junction proper only with the undercoat from these tissues. In this sense, hepatic cells appeared to be appropriate for the isolation of the cell-to-cell adherens junctions. In this study, we have successfully developed a new isolation procedure of the cell-to-cell adherens junctions from rat liver. In the fraction obtained, the adherens junctions were highly enriched both at light and electron microscopic levels. These isolated adherens junctions were characterized by a prominent undercoat and associated short actin filaments. Furthermore, one- and two-dimensional electrophoresis and immunological analyses have revealed that vinculin was highly enriched in this fraction. Given our criteria for the isolation of the adherens junction described in the introduction, we conclude that we have succeeded in isolating the cell-to-cell adherens junction from rat liver.

Since no biochemical enrichment procedure has been available so far for adherens junctions, the immunological approach was the only way to identify the components of the undercoats of this type of junction. Vinculin is the first protein that has been reported to be localized exclusively in the cell-to-cell and cell-to-substrate adherens junctions (9, 10). α-Actinin was found in both types of adherens junctions, but this protein was also localized in the stress fibers in cultured nonmuscle cells and in the myofibrils in muscle cells (22). The other adherens junction-specific protein reported is $pp60^{src}$, which is the product of the oncogene of RSV and a tyrosine-specific protein kinase (29, 31). This kinase is known to localize in both cell-to-cell and cell-to-substrate junctions. Recently, one of the constituents of the undercoats of desmosomes has been reported to be identified immunologically in the cell-to-cell adherens junctions, but not in the cell-to-substrate adherens junctions (4). However, the detailed biochemical properties of this protein, called 'plakoglobin,' have not yet been clarified. Except for α-actinin, none of these proteins shows a specific affinity for actin filaments. Taken together, the following questions have naturally arisen. Is there a unique protein that specifically interacts with vinculin in the undercoats of the cell-to-cell adherens junction? What type of molecule anchors the actin filaments to the adherens junction? What is the substrate for $pp60^{src}$? How is the adhesion molecule connected to the undercoat and to actin filaments? To answer these questions, more detailed and systematic analyses of the constituents of undercoat of cell-to-cell adherens junctions are needed. Our newly developed isolation method will make such systematic analyses possible.

In the low-salt extract from isolated adherens junction, 10 polypeptides were predominant. Three of them were identified as vinculin (130 kD), α-actinin (100 kD), and actin (43 kD) in this study; all three are known to be localized in or associated with the undercoats of the cell-to-cell adherens junction. Therefore, it is probable that the other seven polypeptides are also the constituents of the undercoats of the cell-to-cell adherens junction. Of course, the possibility can

not be excluded that some of these proteins are nonspecifically associated with adherens junctions through the isolation procedure. Actually, in this study, it was suggested that the 55 and 50-kD polypeptides were cytokeratins, although at present it is not clear whether the occurrence of these cytokeratins is specific or nonspecific. Therefore, in the next step, antibodies specific for each polypeptide (seven polypeptides) must be developed to localize each protein. In our laboratory, recently, we have succeeded in obtaining specific antibodies for the 400-, 235-, 82-, and 70-kD polypeptides, and succeeded in showing that these four polypeptides are localized in the undercoat of cell-to-cell adherens junction, not only in the liver but also in various types of tissues (manuscripts in preparation).

Our present data appear to suggest that the molecular organization of the undercoats of adherens junction is not simple. However, we believe our newly developed isolation procedure will lead us to a better understanding of the molecular mechanism of formation and destruction of adherens junctions and of the regulation of cell-to-cell adhesion through the underlying cytoskeleton.

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