Monoclonal Antibody 7H6 Reacts with a Novel Tight Junction-Associated Protein Distinct from ZO-1, Cingulin and ZO-2

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Abstract. The tight junction is an essential element of the intercellular junctional complex; yet its protein composition is not fully understood. At present, only three proteins, ZO-1 (Stevenson, B. R., J. D. Siliciano, M. S. Mooseker, and D. A. Goodenough. 1986. *J. Cell Biol.* 103:755–766), cingulin (Citi, S., H. Sabanay, R. Jakes, B. Geiger, and J. Kendrick-Jones. 1988. *Nature (Lond.).* 333:272–275) and ZO-2 (Gumbiner, B., T. Lowenkopf, and D. Apatira. 1991. *Proc. Natl. Acad. Sci. USA.* 88:3460–3464) are known to be associated with the tight junction. We have generated a monoclonal antibody (7H6) against a bile canaliculus-rich membrane fraction prepared from rat liver. This 7H6 antigen was preferentially localized by immunofluorescence at the junctional complex regions of hepatocytes and other epithelia, and 7H6-affiliated gold particles were shown electron microscopically to localize at the periphery of tight junctions. Immunoblot analysis of a bile canaliculus-rich fraction of rat liver using 7H6, anti-ZO-1 antibody (R26.4C), and anti-cingulin antibody revealed that 7H6 reacted selectively with a 155-kD protein, whereas R26.4C reacted only with a 225-kD protein. Anti-cingulin antibody reacted solely with 140 and 108-kD proteins, indicating that the protein recognized by 7H6 is immunologically different from ZO-1 and cingulin. Immunoprecipitation of detergent extracts obtained from metabolically labeled MDCK cells with R26.4C coprecipitated a 160-kD protein, which corresponds to ZO-2, with ZO-1. However, 7H6 did not react with the 160-kD protein. These results strongly suggest that the 7H6 antibody recognizes a novel tight junction-associated protein different from ZO-1, cingulin and ZO-2.

THE tight junction, or zonula occludens, is an essential element of the intercellular junctional complex, which functions as a selective permeable barrier to ions and small molecules (Farquhar and Palade, 1963; Staehlin, 1973; Gumbiner, 1987; Stevenson et al., 1988; Cereijido et al., 1989). The tight junction also acts as a barrier to proteins and some lipids of plasma membranes, thus, acting to maintain the polarized cell surface domains (Martinez-Paloma and Erlij, 1975; Dragsten et al., 1981; Roman and Hubbard, 1984; Van Meer et al., 1986).

Earlier electron microscopic studies of freeze-fracture replicas revealed that the tight junction is composed of a belt-like band of anastomotic strands (Goodenough and Revel, 1970; Claude, 1973; Claude and Goodenough, 1978; Pinto de Silva and Kachar, 1982; Stevenson and Goodenough, 1984). This structure is maintained even after exposure of membrane fractions to detergents (Stevenson and Goodenough, 1984; Anderson et al., 1988), which suggests that the fundamental structure of the tight junction consists of integral membrane proteins. However, the protein composition of the tight junction is not yet fully understood.

Recent studies have disclosed three tight junction-associated proteins, ZO-1, cingulin and ZO-2. ZO-1 (Stevenson, 1986) is a high-molecular-weight (210–225 kD) protein localized at the cytoplasmic surface of the tight junction membrane, originally identified in the mouse liver. ZO-1 is found in a wide variety of mammalian organs (Anderson et al., 1988, 1989; Schnabel et al., 1990). Although the molecular weight of ZO-1 varies slightly depending on the species of origin, ZO-1 from rat organs is a 225-kD polypeptide. Cingulin (Citi et al., 1988) is a 140- and 108-kD protein originally isolated from the chicken intestinal brush border fraction. Cingulin is localized near the vinculin-rich cytoskeletal bundle at the periphery of tight junctions in avian organs (Citi et al., 1989), and is also found in such mammalian cells as MDCK cells (Stevenson et al., 1989), and normal and neoplastic human colonic epithelial cells (Citi et al., 1991). ZO-2 is a 160-kD tight junction-associated protein, recently identified by coimmunoprecipitation with ZO-1 from metabolically labeled MDCK cells (Gumbiner et al., 1991), but its tissue distribution is not yet known.

In this paper, we report evidence that a new monoclonal

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antibody we have generated recognizes a tight junction-associated protein of 155 kD in rat organs and 175 kD in MDCK cells which is different from ZO-1, cingulin and ZO-2, in its molecular weight and immunoreactivity.

Materials and Methods

Preparation of Bile Canaliculus-Rich Fraction

A bile canaliculus-rich fraction was prepared from the livers of 8-wk-old Fischer 344 (F344) rats (Charles River Japan, Inc., Yokohama, Japan) according to the method of Song et al. (1969), with a slight modification. In each isolation, three rats were used. All procedures were carried out at 4°C.

Approximately 20 g of liver tissues was homogenized in 1 mM $NaHCO_3$ (pH 8.0) containing 2 μg/ml leupeptin, 0.5 μg/ml pepstatin and 0.5 μg/ml chymostatin (hypotonic solution), which was diluted with a hypotonic solution to 400 ml, filtered twice through four layers of gauze, and centrifuged at 1,500 *g* for 10 min. The pellet was resuspended in the hypotonic solution to 13.1 ml, to which 96.9 ml of 55% sucrose solution was added to produce a final sucrose concentration of 48.45%. The sample was placed under a 42.9% sucrose solution in 25 × 75 mm tubes, and centrifuged for 60 min at 10,000 *g*. The layer at the 42.9/48.5% interface was collected and resuspended in an equal volume of extraction buffer (EDTA), 1.0 μg/ml leupeptin, 0.5 mM PMSF, 2 mM Tris-HCl, pH 9.2 and then was centrifuged for 30 min at 2,600 *g*, after which the precipitate was immediately frozen at −70°C, until it was used as an immunogen. All chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

Monoclonal Antibody Production

Monoclonal antibodies were generated essentially according to the procedure of Köhler and Milstein (1975). BALB/c mice were immunized intraperitoneally with 1.0 mg of the above-mentioned immunogen. Splenocytes isolated from two mice were fused with NS-1 myeloma cells, and the resultant hybrid cells were transferred to 96-well plates. The supernatants were screened by ELISA using the immunogen and by indirect immunofluorescence of frozen sections of F344 rat liver. A subsequent indirect immunofluorescence assay of ELISA-positive clones identified 7H6 as an antibody which reacted with the junctional-complex regions of hepatocytes.

Gel Electrophoresis and Immunoblotting

SDS-PAGE analysis was carried out based on the discontinuous Tris-glycine system of Laemmli (1970) and Matsudaira and Burgess (1978). Freshly prepared bile canaliculus-rich fractions of the liver, and whole homogenates of the liver, intestine, and kidney of F344 rats were placed in SDS sample buffer containing protease inhibitors (Citi et al., 1986) and immediately boiled for 3 min. After applying the samples to polyacrylamide gradient gels (4–20%) to undergo electrophoresis, the gels were either stained with Coomassie brilliant blue R-250, or transferred electrophoretically onto a 0.45 μm nitrocellulose membrane for immunoblot analysis (Towbin et al., 1979; Johnson et al., 1984). Protein was determined according to the method of Hartree (1972).

For immunoblot analysis, 7H6 supernatant was diluted 1:10 with PBS. The anti-ZO-1 monoclonal antibody (R26.4C) purchased from Chemicon International Inc. (Temecula, CA) was diluted 1:100 to 1:500 with PBS. The anti-cingulin polyclonal antibody, kindly provided by Dr. S. Citi, Department of Cell Biology and Anatomy, Cornell University Medical College (New York), was diluted 1:5,000 to 1:10,000 with PBS. The second antibody was an affinity-purified goat antiserum to mouse IgM, rat IgG, or an affinity-purified swine antiserum to rabbit IgG conjugated with HRP, diluted 1:1,000 to 1:5,000 with PBS. The immunoblot was detected by the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham International PLC. Amersham, UK), being covered with the detection reagents for 1 min at room temperature, and then exposed to Hyperfilm™ (Amersham) for 3 min.

Immunohistochemistry

Light microscopy: Frozen sections (4–6 μm thick) of rat tissues including liver, kidney, intestine, lung, uterus, testis, and brain, applied to albumin-coated glass slides were dipped in acetone at −20°C for 20 min, and incubated with 7H6 supernatant, R26.4C diluted to 1:100 or anti-cingulin antibody diluted to 1:500 for 1 h. After several washes with PBS, the second antibody, an FITC-conjugated goat anti-mouse IgM, goat anti-rat IgG, or swine anti-rabbit IgG (Amersham) diluted to 1:50 with PBS, was applied for 1 h.

Electron microscopy: an isolated bile canaliculus-rich fraction of rat liver was centrifuged at 15,000 *g* for 10 min, resuspended in TBS (50 mM NaCl in 10 mM Tris-HCl at pH 8.0) and pelleted again. The fraction was resuspended and incubated with 7H6 supernatant for 3 h. After washing with TBS for 10 min, three times, the fraction was incubated with the second antibody (goat anti-mouse IgM) coupled with 5 nm gold particles (Amersham) for 2 h. After washing with TBS for 10 min three times, the fraction was fixed for 2 h with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4, postfixed with 1% OsO_4 for 1 h, and stained with 1% aqueous uranyl acetate. The fraction was then dehydrated with ascending concentrations of ethanol, and embedded in Epon 812. Thin sections were examined with a 1200 EX electron microscope (JEOL, Tokyo, Japan) after lead citrate staining.

Cell Culture and Labeling

MDCK cells were obtained from the American Type Culture Collection (Rockville, MD) and were cultured in DME supplemented with 5% FCS (Filtron PTY. LTD., Victoria, Australia) and penicillin-streptomycin (40 iu-0.1 mg/ml). When the cells were completely confluent (-7×10^7 cells/dish), they were incubated in methionine-depleted DME supplemented with 10% FCS for 30 min. They were then metabolically labeled for 12 h with [^{35}S]methionine (Amersham) at 50 μci/ml in the above-mentioned medium (4 ml of labeling medium per 75 cm^2 of cells). The cells were then washed twice with ice-cold PBS and used for immunoprecipitation.

Immunoprecipitation

Immunoprecipitation was performed according to the method of Gumbiner et al. (1991). The R26.4C hybridoma producing the anti-ZO-1 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA) and the monoclonal antibody was purified from hybridoma culture supernatants on protein G-Sepharose (Pharmacia LKB, Uppsala, Sweden) according to the manufacturer's instructions. It was coupled to CNBr-activated Sepharose CL-4B (Pharmacia) according to the instructions. 7H6 (IgM) was purified on Sepharose 6B according to the method of Hardy (1986), and coupled to CNBr-activated Sepharose CL-4B as described above. Sepharose without conjugation with anti-ZO-1 and 7H6 monoclonal antibodies was served as the control. For immunoprecipitations with anti-ZO-1-Sepharose and 7H6-Sepharose, MDCK cells (-7×10^7 cells/dish) were extracted with 1 ml of immunoprecipitation (IP) buffer (1% [vol/vol] Triton X-100, 0.5% [wt/vol] sodium deoxycholate, 0.2% [wt/vol] SDS, 150 mM NaCl, 10 mM Hepes, pH 7.4) containing the protease inhibitors PMSF 1 mM, iodoacetamide 0.5 mM, pepstatin A 1 μg/ml, leupeptin 2 μg/ml, aprotinin 4 μg/ml, antipain 10 μg/ml, benzamidine 50 μg/ml and soybean trypsin inhibitor 10 μg/ml). The extract was centrifuged at 10,000 *g* for 30 min by microcentrifugation. For preclear supernatant, a half-packed volume of activated Sepharose CL-4B was added to the extract and shook for 2 h. After centrifugation, an aliquot containing ~10^6 cpm was poured into a test tube precoated with IP buffer to which 10 μl of anti-ZO-1-Sepharose or 7H6-Sepharose was added. After shaking for 2 h at 4°C, they were pelleted and washed by microcentrifugation. Polypeptides were analyzed by SDS-PAGE and fluorography. For fluorography, the gel was fixed for 1 h in 25% isopropane/10% acetic acid, treated with Amplify™ (Amersham) for 30 min, vacuum dried and exposed to Hyperfilm at −70°C.

Immunoblotting was carried out as described above and binding of the anti-ZO-1 antibody or 7H6 was detected by the ECL Western blotting detection system.

Results

Immunofluorescent Localization of 7H6

The localization of 7H6 using indirect immunofluorescence on a cryostat section of rat liver is shown in Fig. 1. Characteristic staining of junctional complex regions of hepatocytes was seen. In the longitudinal section of the junctional complex regions, a pair of parallel lines was observed, whereas in the cross-section, a pair of intensely stained dots was seen.

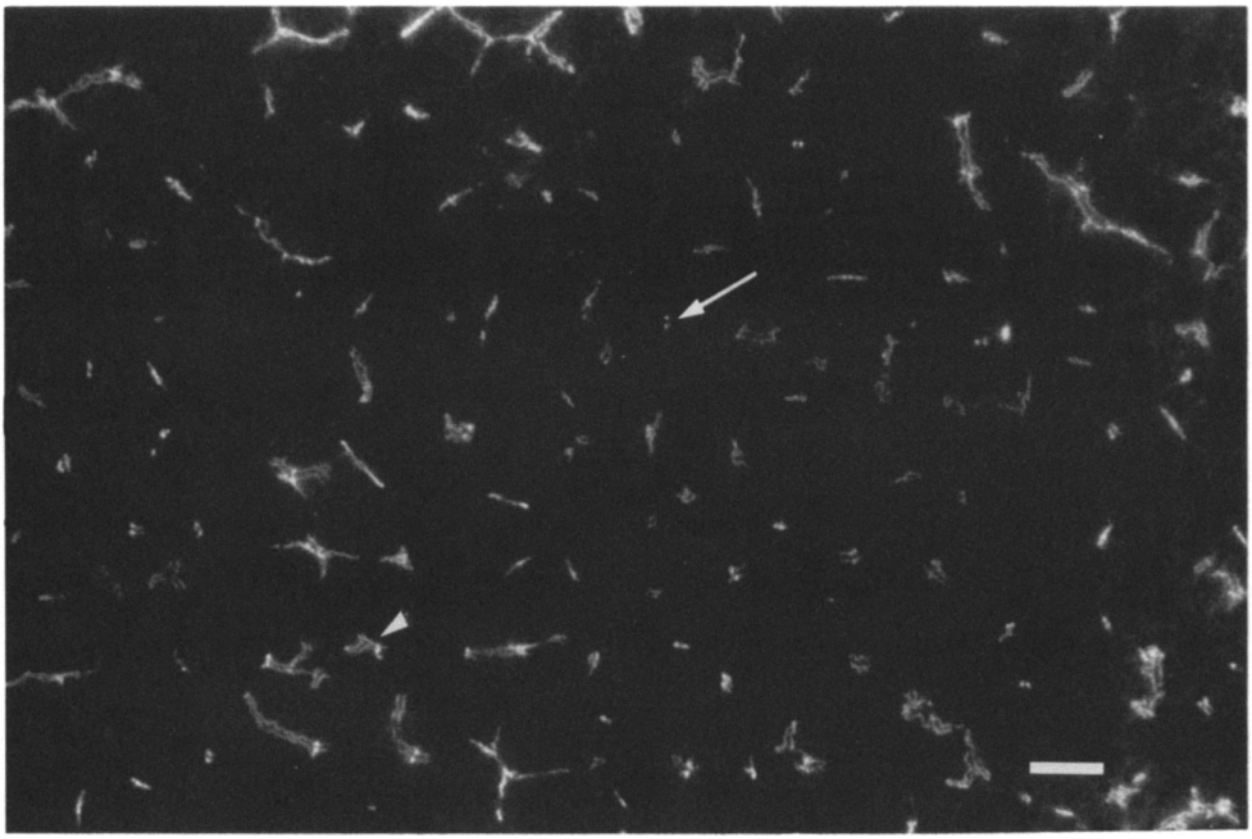

Figure 1. Immunofluorescent localization of 7H6 in a frozen section of rat liver. Intensive staining of the junctional complex regions along the bile canaliculi is seen. In cross section, a pair of dots (*arrow*) is observed, whereas in longitudinal section a pair of parallel lines (*arrowhead*) is visible. Bar, 20 μm.

In the interlobular bile duct, immunofluorescent staining was seen at the apical regions as a honeycomb-like pattern or strands, depending on the angle of sectioning (Fig. 2 *A*).

Such a honeycomb-like or a beltlike array of immunofluorescence at the cell periphery was observed in absorptive cells of the small intestine (Fig. 2 *B*), epithelial cells of the bronchus (Fig. 2 *C*), and in uterus endometrial cells (not shown). Immunofluorescent labeling with 7H6 was also seen as bright dots or strands in the subapical regions of uriniferous tubular epithelial cells of the kidney (Fig. 2 *D*), testis seminiferous tubular epithelial cells and brain ependymal cells (not shown).

Immunoblot Analysis

Immunoblot analysis of the bile canaliculus-rich fraction prepared from rat liver using 7H6 showed that 7H6 reacted with one apparent band at 155 kD (Fig. 3, lane *1'*). A similar band at 155 kD was detected in the whole homogenates of liver (Fig. 3, lane *2'*), intestine (Fig. 3, lane *3'*) and kidney (Fig. 3, lane *4'*) of rats, when the reaction was enhanced by use of an ECL western blotting detection system. These results suggest that the 7H6 antigen is most enriched in the bile canaliculus-rich fraction, but is ubiquitously present in a variety of rat organs.

Immunoelectron Microscopy

The localization of 7H6 using immunogold electron microscopy on a bile canaliculus-rich fraction prepared from rat liver is shown in Figs. 4 and 5. A low-magnification view showed that 7H6-affiliated gold particles were preferentially localized in the tight junction area. Gold particles were not detectable on any other membrane surface, including the gap junction (Fig. 4 *A*) and desmosome (Fig. 4 *B*). At higher magnification, gold particles were found in areas around the precise points of membrane contact (Fig. 5, *A–C*). Gold particles were found in areas adjacent to the tight junction as if 7H6 antigen was linked to the submembranous cytoskeleton of the tight junction (Fig. 5, *A* and *B*). The mean distance of 110 immunogold particles from the center of tight junction membranes measured along a line perpendicular to the plane of the membranes was 41.11 $\pm$ 18.50 nm.

Immunoblot Analysis of the Relationships Among 7H6 Antigen, ZO-1 and Cingulin

Immunoblot analysis of the whole homogenate prepared from rat liver using 7H6, R26.4C (anti-ZO-1) and anti-cingulin antibodies showed that 7H6 reacted only with a band at 155 kD (Fig. 6, lane *2*). On the other hand, R26.4C reacted solely with a band of high-molecular mass (>200 kD) (Fig. 6, lane *3*). Anti-cingulin antibody reacted with two bands at 140 and 108 kD (Fig. 6, lane *4*), but not with those at 155 and 225 kD. These results indicate that 7H6 antigen is immunologically unrelated to ZO-1 and cingulin.

Immunoprecipitation Analysis of the Relationship between 7H6 Antigen and ZO-2

The 7H6 antigen and ZO-1 were extracted from [^{35}S]methi-

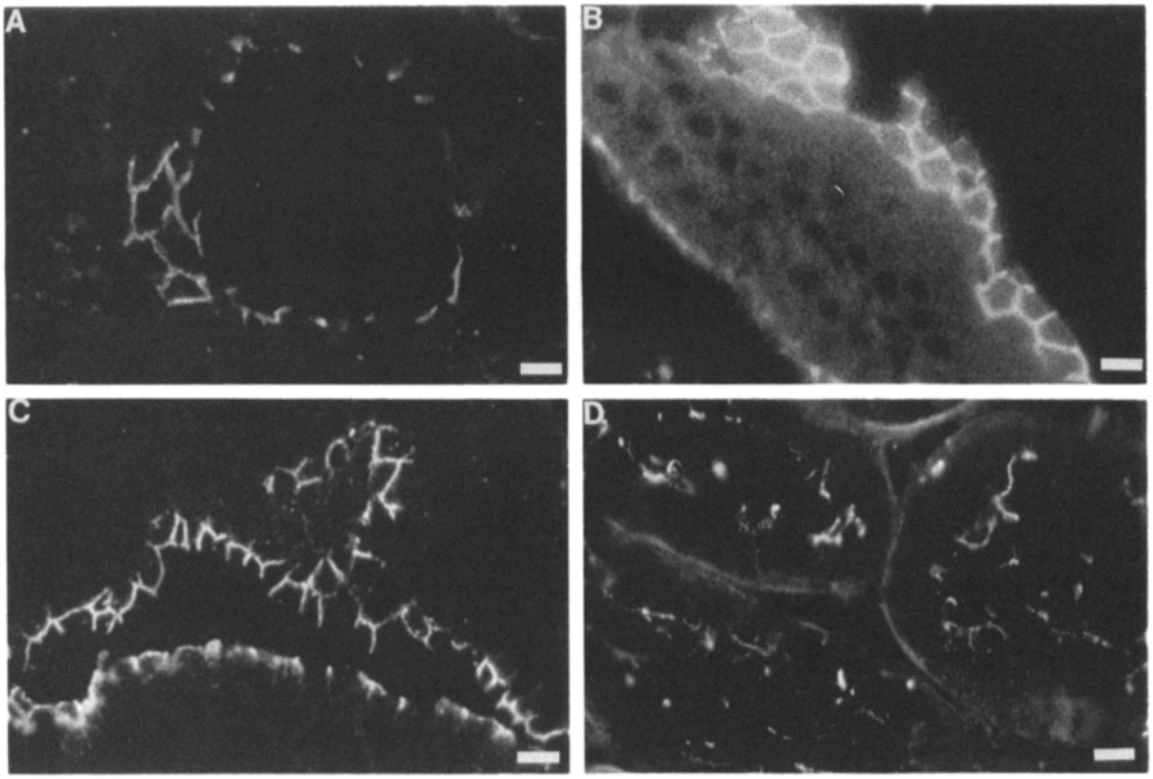

Figure 2. Immunofluorescent localization of 7H6 in a frozen section of rat liver (*A*), small intestine (*B*), lung (*C*), and kidney (*D*). A honeycomb-like fluorescence pattern is detected in liver interlobular bile duct cells (*A*), in the apical brush border of absorptive cells of the intestine (*B*), and in lung bronchial epithelial cells (*C*). Bright dots and strands are seen at the subapical regions of kidney uriniferous tubular epithelial cells (*D*). Bars, 10 μm.

onine-labeled MDCK cells mixed with nonionic detergents, deoxycholate and SDS. When ZO-1 was immunoprecipitated from the extract with the R26.4C antibody directly coupled to Sepharose, a 160-kD protein which corresponded closely with ZO-2 was observed to coimmunoprecipitate (Fig. 7, lane *2*).

The anti-ZO-1 antibody immunoprecipitates that contained the ^{35}S-labeled high molecular mass (ZO-1) and 160-kD protein (ZO-2) were immunoblotted with 7H6. 7H6 detected neither ZO-1 nor ZO-2 (Fig. 7, lane *4*). On the other hand, when the 7H6 antigen was immunoprecipitated from

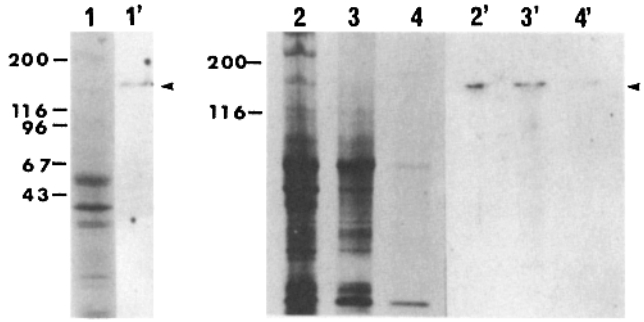

Figure 3. Immunoblot analysis of plasma membrane protein solubilized from rat tissues. Lanes *1'* to *4'* were treated with 7H6, whereas lanes *1* to *4* were stained with Coomassie blue. Lanes *1* and *1'*, the bile canaliculus-rich fraction of liver. Lanes *2* and *2'*, whole liver homogenate. Lanes *3* and *3'*, whole intestine homogenate. Lanes *4* and *4'*, whole kidney homogenate.

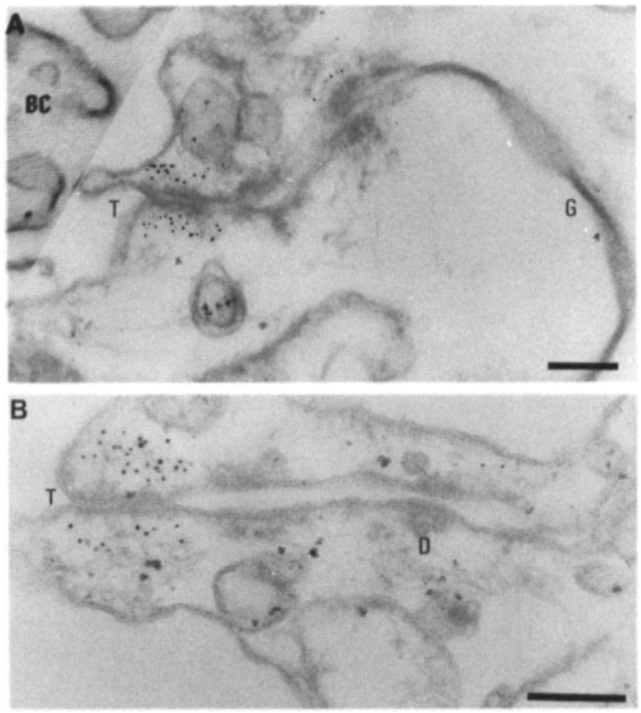

Figure 4. Immunoelectron microscopic localization of 7H6 in the bile canaliculus-rich fraction prepared from rat liver. Colloidal gold particles are heavily localized at the cytoplasmic surface of the tight junction (*T*) near the bile canaliculus (*A* and *B*). No gold particles are found in the gap junction (*G* in 4 *A*), or desmosome (*D* in 4 *B*). Bar, 100 nm.

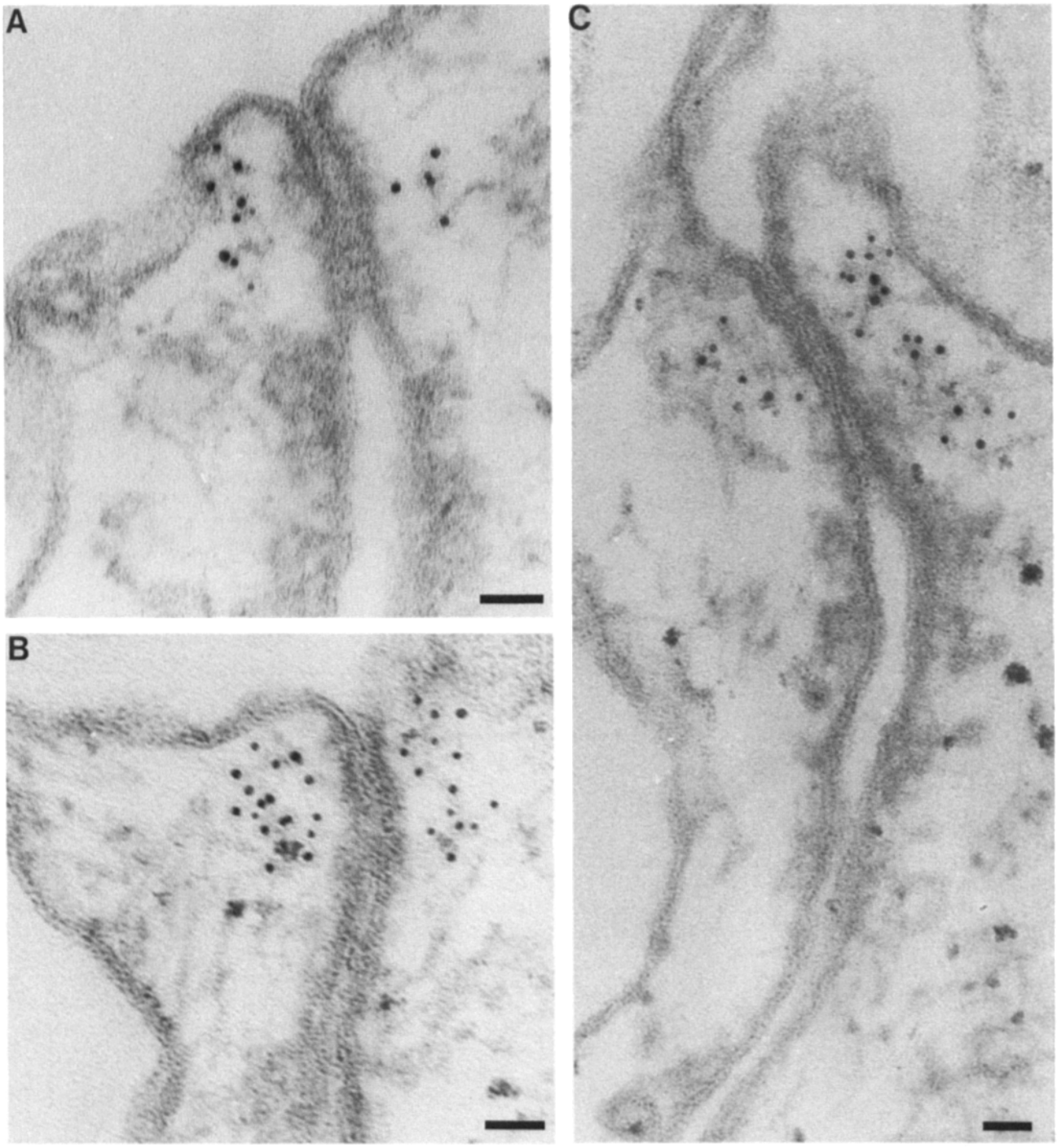

Figure 5. Immunoelectron microscopic localization of 7H6 in the bile canaliculus-rich fraction prepared from rat liver (5, *A–C*). Colloidal gold particles are found along the fine fibrils associated with the tight junction at the precise points of membrane contact (5, *A* and *B*). Bar, 20 nm.

the extract of labeled MDCK cells with 7H6 coupled to Sepharose, neither ZO-1 nor ZO-2 was coimmunoprecipitated (Fig. 7, lane *1*). Immunoblot analysis of the immunoprecipitate showed that the 7H6 antigen in MDCK cells was 175 kD (Fig. 7, lane *3*).

Discussion

We have identified a monoclonal antibody (7H6) recognizing a tight junction-associated protein. This antibody was generated with a bile canaliculus-rich membrane fraction prepared from rat liver as the immunogen. Immunofluorescent localization of 7H6 in frozen sections of rat liver indicated that the 7H6 antigen was preferentially present at the junctional complex regions of hepatocytes. The parallel-line pattern and paired-dot pattern of immunofluorescence were identical with those demonstrated by Stevenson et al. (1986) in frozen sections of rat liver using anti-ZO-1 antibody. A similar immunofluorescence pattern was demonstrated in the chicken liver using anti-cingulin antibody by Citi et al.

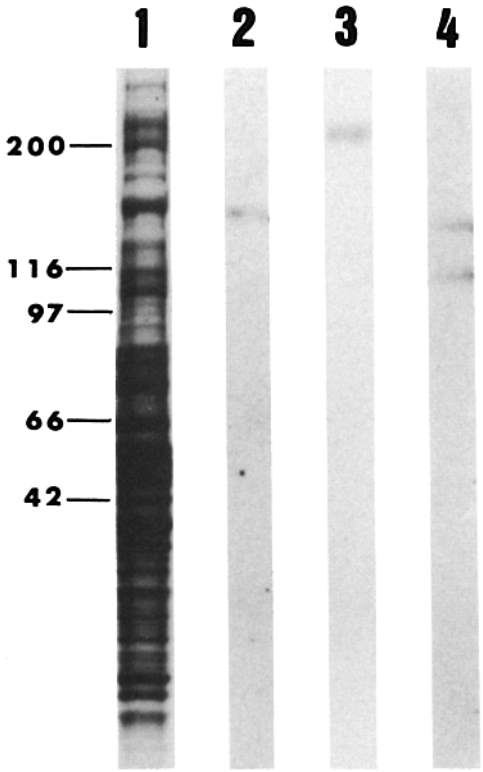

Figure 6. Immunoblot analysis of the whole liver homogenate using 7H6, R26.4C and anti-cingulin antibodies. Lane *1* is stained with Coomassie blue. Lane *2* is probed with 7H6, which reacts solely with a 155-kD protein. Lane *3* is probed with R26.4C, which reacts with a 225-kD ZO-1 protein. Lane *4* is probed with anti-cingulin antibody. Anti-cingulin antibody reacted with two bands at 140 and 108 kD, but not with the 155-kD protein.

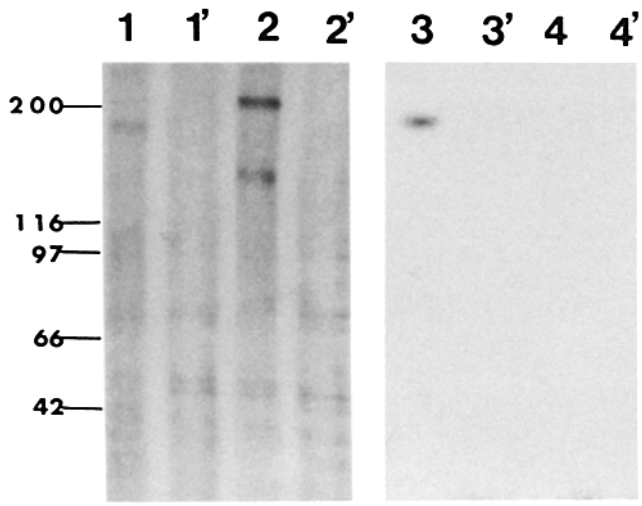

Figure 7. Immunoprecipitation analysis of the extract from [^{35}S]-methionine-labeled MDCK cells using 7H6 and R26.4C. Lanes *1* and *2* are fluorographs of 7H6 and anti-ZO-1-immunoprecipitates, respectively. Lanes *1'* and *2'* are fluorographs of precipitates using Sepharose without 7H6 or R26.4C antibodies. Lanes *3* and *4* are immunoblots of the immunoprecipitates shown in lanes *1* and *2*, probed with 7H6. Lanes *3'* and *4'* are immunoblots of the precipitates shown in lanes *1'* and *2'*, probed with 7H6.

(1989). The 7H6 produced a honeycomb-like immunofluorescence pattern at the sub-brush border regions in the absorptive epithelial cells of rat intestine that was quite identical to the immunolocalization of cingulin in the chicken intestine demonstrated by Citi et al. (1988). The tiny dots and short strings observed by indirect immunofluorescence using 7H6 in the uriniferous tubules of rat kidney were identical to the immunolocalization of ZO-1 reported by Schnabel et al. (1990) in rat kidney. Such immunofluorescent localization of 7H6 suggests the possibility that 7H6 reacts with a component related to the tight junction.

Immunoelectron microscopy of isolated bile canaliculus-rich membrane fractions stained in suspension revealed that 7H6-affiliated gold particles were localized preferentially in tight junction areas. Ultrastructural localization of 7H6-affiliated gold particles suggested that the protein was distributed in areas immediately adjacent to the tight junction membrane. Measurement of the distances of these gold particles from the tight junction membrane indicated that the mean distance from the junctional membrane was 41.11 ± 18.50 nm. This distance for 7H6 was equivalent to that for cingulin (Stevenson et al., 1989). Although such values may change depending on variations in tissue preparation or in tissues examined, such a close localization of 7H6 at the tight junction strongly suggests that 7H6 recognizes a component associated with the tight junction. Because the antigenicity of the epitope immunoreactive to 7H6 was easily destroyed by fixation of tissues, only a few gold particles were found in the sections of rat hepatocytes fixed in situ. However, 7H6-affiliated gold particles were observed in tight junction areas of hepatocytes in situ (not shown).

Immunoblot analysis showed that 7H6 reacted with a distinctive molecular mass of 155 kD in rat tissues. The 7H6 antigen was present in the greatest amount in the bile canaliculus-rich fraction of the liver, but was also detectable in the intestine and kidney of rat. Indirect immunofluorescence showed that the 7H6 antigen was present in bile duct cells, absorptive cells of the intestine, uterus endometrial cells, lung bronchial mucosal cells, and kidney uriniferous tubule epithelial cells, suggesting ubiquitous distribution of this protein in rat organs. The 7H6 antigen was also detected in MDCK cells but the molecular size of the antigen in MDCK cells was 175 kD as determined by SDS-PAGE/immunoblot analysis. Such a variation of molecular mass depending on the species of origin is also reported for ZO-1 (Anderson, 1988).

Peripheral localization of 7H6 at the tight junction and the nearly identical molecular weights of the 7H6 antigen and cingulin suggest that 7H6 may react with cingulin. However, the antigen recognized by 7H6 was not related to cingulin. Immunoblot analysis of the whole rat liver homogenate using 7H6, anti-ZO-1 and anti-cingulin antibodies showed that these antibodies did not cross-react with each other, indicating that the 7H6 antigen is immunologically different from cingulin.

Recently, Gumbiner et al. (1991) reported a new tight junction-associated protein designated ZO-2. ZO-2 was identified by coimmunoprecipitation with ZO-1 from the detergent extracts of metabolically labeled MDCK cells. Because ZO-2 is reported to be 160 kD, the indistinguishable close molecular weight of the 7H6 antigen would suggest that the two proteins may be identical. However, the present immunoprecipitation analysis showed that the 160-kD protein

coprecipitated with ZO-1 from the detergent extracts of metabolically labeled MDCK cells was not reactive for 7H6. Conversely, immunoprecipitation with 7H6 did not precipitate the 160-kD protein. These results suggest that the 7H6 antigen and ZO-2 are immunologically different.

In our experimental conditions, we could not demonstrate the 7H6 antigen in the immunoprecipitation with ZO-1. This may be attributed to the immunoprecipitation buffer used in the present study. If this is the case, ZO-2 is suggested to associate with ZO-1 more tightly than the 7H6 antigen.

In conclusion, we propose that the 7H6 monoclonal antibody we have generated recognizes a novel tight junction-associated protein different from ZO-1, cingulin and ZO-2. Because the protein composition of the tight junction is not yet fully understood, 7H6 is expected to serve as an additional tool for studies of the structure and function of the tight junction.

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