

The 220-kD Protein Colocalizing with Cadherins in Non-Epithelial Cells Is Identical to ZO-1, a Tight Junction-associated Protein in Epithelial Cells: cDNA Cloning and Immunoelectron Microscopy

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Abstract. We previously identified a 220-kD constitutive protein of the plasma membrane undercoat which colocalizes at the immunofluorescence microscopic level with cadherins and occurs not only in epithelial but also in nonepithelial cells such as fibroblasts (Itoh, M., S. Yonemura, A. Nagafuchi, Sa. Tsukita, and Sh. Tsukita. 1991. *J. Cell Biol.* 115:1449–1462). To clarify the nature and possible functions of this protein, we cloned its full-length cDNA and sequenced it. Unexpectedly, we found mouse 220-kD protein to be highly homologous to rat protein ZO-1, only a part of which had been already sequenced. This relationship was confirmed by immunoblotting with anti-ZO-1 antibody. As protein ZO-1 was originally identified as a component exclusively underlying tight junctions in epithelial cells, where cadherins are not believed to be localized, we analyzed the distribution of cadherins and the

220-kD protein by ultrathin cryosection immunoelectron microscopy. We found that in non-epithelial cells lacking tight junctions cadherins and the 220-kD protein colocalize, whereas in epithelial cells (e.g., intestinal epithelial cells) bearing well-developed tight junctions cadherins and the 220-kD protein are clearly segregated into adherens and tight junctions, respectively. Interestingly, in epithelial cells such as hepatocytes, in which tight junctions are not so well developed, the 220-kD protein is detected not only in the tight junction zone but also at adherens junctions. Furthermore, we show in mouse L cells transfected with cDNAs encoding N-, P-, E-cadherins that cadherins interact directly or indirectly with the 220-kD protein. Possible functions of the 220-kD protein (ZO-1) are discussed with special reference to the molecular mechanism for adherens and tight junction formation.

CALCIUM-dependent cell-cell adhesion mechanisms play a key role in morphogenesis and carcinogenesis in a multicellular system (Takeichi, 1988). Cadherins are a family of transmembrane glycoproteins responsible for calcium-dependent cell-cell adhesion (Takeichi, 1991) which includes E-cadherin/uvomorulin (Nagafuchi et al., 1987; Ringwald et al., 1987), N-cadherin/A-CAM (Hatta et al., 1988), P-cadherin (Nose et al., 1987), L-CAM (Gallin et al., 1987), and several other cadherins have recently been identified in rat and human by polymerase chain reaction (Suzuki et al., 1991). Furthermore, desmosomal adhesion molecules called desmogleins or desmocollins also have similarity to these cadherins and fall into the cadherin superfamily (Holton et al., 1990; Koch et al., 1990). The pattern of expression and binding specificities of the different types of cadherins are thought to be crucial for the morphogenetic processes involved in the development of an organism.

Accumulating evidence has shown that the adhesion ability of cadherin molecules is regulated by cytoplasmic proteins (Takeichi, 1991; Tsukita et al., 1992). Immunoprecipi-

tation with anti-cadherin antibodies has so far revealed that at least three proteins called α -, β -, and γ -catenin are tightly associated with the carboxyl-terminal region of the cadherin cytoplasmic domain (Ozawa et al., 1989; Vestweber and Kemler, 1984; Peyrieras et al., 1985). Furthermore, when this catenin-binding region is deleted, cadherins can not function as cell adhesion molecules, indicating that catenins act as cytoplasmic regulators of cadherin molecules (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989). Cloning and sequence analyses of these cDNAs revealed that α - and β -catenins are homologous to vinculin and the *armadillo* protein of *Drosophila* (or plakoglobin), respectively (Herrenknecht et al., 1991; MacCrea et al., 1991; Nagafuchi et al., 1991). Recently, catenins have also been shown to be involved in the invasion and metastasis of carcinomas (Shimoyama et al., 1992). However, interestingly, analyses by Kintner (1991) on the involvement of the cytoplasmic domains of cadherin in *Xenopus* embryogenesis suggest the existence of other cytoplasmic proteins responsible for the regulation of cadherin functions.

Recently, using the isolated adherens junction fraction (Tsukita and Tsukita, 1989), we have identified a 220-kD undercoat-constitutive protein, which has been colocalized with the detergent-resistant form of cadherins in non-epithelial cells such as fibroblasts and cardiac muscle cells, at least at the immunofluorescence microscopic level (Itoh et al., 1991). In cardiac muscle cells, this protein appears also at the electron microscopic level at so-called intercalated discs, in the immediate vicinity of the plasma membrane in the undercoat of adherens junctions. Furthermore, this 220-kD protein is tightly associated with spectrin molecules. Therefore, we thought that this 220-kD protein would be a good candidate for binding directly or indirectly, cadherins to the actin-based cytoskeleton and thereby regulating cadherin function. To further characterize this protein, we have cloned a full-length cDNA encoding the 220-kD protein and sequenced it. Unexpectedly, a homology search and immunological analyses have shown that the mouse 220-kD protein is very similar to rat protein ZO-1, which is reportedly concentrated at tight junctions (Anderson et al., 1988; Stevenson et al., 1986) and only a part of which had been sequenced (Willott et al., 1992). Localization analyses of cadherins and the 220-kD protein in various types of tissues have further revealed that the 220-kD protein precisely colocalizes with N- and P-cadherins in non-epithelial cells, and that only in epithelial cells the 220-kD protein and E-cadherins segregate into tight and adherens junctions, respectively. Furthermore, by transfecting mouse L cells with cadherin cDNAs we have demonstrated that cadherins, directly or indirectly, interact with the 220-kD protein in order to colocalize. We believe that this study will cast a new light on the relationship between adherens and tight junctions, as well as on the molecular mechanisms of tight junction formation.

Materials and Methods

Antibodies and Cells

The mouse anti-220-kD protein mAbs, T8-754 and T4-192, were obtained and characterized as described previously (Itoh et al., 1991). In this study, according to the same method, we raised two more mAbs, T8-103 and T8-109, in mouse, which specifically recognize the 220-kD protein. Rat anti-mouse ZO-1 mAb (R40.76) was generously provided by Dr. D. Goodenough (Harvard University, Cambridge, MA). Rabbit anti-chick N-cadherin pAb, anti-mouse P-cadherin pAb, and anti-mouse E-cadherin pAb were kindly provided by Dr. M. Takeichi (Kyoto University, Kyoto, Japan).

Rat 3Y1 fibroblasts were a generous gift from Dr. C. Sato (Aichi Cancer Center Research Institute, Aichi, Japan). Mouse L-cells, their E-, N-, and P-cadherin transfectants (ELs8, mNLβ1, and PLβ1, respectively), and mouse teratocarcinoma F9 cells were kindly provided by Dr. M. Takeichi (Kyoto University). Mouse thymoma cells L5178Y were a generous gift from Dr. I. Yahara (Tokyo Metropolitan Institute for Medical Sciences, Tokyo, Japan).

cDNA Library Screening and DNA Sequencing

Two λgt11 expression libraries made from mouse F9 poly (A)⁺ RNAs were used in the following procedures. In preparing these libraries, either a random mixture of hexanucleotides or oligo (dT) was used as primer for the first-strand synthesis (Nagafuchi et al., 1987). The initial cDNA clone, F4 (see Fig. 2), was isolated from a randomly primed library using T8-754 according to the method previously described (Huyhn et al., 1985). The F4 fragment was then labeled by means of the DIG labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) and used to screen the randomly primed library according to the procedure developed by Boehringer Mannheim Biochemicals. Through rescreening, cDNA clones F6, F11, F14,

F16, F17, F19, F20, F21, F22, F31, and F35 were isolated. Only one clone, 012, was obtained from the oligo(dT) primed library.

All clones were subcloned into pBluescript SK(-) and sequenced with the 7-deaza Sequenase Version 2.0 kit (U. S. Biochemical Corp., Cleveland, OH). Both strands of all clones were sequenced.

Isolation of RNA and Northern Blot Hybridization

Total RNAs from cultured F9 cells and thymoma L5178Y cells were isolated according to the method described by Sambrook et al. (1989). Approximately 8 μg per well of RNA was resolved by electrophoresis and blotted onto a nitrocellulose membrane. An RNA ladder (Bethesda Research Laboratories, Bethesda, MD) was used as a size marker. The F22 fragment was labeled with [α -³²P]dCTP, using the Random Primer DNA Labeling Kit (Takara Shuzo Co., LTD., Kyoto, Japan), and used as a probe. Hybridization proceeded under conditions of high stringency (50% formamide/10× Denhardt's solution without BSA/5× SSC/50 mM phosphate buffer, pH 6.5/100 μg/μl boiled salmon sperm DNA).

Production of Fusion Proteins in *Escherichia coli* and Generation of pAb

F4, F19, F21, and F22, were introduced into the pMAL-CRI (New England Bio-Labs, Beverly, MA) in frame. Fusion proteins were expressed in XL1/Blue synthesized according to the procedure described by the manufacturer, and *Escherichia coli* lysate was separated by SDS-PAGE. The fusion protein from clone F4 was purified electrophoretically, and antiserum to the purified protein was elicited in a rabbit.

Gel Electrophoresis and Immunoblotting

SDS-PAGE was based on the discontinuous Tris-glycine system of Laemmli (1970). Immunoblotting was performed by one-dimensional electrophoresis, followed by electrophoretic transfer to nitrocellulose sheets, which were incubated with various mAbs or pAbs. Antibody was detected, using a blotting detection kit with alkaline phosphatase-conjugated immunoglobulin (Amersham International, Amersham, UK).

Immunofluorescence Microscopy and Immunoelectron Microscopy

For indirect immunofluorescence microscopy of frozen sections and cultured cells, samples were treated as described previously (Itoh et al., 1991; Tsukita et al., 1989a,b). The fixation methods were as follows: Frozen sections (~5 μM) were cut in a cryostat, mounted on glass slides, air dried, and fixed in 95% ethanol at 4°C for 30 min, and then in 100% acetone at room temperature for 1 min. Cells cultured on cover slips were fixed with 1% formaldehyde in PBS for 15 min followed by soaking for 15 min in 0.2% Triton X-100 in PBS.

Immunoelectron microscopy using ultrathin cryosections was performed essentially according to the method developed by Tokuyasu (1980, 1989). Small pieces of rat cardiac muscle, intestine, and liver were fixed in 1% formaldehyde in 0.1 M Hepes (pH 7.5) for 1 h at room temperature. All mAbs for the 220-kD protein used in this study were very sensitive to aldehyde fixation. The staining ability of these mAbs was completely abolished not only by the glutaraldehyde but also by 3% formaldehyde fixation. Therefore, when samples were fixed in aldehyde, all observations in this study were performed in samples fixed with 1% formaldehyde. The fixed samples were infused with 2.0 M sucrose containing 10% polyvinylpyrrolidone at room temperature for 2 h, rapidly frozen using liquid nitrogen, and ultrathin sectioned in the frozen state at ~110°C using glass knives with an FC-4E low temperature sectioning system (Reichert-Jung, Vienna, Austria). The sectioned samples were collected on formvar-film grids, washed three times with PBS containing 30 mM glycine (PBS-glycine), and incubated with PBS-glycine containing 2% goat serum for 3 min. The samples were then incubated with a mixture of anti-220-kD protein mAb (T4-192, T8-109, or T8-754) and anti-cadherin pAb for 15 min. After being washed with PBS-glycine three times, the samples were blocked with PBS-glycine containing 2% goat serum for 3 min, and then incubated with a mixture of goat anti-mouse IgG or IgM coupled to 10 nm gold (GAMIgG G10 or GAMIgM G10) and goat anti-rabbit IgG coupled to 15 nm gold (GARIGG G15; Amersham Corp., Arlington Heights, IL). After being washed with PBS and distilled water, the samples were incubated with distilled water containing 2% uranyl acetate for 10 min and then with distilled water containing 2% polyvinylalcohol and 0.2% uranyl acetate for 10 min, air dried, and examined

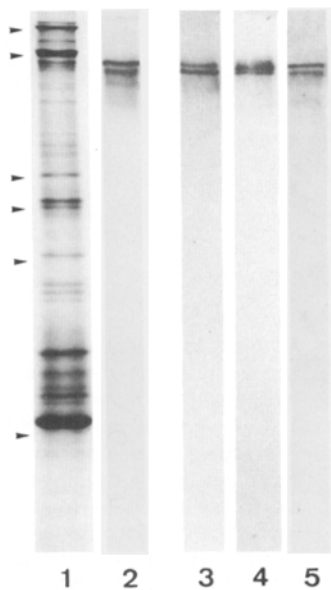


Figure 1. Specificity of the anti-220-kD protein mAbs. (Lane 1) Coomassie blue-stained gel of membrane fractions prepared from isolated rat adherens junctions. (Lane 2) Accompanying immunoblot with anti-220-kD protein mAb (T8-754). (Lanes 3-5) Immunoprecipitants from adherens junction extracts by T8-754 were electrophoresed, and immunoblotted with the other anti-220-kD protein mAbs (lane 3, T4-192; lane 4, T8-103; lane 5, T8-109). All mAbs recognize the antigen that has a molecular mass of 220 kD. Arrowheads indicate molecular sizes as 400, 240, 130, 100, 80, and 40 kD from the top.

in an electron microscope (1200 EX; JEOL, Tokyo, Japan) at an accelerating voltage of 100 kV.

For the preembedding-labeling procedure, cultured 3Y1 cells were fixed with 1% formaldehyde in 0.1 M Hepes (pH 7.5) for 30 min and then soaked with 0.5% Triton X-100 in PBS for 20 min. After being washed three times with PBS, the samples were soaked in PBS containing 1% BSA for 10 min and treated with a mixture of anti-220-kD protein mAb (T8-754) and anti-P-cadherin pAb for 1 h in a moist chamber. The samples were then washed three times with PBS containing 1% BSA, followed by incubation with a mixture of GAMiG G10 and GARiG G15 for 1 h. The samples were washed three times with PBS, fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h, washed three times with 0.1 M cacodylate buffer, postfixed with 1% OsO₄ at 4°C for 30 min, washed with distilled water three times, dehydrated with graded concentration of ethanol, and then embedded in Epon 812. Ultrathin sections were cut with a diamond knife and then doubly stained with uranyl acetate and lead citrate.

Results

Isolation and Sequencing of cDNA Encoding the 220-kD Protein

We have previously described two anti-220-kD protein

mAbs called T4-192 and T8-754 (Itoh et al., 1991). Using the same protocol, we have raised two more mAbs (T8-103 and T8-109). As shown in Fig. 1, the 220-kD protein affinity purified with the use of mAb T8-754 was specifically recognized by the other mAbs in its immunoblot, indicating that all these mAbs specifically recognized the same 220-kD protein.

Using mAb T8-754, we screened $\sim 1.5 \times 10^6$ plaques from a random-primed λ gt11 cDNA library made from mouse teratocarcinoma cells (F9 cells) as described in Materials and Methods, and cloned one positive phage recombinant: F4 (1.4kb) (Fig. 2). To isolate the rest of the 220-kD protein cDNA, the same library was rescreened with probes from either end of the F4 clone. Through a series of rescreening experiments with random- and oligo(dT)-primed λ gt11 cDNA libraries, 13 overlapping clones, including F4, which together span 7,046 bp, were isolated and sequenced in both directions (Fig. 2).

Several criteria confirmed that these clones encoded the 220-kD protein. Firstly, we generated fusion proteins encoded by the 700-bp fragment of F4 (see Fig. 2) in *E. coli* and raised polyclonal rabbit antibodies against this fusion protein. As shown in Fig. 3, this pAb specifically recognized the 220-kD protein band of F9 cells in an immunoblot, and stained the cell-cell adhesion sites in cultured fibroblasts as determined by immunofluorescence microscopy. Secondly, mAbs T4-192, T8-103, and T8-109 specifically recognized the fusion proteins generated in *E. coli* from F21, F22 and F19, respectively. Third, in vitro translation in a mRNA-dependent rabbit reticulocyte lysate system was performed, and the product was electrophoretically identical to the 220-kD protein (data not shown).

The complete nucleotide sequence encoded by the overlapping clones and the predicted amino acid sequence are shown in Fig. 4. The reading frame of the sequence starts at nucleotide 404 and remains open until residue 5638, thereby coding for a protein of 1,745 amino acids with a molecular mass of 195 kD. No ambiguities between regions of clone overlap were detectable with the exception of one missing region of 240 bp shown in Fig. 2, which could be missed due to probable alternative mRNA splicing. This cDNA has no long hydrophobic stretches which could encode a signal pep-

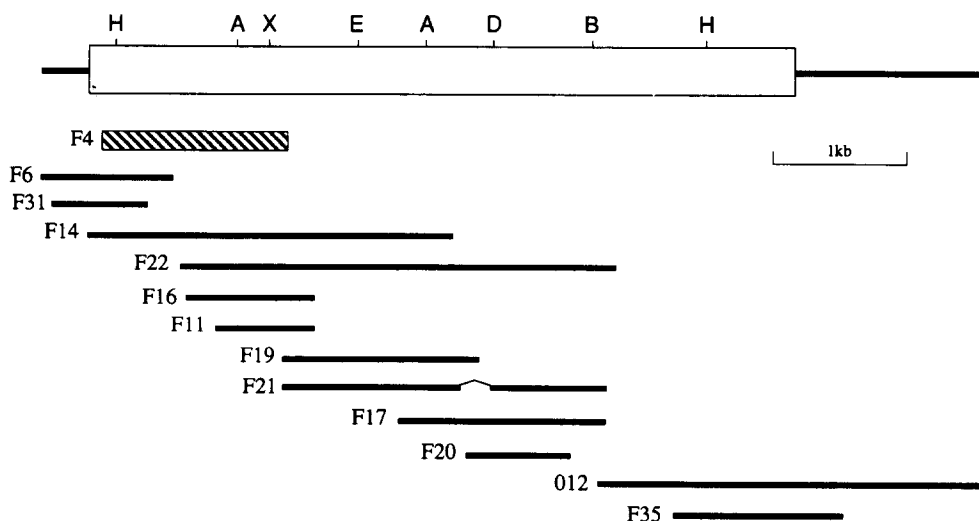


Figure 2. Restriction map and cDNA fragments of the mouse 220-kD protein. Open box indicates the coding region. F4 was first obtained using an anti-220-kD protein mAb. All clones except 012 were obtained from a random primed λ gt11 library made from mouse teratocarcinoma F9 cells, and the 012 clone was from an oligo(dT) primed λ gt11 library. A, AccI; B, BamHI; D, DraI; E, EcoRI; H, HincII; X, XbaI.

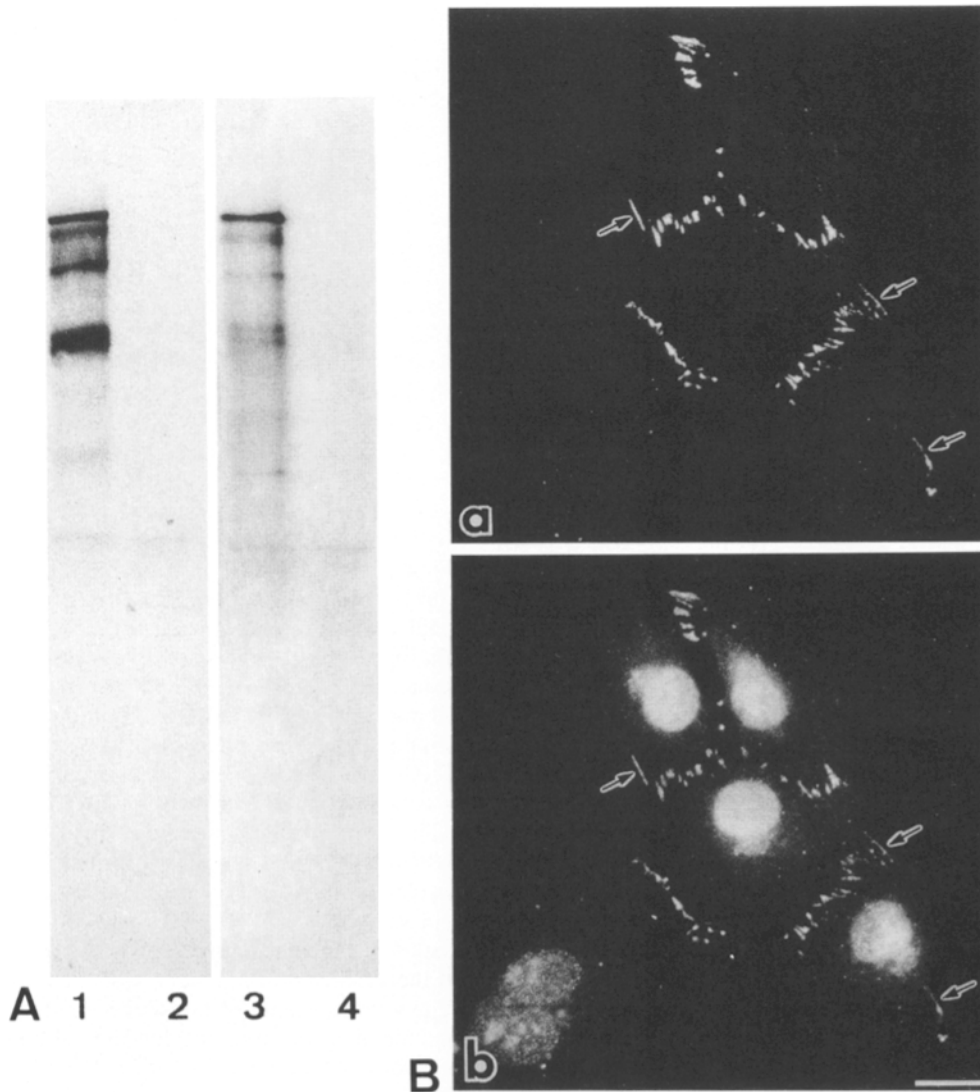


Figure 3. Immunoblotting (A) and indirect immunofluorescence staining (B) with anti-220-kD protein mAb (T8-754) and a polyclonal antibody raised against the fusion protein from clone F4. (A) Mouse teratocarcinoma F9 cells (lanes 1 and 3) and mouse thymoma cells (lanes 2 and 4) were electrophoresed and immunoblotted with anti-220-kD protein mAb (lanes 1 and 2), and anti-fusion protein pAb (lane 3 and 4). Both antibodies recognized the 220 kD-antigen in F9 cells, but did not detect any proteins in thymoma cells lacking the 220-kD protein. (B) Immunofluorescence staining of cultured rat 3Y1 fibroblasts doubly with anti-220-kD protein mAb, T8-754, (a), and anti-fusion protein pAb (b). Both staining profiles at cell-cell adhesion sites coincided completely (arrows), except that the pAb nonspecifically stained nuclei. Bar, 30 μ m.

tide or a transmembrane domain, confirming that this 220-kD protein is neither a secretory nor a transmembrane protein.

The 220-kD protein does not contain repeats as similar motifs (Fig. 5 A), and secondary structure analysis predicts that it has neither a typical α -helix nor a β -sheet region. The most striking feature of the primary structure is the presence of a "leucine zipper"-type motif at amino acids 754-775 (Fig. 5 B) (Landschulz et al., 1988). The protein contains seven cysteine residues among which five are localized at the carboxyl-terminal domain (amino acids 1673-1737).

To detect mRNA encoding the 220-kD protein, Northern blotting was performed using F22 as the probe. As shown in Fig. 6, a band at 7.3 kb was detected in mouse F9 cells. This band was somewhat broad, but was not resolved into two bands (splice and non-splice forms). Furthermore, the 220-kD protein mRNA was not detected in mouse blood cells such as thymoma cells, where neither cadherins nor 220-kD proteins were detected by immunoblotting (Fig. 3 A).

Comparison of the 220-kD Protein with ZO-1

ZO-1 was first identified as a protein exclusively underlying

the tight junctions in human and rat epithelial cells. Recently, a cDNA encoding a part of the human and rat ZO-1 protein was cloned (Willott et al., 1992). It was shown that there are at least two different types of protein ZO-1 due to alternative mRNA splicing, and only short cDNA fragment, including the region missing in one spliced form, have been sequenced and reported. The deduced amino acid sequence of this region was very similar (77.5% identity) to that of the region which is thought to be spliced out of the mouse 220-kD protein (amino acids 922-1001) (Fig. 7 A). This indicates that the 220-kD protein is a mouse homologue for protein ZO-1. This was confirmed by the fact that the fusion protein produced from F22 in *E. coli* is recognized by anti-ZO-1 mAb (R40.76) (Fig. 7 B).

Immunoelectron Microscopic Localization of the 220-kD Protein and Cadherins in Non-epithelial Cells

In a previous study, we found that at the immunofluorescence microscopic level the 220-kD protein colocalizes with cadherins at cell-cell adhesion sites in non-epithelial cells such as fibroblasts, that lack tight junctions (Itoh et al.,

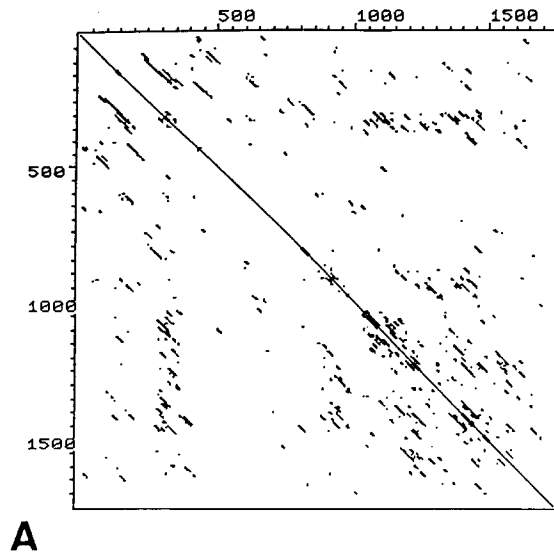
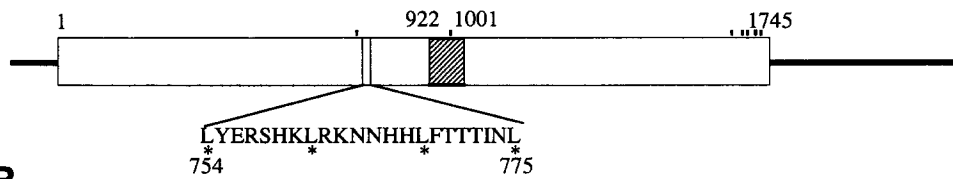


Figure 5. Structure of the mouse 220-kD protein. (A) A diagonal plot of the amino acid sequence of the 220-kD protein with itself. The comparison was made using the HARPLT2 program. The axes are labeled in amino acid residue numbers. The dots correspond to the midpoints of 50-residue spans with at least 10 identical residues between two proteins. Note that there are no significant internal repeats in this protein. (B) The location of the leucine zipper motif (amino acids 754-775), splicing region (▨) and cysteine residues (|) in the open reading frame.



B

highly concentrated at adherens junctions, while the tight junction zone was exclusively labeled by anti-220-kD protein mAb (Fig. 10, *a* and *b*). Evidently, the two proteins were not intermixed.

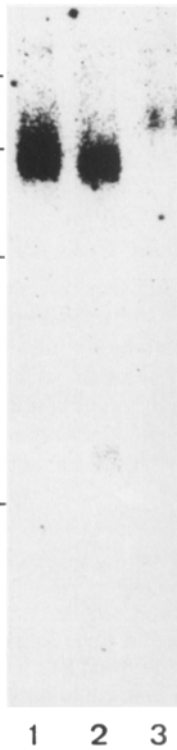


Figure 6. Northern blot of the total RNA isolated from mouse 3Y1 fibroblasts (lane 1), teratocarcinoma F9 cells (lane 2), and L5178Y thymoma cells (lane 3). Total RNA was probed at high stringency with F22 cDNA. The bars on the left indicate the positions of RNA markers of 9.5, 7.5, 4.4, and 1.4 kb (from the top).

Compared with intestinal epithelial cells, tight junctions are not so well developed in liver. In this tissue, the labels for these proteins were occasionally intermixed at the adherens junction zone (Fig. 11). Anti-E-cadherin pAb never labeled the typical tight junctions but clearly the adherens junction zone, whereas the 220-kD protein was reproducibly detected in the tight junction zone and occasionally also in the adherens junction zone.

As far as we examined, the 220-kD protein was never detected in the undercoat of desmosomes.

Localization of the 220-kD Protein in Mouse L Cells Transfected with N-, P-, or E-cadherin cDNAs

Our results indicate that the 220-kD protein (ZO-1), directly or indirectly, interacts with cadherins, especially in non-epithelial cells. To test this idea, we compared the localization of the 220-kD protein in N-, P-, and E-cadherin-transfected L fibroblasts with that in parental L fibroblasts (L cells are known to lack the cadherin expression; Nagafuchi et al., 1987). In L fibroblasts, the 220-kD protein was not concentrated at cell-cell adhesion sites and occasionally appeared to be concentrated at the tip area of cell processes (Fig. 12, *a* and *b*). In sharp contrast, the N-, P-, and E-cadherin-transfected L fibroblasts showed a typical cadherin-based cell adhesion. In these cells, the 220-kD protein was highly concentrated at cell-cell adhesion sites, together with cadherin molecules (Fig. 12, *c-h*). As previously reported and as far as we examined electron microscopically, these transfectants completely lacked tight junctions at their cell-cell contact sites (data not shown).

A

	922	961
mouse 220kD-protein	KAEASSVPYLSPETTPASSASAVNHNVSVTNVSLEEPAP	
	*****.*****.***.***.***.*	
Rat ZO-1	KAEASSVPYLSPETNPASSASAVKHNVLTNVNLEEPTP	
	962	1001
mouse 220kD-protein	APPTSHASQPGCLGAPSAEAAHVGLRGEPPPLPHADPAK	
	*****.***.*****.***.***.***.*	
Rat ZO-1	APPTSHVSQADCLGAPSPPEAPHTMLRDEGVSLPSHVDPK	

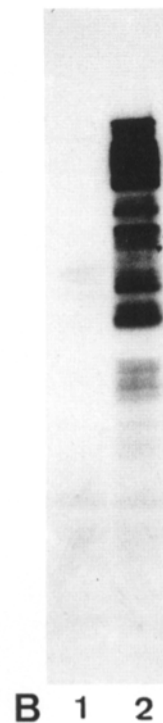


Figure 7. (A) Comparison of the amino acid sequence of a region (amino acids 922-1001) of the mouse 220-kD protein with the corresponding portion of rat ZO-1. The amino acid sequence of only this region of rat ZO-1 has so far been reported (Willott et al., 1992). Identity and homology are indicated by asterisks (*) and dots (.), respectively. (B) Immunoblots analysis of the lysate from control *E. coli* (lane 1) and from *E. coli* generating the fusion protein from clone F22 (lane 2) with anti-ZO-1 mAb, R40.76.

Discussion

In a previous study, we identified and characterized a 220-kD constitutive protein from the undercoat of the plasma membrane present in isolated adherens junctions from rat livers (Itoh et al., 1991). One of the most characteristic aspects of this protein is that it colocalizes by immunofluorescence microscopy with cadherins at cell-cell contact sites, even in non-epithelial cells such as fibroblasts and cardiac muscle cells.

In this study, we describe a cDNA encoding this 220-kD protein. Its sequence was determined from 13 overlapping clones encoding a non-splice (1,745 amino acids) and a splice form (1,665 amino acids). Sequence analysis using this 220-kD protein cDNA led us to conclude that the 220-kD protein has no similarity to ankyrin members (c.f., Lux et al., 1990) but is closely related, if not identical, with protein ZO-1 previously reported to be a constitutive protein of the undercoat material associated with tight junctions (Anderson et al., 1988; Stevenson et al., 1986).

Two forms of rat ZO-1 are known to exist, due to alternative mRNA splicing, and the amino acid sequence of the region that is absent in one of the splice forms has already been determined (Willott et al., 1992). It is highly homologous to that of the mouse 220-kD protein (77.5% identity in the splice variant region). The amino acid sequence of the rest of ZO-1 has not yet been reported. In addition, anti-ZO-1 mAb (R40.76) clearly recognized the fusion protein produced in *E. coli* from one of the cDNA clones (F22) obtained in this study.

The identity of 77.5% appears to be rather low between rat and mouse homologues. However, the tight junction protein, ZO-1, was originally defined as an antigen for the mAbs, R26.4C, R40.40D3, and R40.76 (Anderson et al.,

1988; Stevenson et al., 1986). In this sense, we can say that the 220-kD protein is identical to ZO-1.

Our knowledge of the structure and properties of protein ZO-1 was still limited. Taking the properties of the 220-kD protein that we clarified previously into consideration (Itoh et al., 1991), the fact that the 220-kD protein is identical to ZO-1 leads to the following conclusions. (a) The ZO-1 molecule has some ankyrin-like properties such as globular molecular structures, specific α -spectrin binding and very close localization to the membrane, although it shows no similarity in amino acid sequence with ankyrins. (b) Within this protein, there are no regions containing repeated similarities. (c) One "leucine zipper"-type motif is included at amino acids 754-775. (d) Cysteine residues are concentrated in the carboxyl terminus region (amino acids 1673-1737).

The fact that the 220-kD protein is identical to ZO-1 naturally opens up some debate. The first issue is what the function of ZO-1, a tight junction protein, is in non-epithelial cells. The tight junction is an element of the epithelial junctional complex (Farquhar and Palade, 1963). It seals the epithelial cells together at their apices to create the primary barrier to the diffusion of solutes through the paracellular pathway. It also works as a boundary between the apical and basolateral plasma membrane domains to create the polarization of epithelial cells (Gumbiner, 1987; Stevenson et al., 1988). This type of junction appears to be unnecessary for non-epithelial cells, since its sealing function is not required and the apical and basolateral domains are not differentiated. Actually, tight junctions are by no means detected in non-epithelial cells by EM. However, ZO-1 expression has already been detected by immunoblotting in non-epithelial cells such as fibroblasts, Schwann cells, and glioma cells (Howarth et al., 1992), although, probably due to the stain-

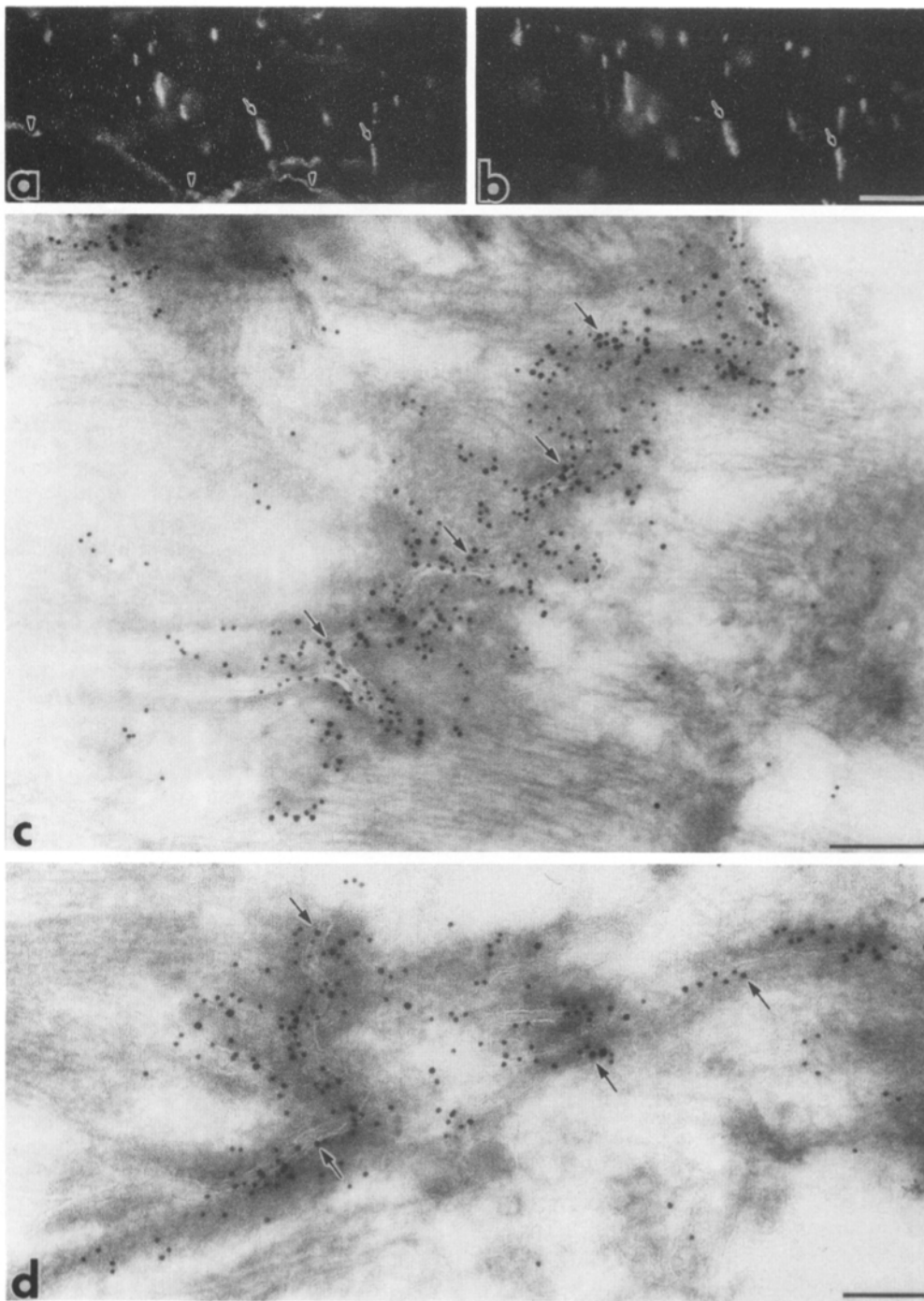


Figure 8. Comparison of the localization of the 220-kD protein (ZO-1) and N-cadherin in chick cardiac muscle cells. (a and b) Double staining of the longitudinally cut frozen section of heart with anti-220-kD protein mAb, T8-754, (a), and anti-N-cadherin pAb (b). The intercalated discs (arrows) are intensely stained with both antibodies. Note that the 220-kD protein staining is also associated with the blood vessels (arrowheads) and that the N-cadherin staining is restricted to intercalated discs. (c and d) Ultrastructural localization. Longitudinal ultrathin cryosections are doubly labeled with anti-220-kD protein mAb (c, T4-192; d, T8-754) (10 nm gold) and anti-N-cadherin pAb (15 nm gold, arrows). Both 10 and 15 nm gold particles have accumulated and intermixed at the cell-to-cell adherens junctions in the intercalated discs. Bars: (a and b) 3 μm ; and (c and d) 0.2 μm .

ing properties of the original anti-ZO-1 mAbs, ZO-1 localization in non-epithelial cells has not been clarified in detail. Both at light and electron microscopic levels, our mAbs for the 220-kD protein specifically stained the cadherin-based cell-cell adhesion sites in fibroblasts and cardiac muscle cells.

Taken all together, the evidence led to the speculation that in non-epithelial cells, ZO-1 has nothing to do with tight junctions or their related functions, but that they play a role in regulating the cell-adhesion ability of cadherin molecules. In fact, as far as we examined, we found that without exception, all cell types expressing cadherins express ZO-1,

whether or not cells bear tight junctions. Furthermore, the precise co-localization of cadherins with ZO-1 in cadherin-expressing L cell transfectants as shown in this study supports the notion that cadherins directly or indirectly interact with ZO-1.

The second issue is whether in epithelial cells, ZO-1 is localized only in tight junctions or in both tight and adherens junctions. The multiplicity of observations using anti-ZO-1 mAbs argue strongly for the occurrence of ZO-1 in tight junctions in epithelial cells, although in these observations, little attention was paid as to whether ZO-1 occurs at adherens junctions or not (Stevenson et al., 1988). On the other

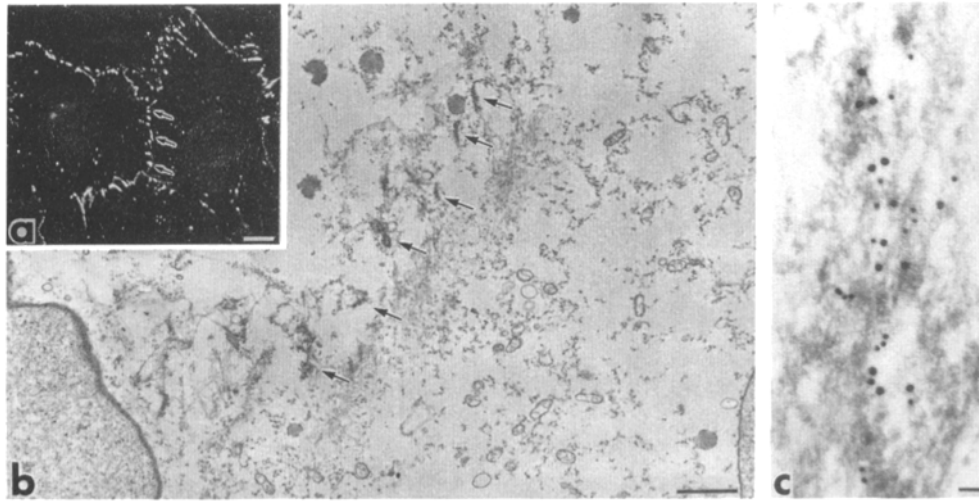


Figure 9. Comparison of the localization of the 220-kD protein (ZO-1) and P-cadherin in rat 3Y1 fibroblasts. (a) Single staining of rat 3Y1 fibroblasts with an anti-220-kD protein mAb, T8-754. The cell-cell adhesion sites are intensely stained (arrows). (b and c) Ultrastructural localization. Cultured cells are fixed with formaldehyde and permeabilized with Triton X-100 before double immunolabeling with mAb T8-754 (10 nm gold) and anti-P-cadherin pAb (15 nm gold). At low magnification (b), the cell-cell adhesion sites are easily identified as electron dense structures (arrows), which are heavily labeled with gold particles. At higher magnification (c), in these electron-dense structures, both 10 and 15 nm gold particles are accumulated and intermixed. Bars: (a) 15 μm ; (b) 4 μm ; and (c) 0.8 μm .

hand, in our previous study, we did not examine the localization of the 220-kD protein in epithelial cells at the electron microscopic level, since immunoelectron microscopic data showing a clear concentration of the 220-kD protein at the intercalated discs of cardiac muscle cells persuaded us to accept an intimate relationship between the 220-kD protein and adherens junctions in general (Itoh et al., 1991). Yet, as

shown in Fig. 10, in intestinal epithelial cells where tight junctions are well developed, the 220-kD protein (ZO-1) was exclusively concentrated at the tight junction zone and was barely detectable in adherens junctions. However, in epithelial cells such as liver cells where tight junctions are not so well developed, ZO-1 was detected not only in the tight junction zone but also in the adherens junction zone, although it

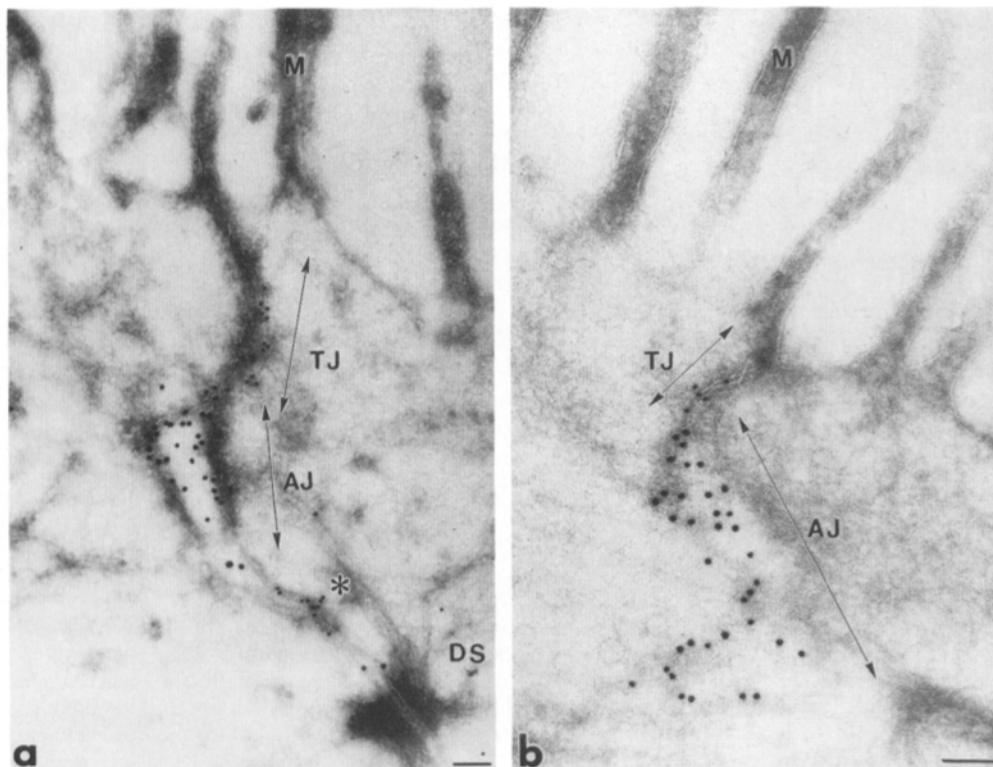


Figure 10. Ultrastructural localization of the 220-kD protein (ZO-1) and E-cadherin. Ultrathin cryosections of rat intestinal epithelial cells are doubly labeled with anti-220-kD protein mAb, T8-109, (10 nm gold) and anti-E-cadherin pAb (15 nm gold). The 220-kD protein is exclusively concentrated at the tight junction zone (TJ), while E-cadherin is highly concentrated at adherens junction zone (AJ). These labels are hardly intermixed. The adherens junctions are artifactually opened, probably due to the formaldehyde fixation. *, ectopic tight junction; DS, desmosome; M, microvilli. Bars: 0.1 μm .

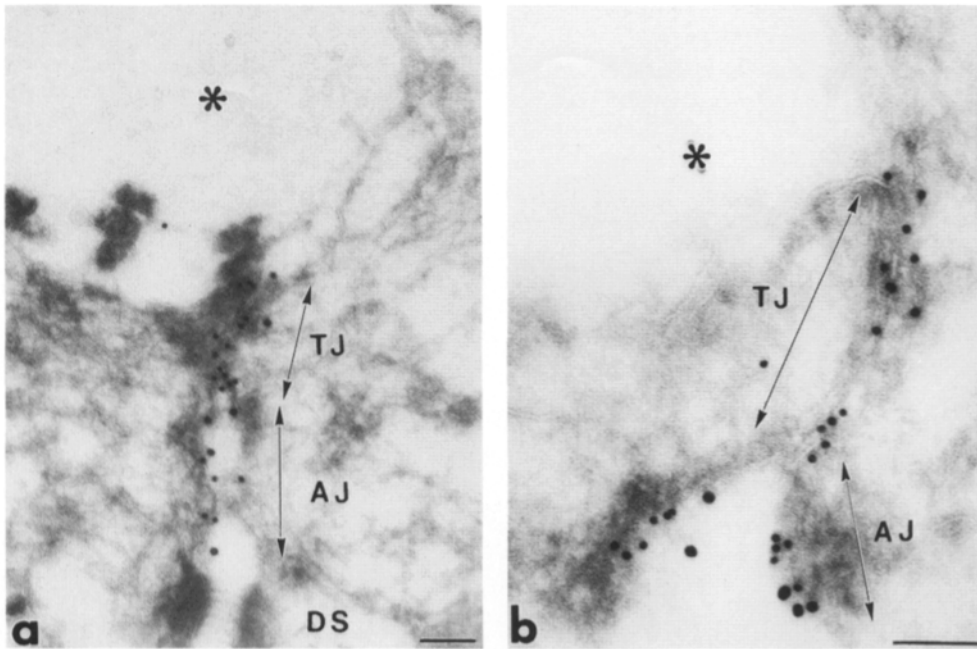


Figure 11. Ultrastructural localization of the 220-kD protein (ZO-1) and E-cadherin. Ultrathin cryosections of rat liver are doubly labeled with the anti-220-kD protein mAb T8-109 (10 nm gold) and anti-E-cadherin pAb (15 nm gold). The 220-kD protein and E-cadherin are enriched at tight junction (TJ) and adherens junction (AJ) zones, respectively. However, in liver, 220-kD proteins (10 nm gold) are occasionally localized at adherens junctions and mixed up with E-cadherin. The adherens junctions are artifactually opened, probably due to the formaldehyde fixation. DS, desmosome; *, bile canaliculus. Bars: 0.1 μ m.

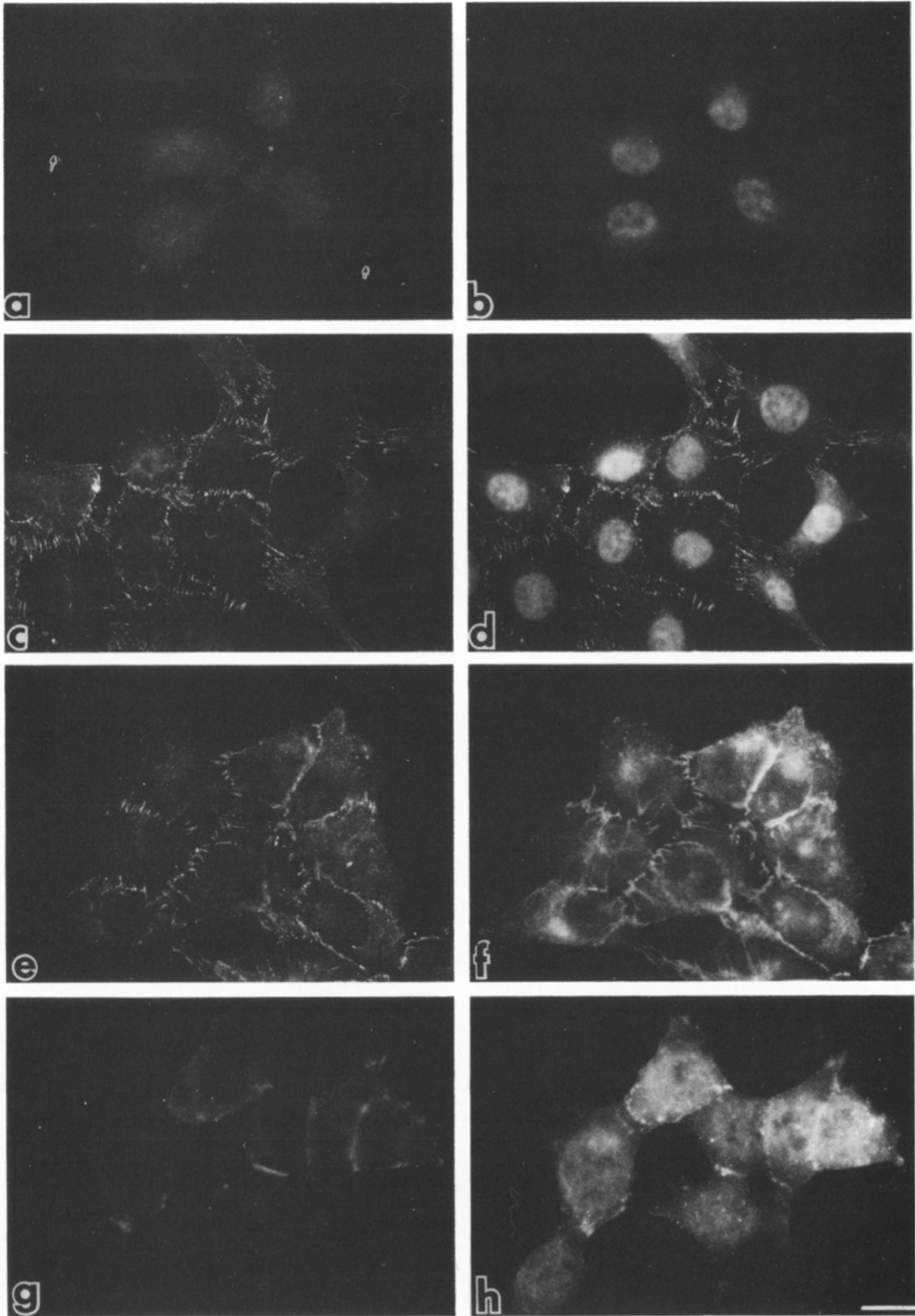
was difficult to determine whether ZO-1 detected in the latter zone was associated with the cadherin-based adherens junction proper or with the tight junction occasionally seen running in the adherens junction zone in these cells.

In summary, we conclude that in non-epithelial cells expressing cadherins, ZO-1 (220-kD protein) is highly concentrated at the cadherin-based cell-cell adhesion sites, and that in epithelial cells, ZO-1 is closely associated with tight junctions although it appears to be occasionally detected at cadherin-based cell-to-cell adherens junctions. It appears that ZO-1 is associated with N- and P-cadherins but not with E-cadherin, so that only in epithelial cells expressing E-cadherin, ZO-1 is not co-localized with cadherins and moves to tight junctions. However, this speculation is not likely, because in the L cell transfectant system, not only N- and P-cadherins but also E-cadherins showed an intimate spatial relationship with ZO-1. At present, it is still premature to further discuss the reason for the peculiar localization of ZO-1 observed in this study, but we consider that ZO-1 is not a specific marker for tight junctions. Actually, ZO-1 is reportedly concentrated along slit diaphragms of the glomerular epithelium where the intercellular spaces are wide open and no fibrils can be seen by freeze fracture (Schnabel et al., 1990; Kurihara et al., 1992).

Our present observations have caused us to reconsider the relationship between adherens and tight junctions, such as, whether or not the tight junction is differentiated from adherens junctions. This relationship has already attracted some

investigators (Gumbiner et al., 1988; Wang et al., 1990). ZO-1 protein (220-kD protein) is located in the immediate vicinity to the plasma membrane, suggesting that it is directly or indirectly associated with an integral membrane protein (Itoh et al., 1991; Stevenson et al., 1986). It is then tempting to speculate that in non-epithelial cells ZO-1 is associated with the detergent-resistant form of cadherins and that, when the putative tight junction membrane protein is expressed in epithelial cells, ZO-1 is transferred from cadherins to the membrane protein. We also speculate that an unidentified type of cadherin is present at the tight junction zone and that it shows higher affinity for ZO-1 than E-cadherins in epithelial cells. To evaluate these speculations, it is prerequisite to identify major constituents of tight junctions. The molecular organization of the tight junction is poorly understood. So far ZO-1 and cingulin were reported as undercoat-constitutive proteins localized at tight junctions (Citi et al., 1988; Stevenson et al., 1986): ZO-1 is located in the immediate vicinity of the plasma membrane, while cingulin is distributed more distantly from the membrane (Stevenson et al., 1989). Most recently, a 160-kD cytoplasmic protein (ZO-2) associated with ZO-1 has been identified (Gumbiner et al., 1991). However, in sharp contrast to adherens junctions and desmosomes, in the case of the tight junctions, there remains no information about the integral membrane proteins of tight junctions, especially adhesion molecules. It remains controversial whether the fibrillate intramembranous elements are predominantly lipidic in nature (Pinto da Silva and Kachar,

Figure 12. Localization of the 220-kD protein (ZO-1) in mouse L cells transfected with mouse N-, P-, and E-cadherin cDNAs. Double staining of L cells (a and b), N-cadherin-transfected L cells (c and d), P-cadherin-transfected L cells (e and f), and E-cadherin-transfected L cells (g and h) with the anti-220-kD protein mAb T8-754 (a, c, e, and g), anti-E-cadherin pAb (b and h), anti- α -catenin rat mAb (d), or anti-P-cadherin pAb (f). Since anti-mouse N-cadherin pAb is not available, anti- α -catenin pAb was used to identify the cadherin-based cell-cell adhesion sites in the N-cadherin-transfected L cells (d). Cadherins are not expressed in L cells (b), but the 220-kD protein is present at the tip areas of cell processes (arrows in a). In all L cell transfectants expressing cadherins (c-h), 220-kD proteins are highly concentrated at cell-cell adhesion sites together with cadherins. Bar, 15 μ m.



1982) or represent a linear aggregation of integral protein units (Staehelin, 1973), although recent evidence strongly favors the latter model (Stevenson and Goodenough, 1984; Van Meer et al., 1986). We believe identification of a tight junction-specific adhesion molecule will lead to a better understanding of the molecular mechanism of tight and adherens junction formation and of the establishment of cell polarity during epithelial differentiation.

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