

Plasticity in Epithelial Cell Phenotype: Modulation by Expression of Different Cadherin Cell Adhesion Molecules

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Abstract. A primary function of cadherins is to regulate cell adhesion. Here, we demonstrate a broader function of cadherins in the differentiation of specialized epithelial cell phenotypes. In situ, the rat retinal pigment epithelium (RPE) forms cell-cell contacts within its monolayer, and at the apical membrane with the neural retina; Na⁺,K⁺-ATPase and the membrane cytoskeleton are restricted to the apical membrane. In vitro, RPE cells (RPE-J cell line) express an endogenous cadherin, form adherens junctions and a tight monolayer, but Na⁺,K⁺-ATPase is localized to both apical and basal-lateral membranes. Expression of E-cadherin in RPE-J cells results in restriction and accumulation of both Na⁺,K⁺-ATPase and the mem-

brane cytoskeleton at the lateral membrane; these changes correlate with the synthesis of a different ankyrin isoform. In contrast to both RPE in situ and RPE-J cells that do not form desmosomes, E-cadherin expression in RPE-J cells induces accumulation of desmoglein mRNA, and assembly of desmosome-keratin complexes at cell-cell contacts. These results demonstrate that cadherins directly affect epithelial cell phenotype by remodeling the distributions of constitutively expressed proteins and by induced accumulation of specific proteins, which together lead to the generation of structurally and functionally distinct epithelial cell types.

CADHERINS comprise a superfamily of more than 30 proteins that mediate calcium-dependent, homophilic cell-cell adhesion (Suzuki et al., 1991; Kessler, 1992; Sano et al., 1993). Cadherins are differentially expressed in development, and expression is often restricted to specific cell types within a tissue (Edelman, 1985; Edelman and Crossin, 1991; Takeichi, 1988, 1990, 1991). The reason a single organism expresses different cadherins is unclear. One proposed function for differential cadherin expression is to mediate cell sorting. Expression of different cadherins in a mixture of cells results in sorting of cells into populations that express the same cadherin (Takeichi et al., 1981; Nose et al., 1988; Takeichi, 1988; Friedlander et al., 1989; Steinberg and Takeichi, 1993). In addition, differential cadherin expression may be important in the regulation of morphogenetic movements of cells during, for example, gastrulation, radial intercalation, and convergent extension in

the embryo (Edelman, 1985; Briehner and Gumbiner, 1994; Gumbiner, 1992).

The restriction of different cadherins to specific cell types indicates that cadherins may also be involved in the differentiation of specialized cell phenotypes (Edelman 1986; Takeichi et al., 1990). However, direct experimental evidence that the expression of different cadherins directly induces formation of structurally and functionally distinct cell types is presently lacking.

Epithelial cells display considerable plasticity in their structural and functional organization in normal tissues and disease states (Fish and Molitoris, 1994; Nelson, 1992; Rodriguez-Boulán and Powell, 1992). Transporting epithelia form monolayers in which the apical and basal-lateral membrane domains face different biological compartments. The distribution of ion channels and transporters between these membrane domains is important in the regulation of vectorial transport of ions and solutes between different compartments separated by the epithelium (Almers and Stirling, 1984). For example, in reabsorptive epithelia, Na⁺,K⁺-ATPase is restricted to the basal-lateral membrane domain (Almers and Stirling, 1984). However, in polycystic kidney disease, renal ischemia and early renal development, Na⁺,K⁺-ATPase is localized to both apical and lateral membrane domains of renal epithelial cells (Avner et al., 1992; Fish and Molitoris, 1994). In the choroid plexus, a secretory

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epithelium, Na⁺,K⁺-ATPase is restricted to the apical membrane domain (Quinton et al., 1973; Wright, 1972; Marrs et al., 1993). Mechanisms underlying these different distributions of Na⁺,K⁺-ATPase are poorly understood, but it has been proposed that cadherin-mediated cell adhesion and assembly of the membrane cytoskeleton are important determinants of this process (Nelson, 1992).

To examine the roles of cadherins in regulating epithelial cell differentiation and Na⁺,K⁺-ATPase distribution, we have studied cells of the retinal pigmented epithelium (RPE)¹. In situ, the RPE comprises a monolayer of epithelial cells that is bounded on the apical membrane by the neural retina, and on the basal membrane by extracellular matrix. Cell-cell adhesion within the RPE monolayer is calcium dependent, and in the chicken, it is mediated by B-cadherin (Murphy-Erdosh et al., 1994). Adhesion between the RPE and the neural retina may be mediated by neural cell adhesion molecule (NCAM) (a calcium-independent cell adhesion protein) (Gundersen et al., 1993), or a cadherin that has not yet been identified. The distribution of Na⁺,K⁺-ATPase is restricted to the apical membrane domain of RPE in situ (Gundersen et al., 1991).

A stable cell line has recently been established from rat RPE (RPE-J) and resembles dissociated RPE in primary culture (Nabi et al., 1993). Although confluent monolayers of RPE-J cells express an endogenous cadherin that is distinct from E-cadherin at the lateral membrane, they display a non-polar steady-state distribution of Na⁺,K⁺-ATPase. However, upon transfection of E-cadherin (producing the RPE-J+EC cell line), we show that Na⁺,K⁺-ATPase distribution becomes restricted to sites of cell-cell contact. The polarization of Na⁺,K⁺-ATPase distribution correlates with induced synthesis of a different isoform of ankyrin, and the accumulation and colocalization of the membrane cytoskeleton at cell-cell contacts. In addition, E-cadherin expression induces the assembly of desmosomes at sites of cell-cell contact, which are not observed in either the parental RPE-J cells or in rat RPE in situ. These data demonstrate directly that although different cadherins share the ability to form calcium-dependent cell-cell contacts and organize cells into epithelial monolayers, they differ in their capacities to induce distinct structural and functional polarity in epithelial cells. It has been suggested that cadherins are morphoregulatory molecules (Edelman, 1985; Takeichi, 1988), and our study provides strong experimental support of this concept.

Materials and Methods

Cell Culture and Antibodies

RPE-J and RPE-J+EC cells were maintained at 33°C in DME supplemented with 4% FBS, glutamine, and nonessential amino acids (Nabi et al., 1993). For experiments, cells were plated at 300,000–350,000 cells/cm² on either Millicell HA nitrocellulose filters (Millipore, Bedford MA), on glass coverslips coated with Matrigel (Collaborative Research, Bedford, MA), or on uncoated Transwell filters (Costar Corp., Cambridge MA) (for metabolic labeling/biotinylation experiments of RPE-J+EC cells only), and cells were then cultured in the presence of 10⁻⁸ M all-trans-retinoic acid (Sigma Immunochemicals, St. Louis MO). After 6–7 d, the cells were incubated at 40°C for 33 h (Nabi et al., 1993).

1. *Abbreviations used in this paper:* RPE, retinal pigment epithelium; RT-PCR, reverse transcriptase PCR.

Monoclonal antibody against E-cadherin was a generous gift from Dr. Barry Gumbiner (Memorial Sloan Kettering Cancer Center, New York), and monoclonal antibody against the α -subunit of Na⁺,K⁺-ATPase was a generous gift from Dr. Mike Caplan (Yale University, New Haven, CT). Rabbit polyclonal antibody raised to the cytoplasmic domain of E-cadherin was previously described (Marrs et al., 1993). Rabbit polyclonal antibody raised to the α -subunit of Na⁺,K⁺-ATPase purified from dog kidney (as previously described; Hammerton et al., 1991), and rabbit polyclonal antibody to bovine lens fodrin were provided by Kathy Siemers (Stanford University, Stanford, CA). Rabbit polyclonal antibodies to ankyrin were generously provided by Drs. Ken Beck (Stanford University; raised to erythroid ankyrin from dog), Van Bennett (Duke University, Durham, NC; raised to the spectrin binding domain of human erythroid ankyrin), and Luanne Peters and Samuel Lux (Harvard University, Boston, MA; raised to the spectrin binding domain of ankyrin-3 from human). Rabbit polyclonal antibodies raised to desmoplakins I/II, and desmoglein antibodies were provided by Dr. Manijeh Pasdar (University of Alberta, Alberta, Canada). AE1/AE3 and Troma-1 monoclonal antibodies to keratin were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and Developmental Studies Hybridoma Bank (Iowa City, IA), respectively. Anti-mouse IgG and anti-rabbit IgG secondary antibodies conjugated to either FITC or rhodamine were purchased from Boehringer Mannheim, Jackson Laboratories (Bar Harbor, ME), and Cappel Laboratories (Cochranville, PA).

Transfection

Canine E-cadherin cDNA driven by the RSV promoter in the plasmid pR2/RSV was a kind gift from Dr. Lee Rubin (Eisai London Research Laboratories, University College, London, UK). The plasmid pMV6, which contains a neomycin resistance gene, was used for cotransfection. RPE-J cells were plated subconfluently and grown overnight in petri dishes at 33°C. Cells were rinsed twice with DME and a mixture of DNA-Lipofectin (15 μ g DNA/50 μ l Lipofectin [GIBCO BRL, Gaithersburg MD]) in 5 ml RPE growth medium was added to each dish. The contents were mixed gently and cultured at 33°C for 10 h. The next day, the medium was replaced with RPE growth medium, and the cells were cultured at 33°C until confluent. Colonies were selected by growing cells in medium containing G418 (400 μ g/ml). Detailed studies were performed using RPE-J and RPE-J+EC cells, but the results have been independently replicated in several clones of cells.

SDS-PAGE and Immunoblotting

Protein samples were analyzed in SDS polyacrylamide gels (7.5% or 9%) as previously described (Laemmli, 1970; Nelson and Veshnock, 1986). Molecular mass standards used, 205 kD (myosin), 116 kD (β -galactosidase), 97 kD (phosphorylase b), 66 kD (serum albumin), 45 kD (egg albumin), and 29 kD (carbonic anhydrase), were purchased from Sigma. For fluorography, gels were treated with Amplify (Amersham Corp., Arlington Heights, IL), and they were dried under vacuum before autoradiography using XAR-5 film (Eastman Kodak Co., Rochester, NY) at -80°C with intensifying screens (Du Pont Pharmaceuticals, Wilmington, DE). For immunoblotting experiments, gels were transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH), and they were incubated with primary and secondary antibodies as previously described (Nelson and Veshnock, 1986), except that for monoclonal antibodies, a sheep anti-mouse ¹²⁵I-labeled secondary antibody was used for detection (Amersham). Dried nitrocellulose filters were exposed to XAR-5 film (Eastman Kodak) at -80°C with intensifying screens. Scanning densitometry was used to quantify autoradiograms (model GS300; Hoefer Scientific Instruments, San Francisco, CA).

RNA Preparation, Reverse Transcriptase PCR, and Northern Blotting

Total cellular RNA was prepared from RPE-J and RPE-J+EC cells using RNAzol B (Tel-Test Inc., Friendswood, TX) according to manufacturer's instructions. RNA was further treated with RNase-free DNase (Promega Corp., Madison, WI) for 1 h at 37°C, extracted with phenol/chloroform/isoamyl alcohol, and ethanol precipitated. Reverse transcriptase PCR (RT-PCR) was performed using a GeneAmp kit (Perkin-Elmer Corp., Norwalk, CT) using primers and PCR conditions from Suzuki et al. (1991). PCR products were cloned using pCR-script cloning vector (Stratagene, La Jolla, CA). DNA sequencing was performed using sequenase (United States Biochemical Corp., Cleveland, OH).

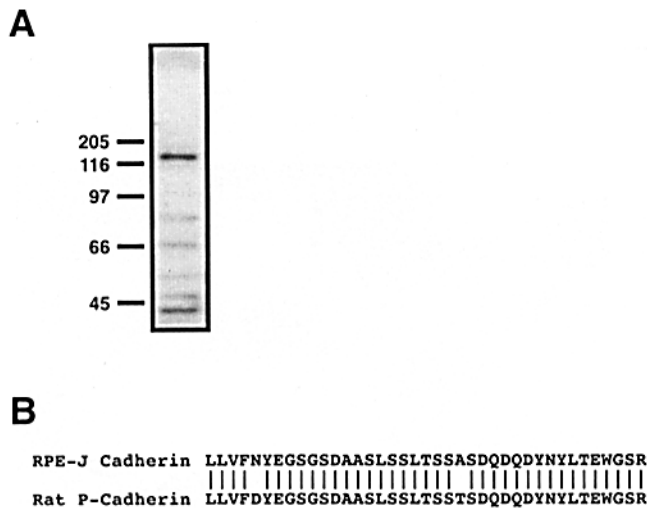


Figure 1. Cadherin expression in the retinal pigment epithelium cell line RPE-J. (A) Immunoblot analysis of total cellular proteins from RPE-J cells using a pan-cadherin antibody. A polypeptide with apparent migration of 125 kD was recognized in these cells, which represents the endogenous cadherin. Other polypeptides recognized in this immunoblot are most likely degradation products, but they may also be a consequence of nonspecific recognition by primary antibody or [¹²⁵I]protein A. Molecular mass markers are 205, 116, 97, 66, and 45 kD. (B) Identification of endogenously expressed cadherin in RPE-J cells by RT-PCR. One cadherin sequence was cloned multiple times from RPE-J cells (*top sequence*), which was found to be most closely related (95% identity) to rat P-cadherin (*bottom sequence*) (Suzuki et al., 1991).

15 μ g of total RNA prepared as described above and high molecular weight RNA standards (GIBCO BRL) were separated on formaldehyde/agarose gels; RNA was transferred to Hybond-N (Amersham) nylon membrane, prehybridized, hybridized, and washed according to standard procedures (Sambrook et al., 1989). Low stringency washing procedure was used: one wash with 1 \times SSC, 0.1% SDS for 20 min at room temperature, three washes with 1 \times SSC for 20 min at 68°C, and one wash with 0.5 \times SSC for 10 min at 68°C. A human desmoglein 2 cDNA clone (Schäfer et al., 1994; generously provided by Dr. Werner Franke, German Cancer Research Center, Heidelberg, Germany) was ³²P labeled using an oligonucleotide primer labeling kit (Pharmacia Fine Chemicals, Piscataway, NJ). Membranes were exposed to XAR-5 film (Eastman Kodak) at -80°C with intensifying screens.

Metabolic Labeling, Biotinylation, and Immunoprecipitation

Cells were starved in methionine-free medium for 15–30 min, then metabolically labeled using 200–500 μ Ci [³⁵S]methionine/cysteine (PRO-MIX; Amersham) in methionine-free medium. Metabolic labeling was performed for various times (5–15-min pulses for ankyrin and fodrin immunoprecipitations, and 1 h or overnight pulses for cadherin and Na⁺,K⁺-ATPase immunoprecipitations).

Biotinylation of RPE-J and RPE-J+EC cell monolayers on Transwell or nitrocellulose filters was performed using sulfo-NHS-biotin (sulfosuccinimidobiotin), NHS-LC-biotin [sulfosuccinimidyl 6 (biotinamido) hexanoate] (steady-state biotinylation), or NHS-SS-biotin [sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate] (newly synthesized and residence time experiments) as previously described (Sargiacomo et al., 1989; Hammerton et al., 1991; Wollner et al., 1992). After biotinylation, cells were processed for immunoprecipitation as described earlier (Le Bivic et al., 1989; Wollner et al., 1992; Siemers et al., 1993). The biotinylation were performed independently in both the Nelson and Rodriguez-Boulan laboratories, and cells were either extracted on Transwell filters (Costar) that had been excised from the plastic collar, or they were extracted in situ; identical results were obtained with either procedure (Siemers et al., 1993). For steady-state biotinylation, immunoprecipitated proteins were processed

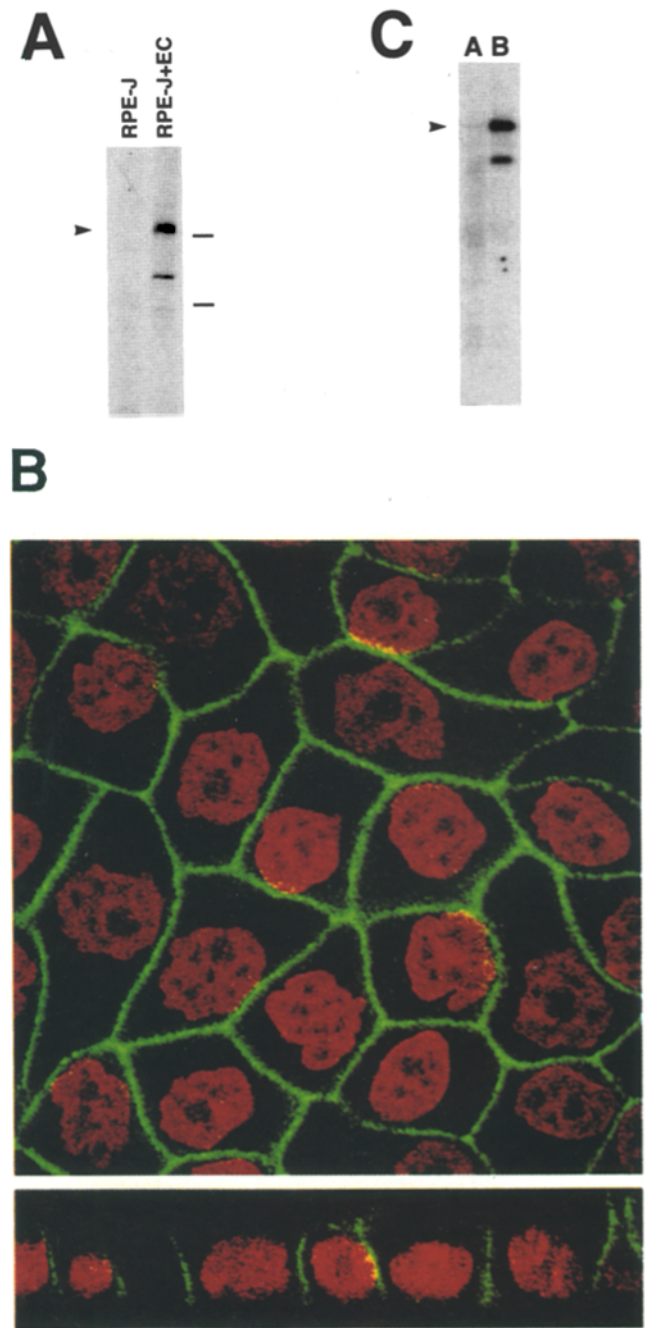


Figure 2. E-cadherin expression in retinal pigment epithelial cells. (A) RPE-J cells transfected with E-cadherin cDNA (*RPE-J+EC cells*) specifically expressed this cell adhesion molecule. In RPE-J cells (lane 1), E-cadherin was not detected, but, in RPE-J+EC cells (lane 2), E-cadherin (*arrowhead*), along with the coprecipitated β -catenin was detected. Molecular mass markers are 116 and 84 kD. (B) Localization of E-cadherin in RPE-J+EC cells. En face (*top panel*) and *x-z* reconstruction (*bottom panel*) by laser scanning confocal immunofluorescence microscopy showed that E-cadherin is localized to sites of cell-cell contact in the transfected RPE-J+EC cells. Bar, 10 μ m. (C) Domain selective biotinylation on either the apical (lane A) or basal-lateral (lane B) cell surface of RPE-J+EC cells shows a basal-lateral distribution for E-cadherin (*arrowhead*).

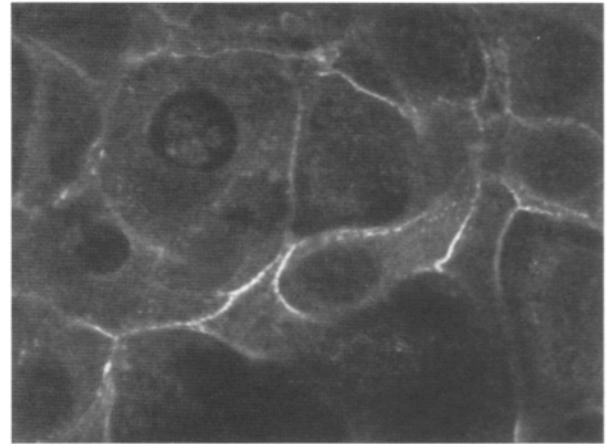
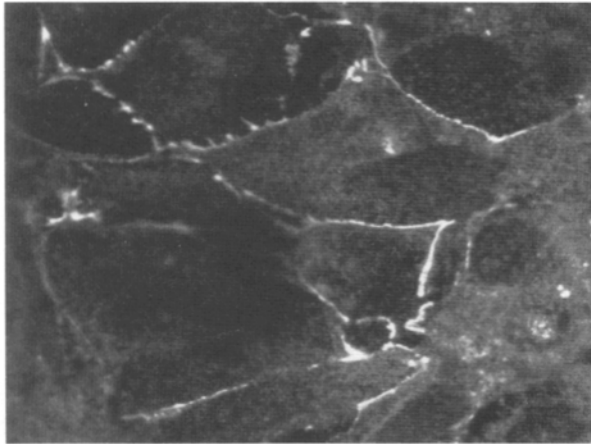
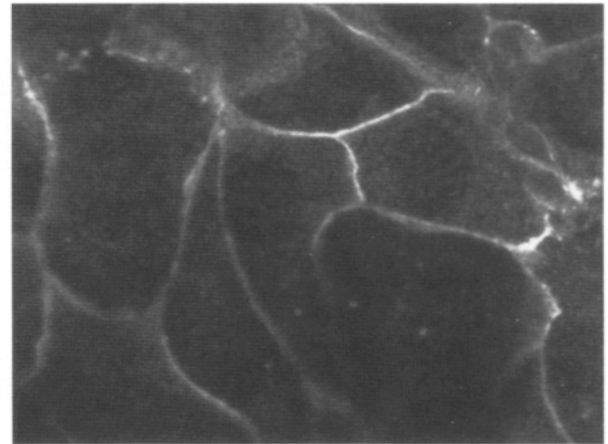
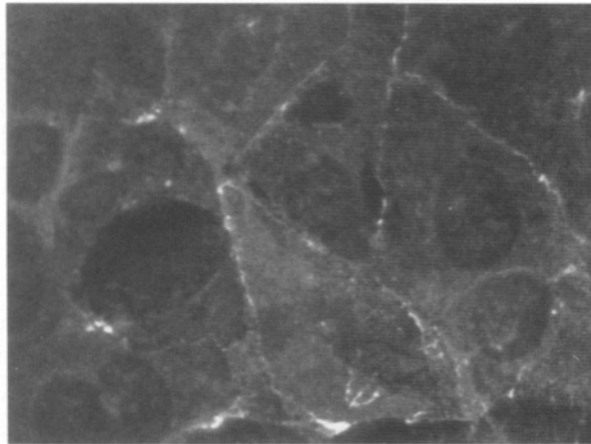
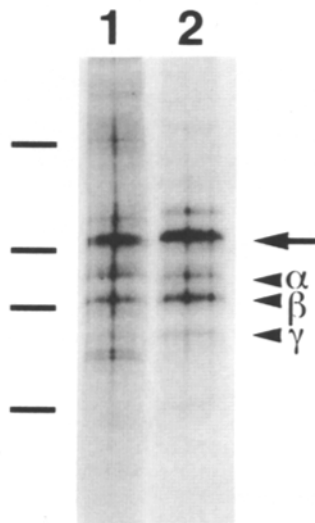
A**RPE-J****RPE-J+EC****Cadherin****Plakoglobin****B**

Figure 3. Comparison of cadherin expression in RPE-J and RPE-J+EC cell lines. (A) Localization of cadherin and catenin proteins in RPE-J cells and RPE-J+EC cells. RPE-J cells (*left panels*) and RPE-J+EC cells (*right panels*) were stained with pan-cadherin (*top panels*) and plakoglobin (*bottom panels*) antibody. Bar, 10 μ m. (B) Coimmunoprecipitation of cadherin (*arrow*) and catenins (*arrowhead*) from Triton X-100 extracts of RPE-J (lane 1) and RPE-J+EC cells (lane 2).

for SDS-PAGE, transferred to nitrocellulose, and detected with streptavidin (Le Bivic et al., 1989; Wollner et al., 1992; Siemers et al., 1993). To detect newly synthesized (35 S-labeled) proteins, immune complexes were dissociated, then precipitated using avidin-agarose before SDS-PAGE and fluorography (Hammeron et al., 1991; Wollner et al., 1992).

Immunofluorescence Microscopy

For *x-z* confocal images, cells were grown on matrigel-coated filters and processed for indirect immunofluorescence microscopy as described previously (Nabi et al., 1993). Nuclei were stained with propidium iodide. Im-

ages were collected using a dual channel laser scanning confocal microscope (Saraströ; Molecular Dynamics, Inc., Sunnyvale, CA) equipped with a Screenstar camera. Otherwise, cells were grown on matrigel-coated coverslips, and they were processed for indirect immunofluorescence microscopy as described previously (Marrs et al., 1993). Images were collected on a laser scanning confocal microscope on a Zeiss IM35 (Carl Zeiss, Oberkochen, Germany; for details see McNeill et al., 1993), except double immunofluorescence images of keratin and desmoplakins I/II, which were collected using a Zeiss Axiophot microscope equipped with epifluorescence (Carl Zeiss).

Electron Microscopy

Transmission electron microscopy of thin sections of RPE-J and RPE-J+EC cells grown on nitrocellulose filters and ruthenium red staining was performed as previously described (Nabi et al., 1993). Sections were viewed at 80 kV with an electron microscope (JEOL-100 CXII; JEOL U.S.A., Inc., Peabody, MA).

Results

Cadherin Expression in RPE-J Cells

Cadherins share a high degree of sequence similarity (Kemler, 1992). The most highly conserved region of cadherins is the cytoplasmic domain, and different members of the cadherin superfamily can be detected using antisera raised to this conserved domain (Marrs et al., 1993). We detected an endogenous cadherin in the RPE-J cells by immunoblot of whole-cell protein (Fig. 1 A). To identify the cadherin(s) expressed in RPE-J cells, we amplified the sequence of the endogenous cadherin using degenerate oligonucleotides corresponding to sequences within the highly conserved cytoplasmic domain. A PCR product of the expected size was isolated from multiple clones and sequenced. The deduced amino acid sequence (Fig. 1 B) shares 95% identity with the corresponding region of rat P-cadherin (Suzuki et al., 1991), and 82% identity with rat E-cadherin (Suzuki et al., 1991). B-cadherin is expressed in chicken RPE cells, but the sequence for the rat homologue is currently unknown; the endogenous rat RPE cadherin shows 79% identity to chick B-cadherin (Napolitano et al., 1991). Other cadherin sequences, particularly E-cadherin sequences, were not recovered from RPE-J cell RNA. These data are sufficient to distinguish the endogenous RPE-J cadherin from E-cadherin. Further sequence analysis of this cadherin is currently ongoing.

In RPE cells *in situ* and in RPE-J cells, E-cadherin was not immunologically detected with a specific antibody (Fig. 2 A) (Gundersen et al., 1993), and E-cadherin mRNA was not detected by RT-PCR (see above). RPE-J cells were transfected with a cDNA encoding canine E-cadherin under the control of the constitutive RSV promoter, producing the cell line RPE-J+EC. Although subsequent detailed studies were performed using the RPE-J and RPE-J+EC cell lines, the results have been replicated independently in several clones.

E-cadherin expression in RPE-J+EC cells was detected by protein immunoprecipitation using an E-cadherin-specific monoclonal antibody (Fig. 2 A). Confocal immunofluorescence microscopy showed that E-cadherin was localized to sites of cell-cell contact (Fig. 2 B), and membrane domain-selective biotinylation showed that E-cadherin distribution was restricted to the basal-lateral membrane (Fig. 2 C; cell surface biotinylation can not distinguish between lateral and basal membrane proteins).

Three cytoplasmic proteins, termed α -, β -, and γ -catenins, have been shown previously to bind to cadherins (Kemler, 1992). By immunofluorescence, cadherin and the three catenins localized to sites of cell-cell contact in both RPE-J and RPE-J+EC cells; the localization of plakoglobin (γ -catenin) is shown as an example of catenin distribution (Fig. 3 A). In addition, α -, β -, and γ -catenin were coimmunoprecipitated in a complex with cadherin using an antiserum to the conserved cytoplasmic domain of cadherins, from both RPE-J and RPE-J+EC cells (Fig. 3 B).

E-Cadherin Induces (Basal-)Lateral Membrane Distribution of Na^+ , K^+ -ATPase in RPE Cells

In RPE-J cells, Na^+ , K^+ -ATPase was localized to the cell interior, and to both apical and basal-lateral membranes (Fig. 4 A). In contrast, in RPE-J+EC cells, Na^+ , K^+ -ATPase distribution was restricted to sites of cell-cell contact, and colocalized with E-cadherin (compare with Fig. 2 B); little or no Na^+ , K^+ -ATPase was detected on either apical or basal membranes (Fig. 4 A). Differences in Na^+ , K^+ -ATPase distributions between RPE-J and RPE-J+EC cells were further examined by membrane domain-selective protein biotinylation. In RPE-J cells, Na^+ , K^+ -ATPase was detected in both apical and basal-lateral membranes (63% apical, 37% basal-lateral). In contrast, in RPE-J+EC cells, Na^+ , K^+ -ATPase was detected only in the basal-lateral membrane (Fig. 4 B).

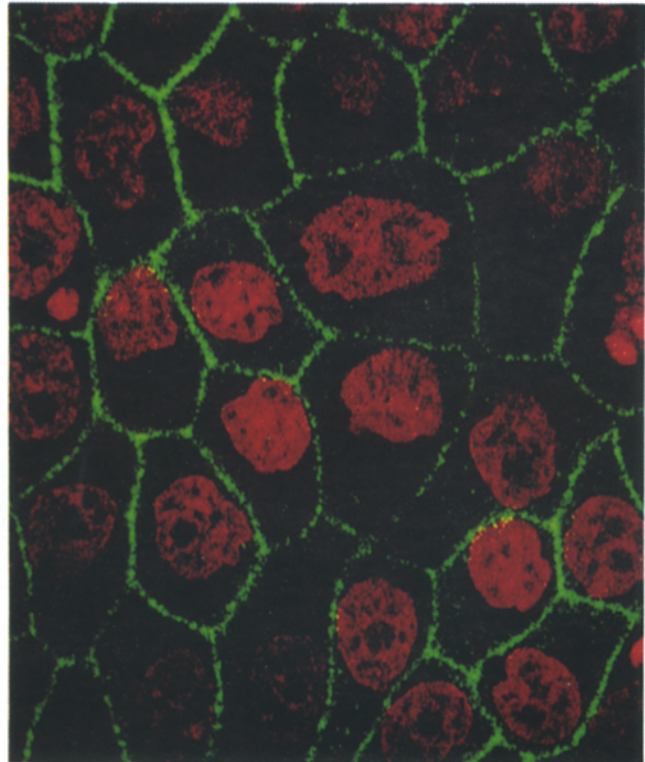
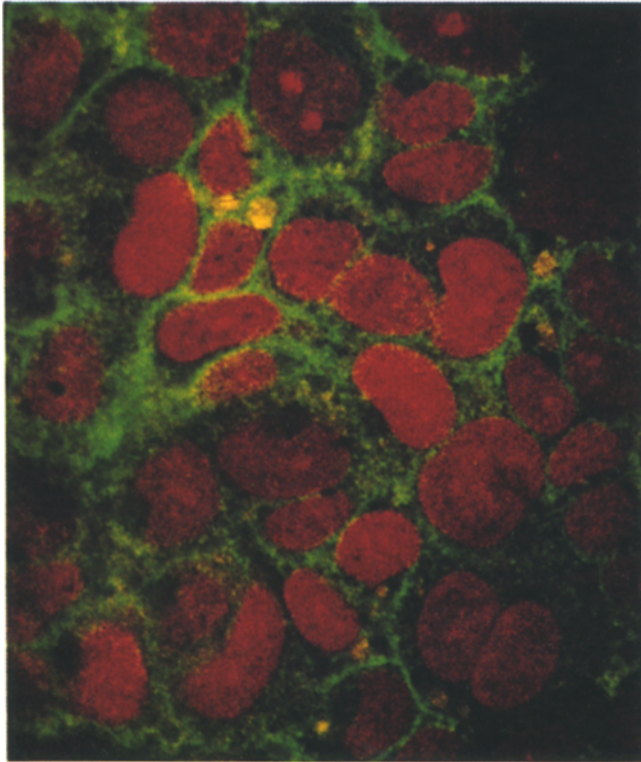
These biotinylation data must be considered in the context of the distribution of the tight junction in RPE-J and RPE-J+EC cells. A previous study showed that the RPE-J cell monolayer develops a high electrical resistance, indicative of tight junction integrity, but the tight junction localized to variable sites along the length of the lateral membrane (Nabi et al., 1993). In contrast, we found that ruthenium red staining of the apical surface of RPE-J+EC cells was blocked at the apico-lateral membrane junction and did not penetrate any area of the lateral membrane (data not shown). These data show that E-cadherin expression induced the relocation of tight junctions to a location similar to that in most epithelial cell types. An important consequence of tight junction location in RPE-J+EC cells is that biotinylation through the apical compartment of the Transwell filter will label only apical membrane proteins, and biotinylation through the basal compartment will label only basal and lateral membrane proteins.

We next investigated mechanisms involved in restricting Na^+ , K^+ -ATPase distribution to the (basal-)lateral membrane in RPE-J+EC cells. The delivery of newly synthesized Na^+ , K^+ -ATPase to the cell surface was examined by [^{35}S]methionine/cysteine pulse labeling followed by membrane domain-selective biotinylation. Results showed delivery of newly synthesized Na^+ , K^+ -ATPase to both apical and basal-lateral membranes, although proportionally more (67%) was delivered to the apical membrane (Fig. 4 C). Note, however, that despite delivery to both domains, Na^+ , K^+ -ATPase distribution is restricted to the basal-lateral membrane of RPE-J+EC cells at steady state (Fig. 4). Using [^{35}S]methionine/cysteine pulse-chase analysis and membrane domain-selective protein biotinylation, we examined the fate of newly synthesized Na^+ , K^+ -ATPase delivered to different membrane domains. Results showed that Na^+ , K^+ -ATPase delivered to the apical membrane had a shorter half-

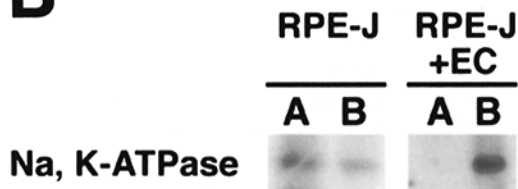
A

RPE-J

RPE-J+EC



B



C



D

Time of Chase	2		5		21	
Membrane Domain	Ap	B-L	Ap	B-L	Ap	B-L
TX-100 Solubility	S	P	S	P	S	P
Na, K-ATPase						
	50	50	29	71	25	75

Figure 4. E-cadherin expression in retinal pigment epithelial cells induces basal-lateral distribution of Na⁺,K⁺-ATPase. (A) Localization of Na⁺,K⁺-ATPase in RPE-J (left panels) and RPE-J+EC (right panels) cells shows an E-cadherin-induced redistribution of this protein. Laser scanning confocal microscope images are shown in en face (top panel) and x-z reconstruction (bottom panels) orientations. (B)

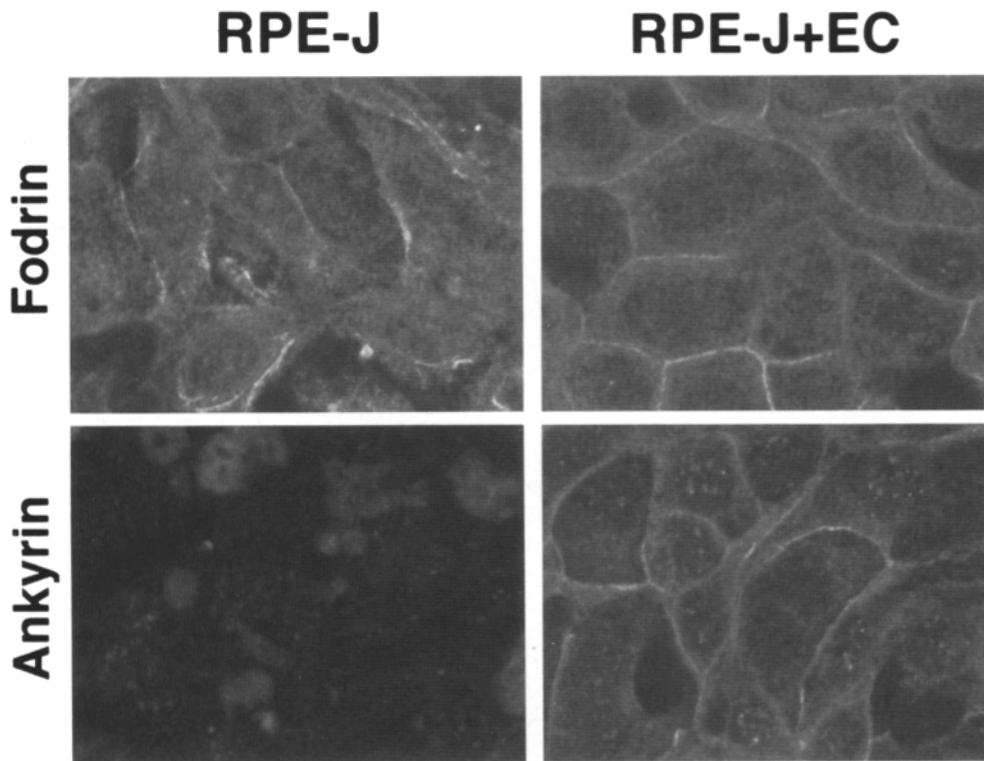


Figure 5. Distribution of membrane cytoskeletal proteins fodrin and ankyrin in RPE-J and RPE-J+EC cells. Laser scanning confocal immunofluorescence microscopy of RPE-J (left panels) and RPE-J+EC (right panels) cells with antibodies raised to fodrin (top panels) and the spectrin-binding domain of erythrocyte ankyrin (bottom panels), which recognizes ankyrins-1/2. Bars, 10 μm .

life than that which arrived at the basal-lateral plasma membrane (Fig. 4 D).

E-Cadherin Induces Synthesis and Accumulation of a Different Ankyrin Isoform Concomitant with the Redistribution of Na^+, K^+ -ATPase

We investigated whether Na^+, K^+ -ATPase redistribution to, and increased retention time in, the basal-lateral membrane of RPE-J+EC cells coincided with reorganization and assembly of the membrane cytoskeleton. Immunofluorescence microscopy showed that fodrin localized to sites of cell-cell contact in both RPE-J and RPE-J+EC cell lines (Fig. 5), although the staining intensity at the membrane of RPE-J+EC cells was stronger than that in RPE-J cells. Immunoblots of equal amounts of total cellular protein from RPE-J and RPE-J+EC cells showed that significantly more fodrin was present in RPE-J+EC cells (17-fold increase) than in RPE-J cells (Fig. 6 A). [^{35}S]methionine/cysteine pulse labeling experiments showed that fodrin was synthesized at approximately the same rate in RPE-J and RPE-J+EC cells (Fig. 6 B). However, [^{35}S]methionine/cysteine pulse-chase analysis showed that the metabolic stability of fodrin is greater in RPE-J+EC cells ($t_{1/2} = 8\text{--}10$ h) than in RPE-J cells ($t_{1/2} = 3\text{--}5$ h) (Fig. 6 C).

We next analyzed the expression of ankyrin isoforms using

antibodies specific for ankyrins-1/2 or ankyrin-3 isoforms. RPE-J and RPE-J+EC cells expressed the ankyrin-3 isoform which, in both cases, colocalized with fodrin at sites of cell-cell contact (Marrs, J. A., unpublished observations). In contrast, a difference in the expression pattern of ankyrins-1/2 was observed between the two cell lines. Expression of these isoforms was not detected in RPE-J cells, whereas RPE-J+EC cells expressed ankyrins-1/2 at sites of cell-cell contact (Fig. 5). E-cadherin-induced accumulation of ankyrins-1/2 in RPE-J+EC cells is shown clearly in an immunoblot of equal amounts of total cellular protein from RPE-J and RPE-J+EC cells (Fig. 6 D).

We examined whether changes in the pattern of ankyrin isoform accumulation resulted from the induction of protein synthesis and/or decreased turnover and accumulation. [^{35}S]methionine/cysteine pulse labeling, followed by protein immunoprecipitation with a pan-ankyrin antibody, revealed a distinct pattern of expression of ankyrin isoforms in the two cell lines (Fig. 6 E). In RPE-J cells, two ankyrin proteins were immunoprecipitated which migrated in SDS-polyacrylamide gels with apparent molecular masses of 220 and 280 kD (Fig. 6 E). A different profile of ankyrin isoform expression was detected in RPE-J+EC, and it comprised proteins with apparent molecular masses of 220 and 240 kD (Fig. 6 E); the 280 kD isoform was not detected. Polypeptides of lower apparent molecular weight are ankyrin degra-

Steady-state distribution of Na^+, K^+ -ATPase on either the apical (lane A) and basal-lateral (lane B) plasma membrane domains of RPE-J (left panel) and RPE-J+EC (right panel) cells. (C) Newly synthesized Na^+, K^+ -ATPase is delivered to the apical (Ap) and basal-lateral (B-L) plasma membrane domains of RPE-J+EC cells: S, Triton X-100-soluble extract; P, Triton X-100-insoluble extract. Quantification of the amount of newly synthesized α -subunit of Na^+, K^+ -ATPase is presented as a percentage of the total. (D) Fate of newly synthesized Na^+, K^+ -ATPase delivered to the apical (Ap) or basal-lateral (B-L) plasma membrane of RPE-J+EC cells. Quantification of Na^+, K^+ -ATPase is presented as a percentage of the total. The times of chase are in hours. Bar, 10 μm .

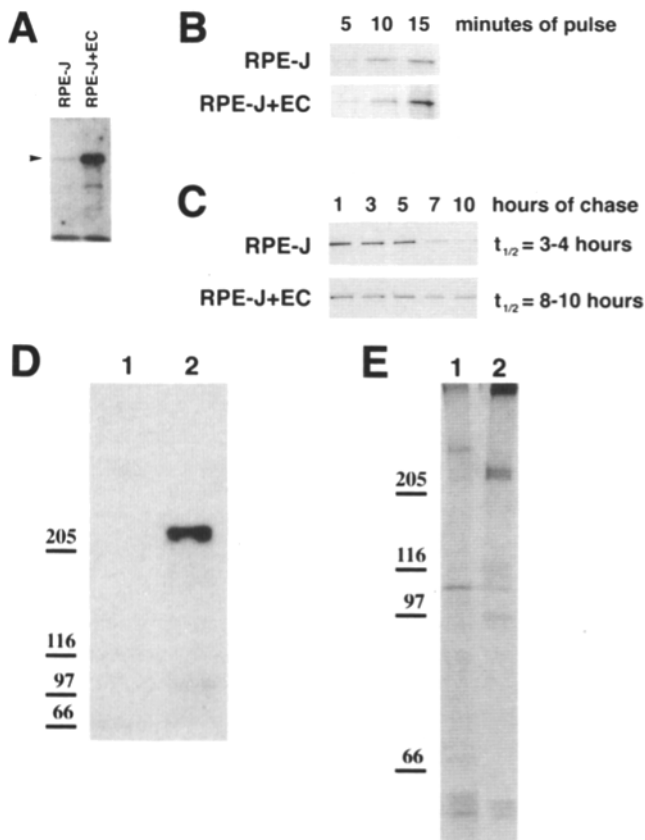


Figure 6. Fodrin and ankyrin expression in retinal pigment epithelial cells. (A) Immunoblot analysis of total proteins from RPE-J (left lane) and RPE-J+EC (right lane) cells using antifodrin antiserum. (B) Synthesis of fodrin in RPE-J (top panel) and RPE-J+EC (bottom panel) cells. (C) Turnover of newly synthesized fodrin in RPE-J (top panel) and RPE-J+EC (bottom panel) cells. (D) E-cadherin-induced accumulation of ankyrins-1/2 in RPE cells. Immunoblot of total proteins from RPE-J (lane 1) and RPE-J+EC (lane 2) cells with antiserum raised to the spectrin-binding domain of erythrocyte ankyrin, which recognizes ankyrins-1/2. (E) Different patterns of ankyrin isoform synthesis in RPE-J (lane 1) and RPE-J+EC (lane 2) cells.

dation products. [³⁵S]methionine/cysteine pulse-chase analysis showed that the turnover and accumulation of ankyrin isoforms expressed were similar to those of fodrin in RPE-J and RPE-J+EC cells (data not shown).

E-Cadherin Induces the Assembly of Desmosomes and Keratin Filaments in Rat Retinal Pigment Epithelial Cells

Unlike RPE cells from other mammalian species (Owaribe et al., 1988), neither rat RPE in situ nor RPE-J cells (Fig. 7, A and B) assemble desmosomal junctional complexes. However, examination of electron micrographs of RPE-J+EC cells reveals electron-dense plaques at cell-cell contacts with some associated cytoplasmic tonofilaments that are characteristic of desmosomes (Fig. 7, C and D).

The expression of desmosomal proteins in RPE-J and RPE-J+EC cells was examined by immunofluorescence with antibodies specific for desmosomal plaque proteins, des-

moplakins I/II, and the desmosomal cadherin, desmoglein (Fig. 8 A). In RPE-J cells, the staining pattern of desmoplakins I/II was diffuse throughout the cytoplasm. In contrast, in RPE-J+EC cells, desmoplakins I/II were localized in punctate structures at cell-cell contacts (Fig. 8 A); this staining pattern is characteristic of desmoplakins I/II assembled in desmosomes, and is similar to that found in other epithelial cell types. Desmoglein staining was not detected in RPE-J cells (Fig. 8 A), nor was desmoglein detected by immunoblotting extracts of RPE-J total cell protein (data not shown). However, in RPE-J+EC cells, a punctate desmoglein staining pattern was found at cell-cell contacts, similar to that of desmoplakins I/II (Fig. 8A).

To determine whether E-cadherin-induced accumulation of desmoglein was controlled at the mRNA level, equal amounts of total RNA from RPE-J and RPE-J+EC cells were probed using a human desmoglein 2 cDNA; desmoglein-2 is the isoform expressed in simple epithelia, such as the RPE (Schäfer et al., 1994). Fig. 8 B shows that little or no desmoglein mRNA was detected in RPE-J cells. However, abundant desmoglein mRNA was detected in RPE-J+EC cells. The desmoglein 2 mRNA detected in RPE-J+EC cells was approximately the same size as that found previously (6.1 kb; Schäfer et al., 1994).

Since desmosomes are membrane attachment sites for the keratin filament cytoskeleton in epithelial cells (Schwarz et al., 1990; Kouklis et al., 1994), we investigated the consequence of E-cadherin expression on the expression and localization of keratin intermediate filaments. In RPE-J cells, keratin filaments were not detected with either a mixture of monoclonal antibodies AE1 and AE3 (Fig. 9 A) nor the Troma-1 monoclonal antibody that detects K8 (data not shown). In contrast, RPE-J+EC cells stained strongly with these keratin antibodies, and filaments were detected radiating from the nucleus, throughout the cytoplasm, to the plasma membrane at sites of cell-cell contact (Fig. 9 A). Immunoblots of equal amounts of total cell proteins from RPE-J and RPE-J+EC cells probed with antikeratin monoclonal antibodies (AE1 and AE3) demonstrated that keratin protein was detectable in RPE-J cells, but in comparison, keratin proteins accumulated significantly in RPE-J+EC cells (Fig. 9 B).

Discussion

Since expression of different cadherin family members is temporally and spatially regulated during embryogenesis and restricted to specific tissues in the adult, it has been suggested that cadherins perform important roles in morphogenesis and differentiation (Edelman, 1985; Takeichi, 1988). One function of cadherins appears to be in regulating the sorting of different cell populations from one another, which gives rise to the spatial segregation of cell types in complex tissues (Edelman, 1986; Takeichi, 1988). Here, we present strong evidence that cadherins have different capacities to induce specialized structural and functional features of the polarized epithelial cell phenotype. In addition, we show that changes in cell phenotype are caused by remodeling the distribution of constitutively expressed proteins, and induction in accumulation of new proteins.

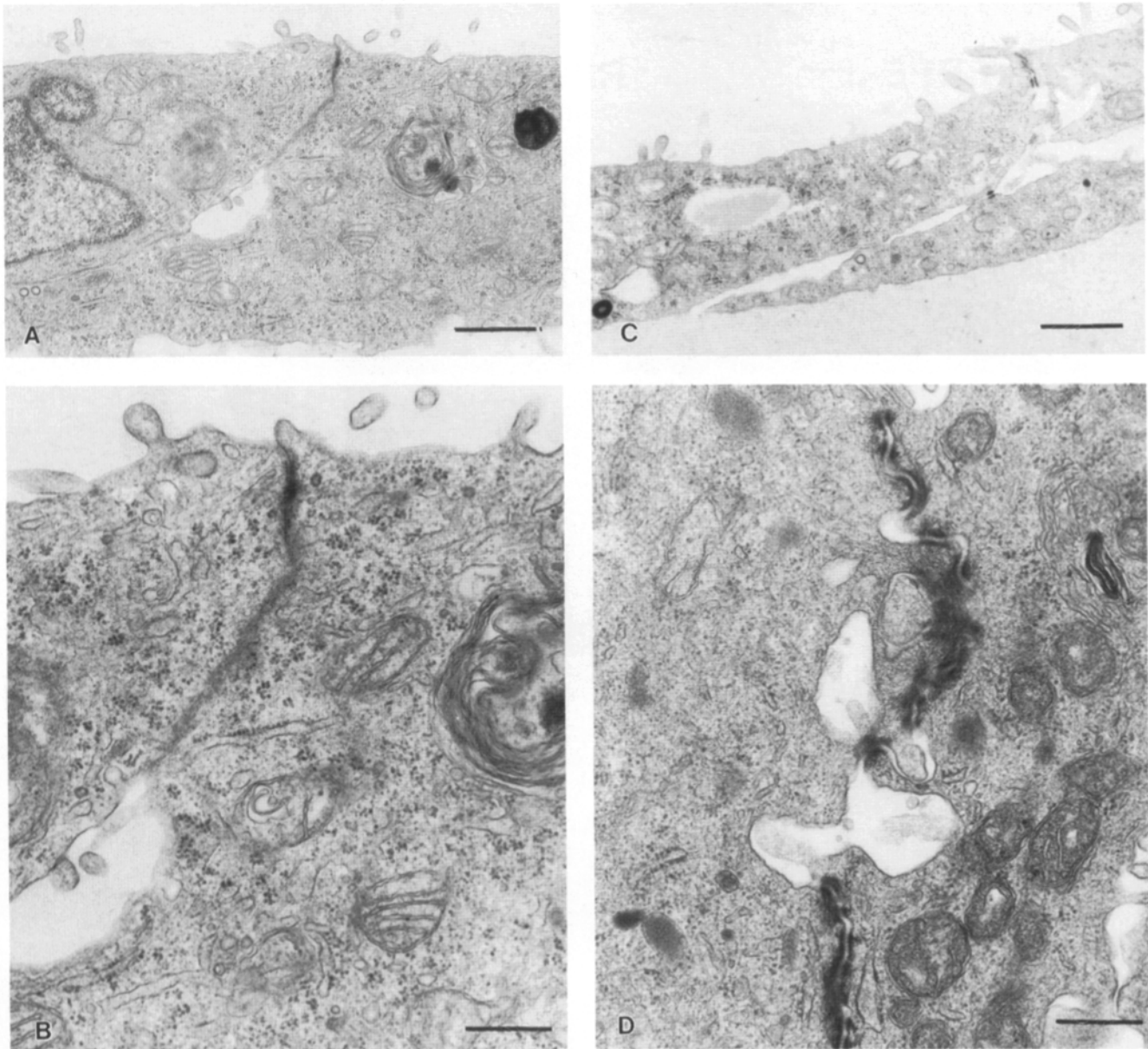


Figure 7. E-cadherin induces the assembly of desmosomes in retinal pigment epithelial cells. Electron micrographs of RPE-J (A) and RPE-J+EC (C) cells shows that desmosomes are only observed in RPE-J+EC cells. (B and D) Higher magnification of RPE-J and RPE-J+EC cells, respectively. Desmosomes in RPE-J+EC cell displays the characteristic trilaminar morphology. Bar, 1 μm in A and B, and 0.5 μm in C and D.

Roles of Cadherins in Generating Plasticity in Polarized Epithelial Cell Organization

The plasma membrane distribution of Na^+, K^+ -ATPase in polarized, transporting epithelia is important in determining the direction of vectorial ion transport (Almers and Stirling, 1984; Rodriguez-Boulant and Nelson, 1989). Depending on the cell type, Na^+, K^+ -ATPase distribution can be restricted to the apical (RPE, choroid plexus), apical and lateral (early collecting duct, polycystic kidney), lateral (MDCK cells), or basal-lateral membranes (renal, intestinal epithelia). Our present study provides new insight into the role of cadherins in generating these different distributions of Na^+, K^+ -ATPase.

In situ, the RPE is located between the neural retina and the choroid. Na^+, K^+ -ATPase is located at the apical membrane of the RPE at the sites of contact with the neural retina. In RPE cells in dissociated cell culture and the cell line RPE-J, Na^+, K^+ -ATPase is localized to both apical and basal-lateral membranes (Rizzolo, 1990, 1991; Nabi et al., 1993).

The plasma membrane distribution of Na^+, K^+ -ATPase in RPE-J cells is a reflection of the delivery of newly synthesized protein to both apical and basal-lateral membranes. However, the restriction of Na^+, K^+ -ATPase to the lateral membrane in RPE-J+EC cells is not a consequence of a re-orientation of the sorting mechanism in the Golgi complex since Na^+, K^+ -ATPase is still delivered from the Golgi complex to both membrane domains. In fact, a higher proportion

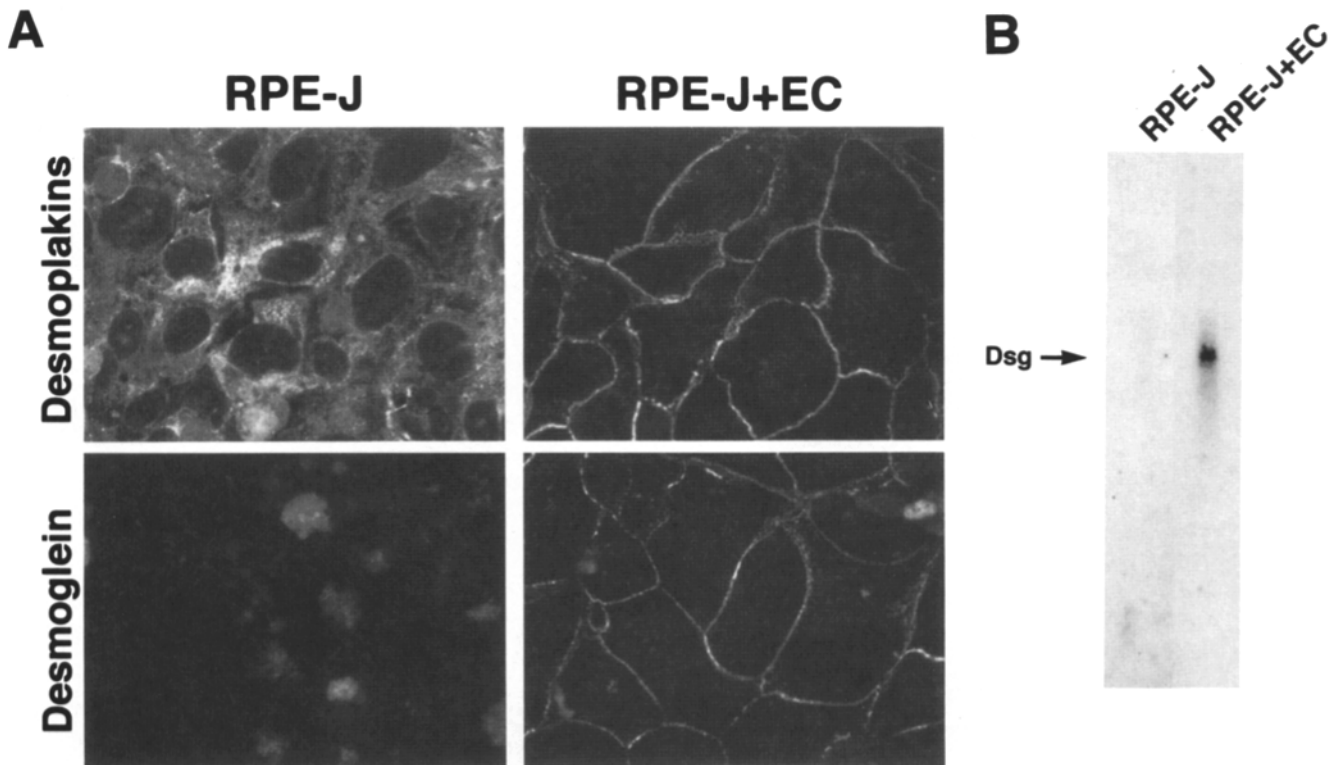


Figure 8. E-cadherin-induced accumulation and relocalization of desmosomal components is in part caused by accumulation of desmoglein mRNA. (A) Localization of desmosomal components desmoplakins I/II (top panels) and desmoglein (bottom panels) by laser scanning confocal immunofluorescence microscopy of RPE-J (left panels) and RPE-J+EC (right panels) cells. Bar, 10 μ m. (B) Northern blot analysis of equal amounts of total RNA from RPE-J (left lane) and RPE-J+EC (right lane) cells probed with a human desmoglein 2 cDNA clone (Schäfer et al., 1994) using low stringency conditions (see Materials and Methods).

(67%) of newly synthesized Na^+, K^+ -ATPase is delivered to the apical membrane. However, we showed that Na^+, K^+ -ATPase delivered to the apical membrane has an apparently shorter half-life than that of Na^+, K^+ -ATPase delivered to the basal-lateral membrane.

The increased stability and accumulation of Na^+, K^+ -ATPase in the basal-lateral membrane in RPE-J+EC cells correlates with the redistribution and accumulation of fodrin, and the synthesis and accumulation of ankyrins-1/2. The accumulation of these proteins is significant since they have been shown to form a supramolecular complex with Na^+, K^+ -ATPase (Nelson and Veshnock, 1987; Koob, et al., 1987; Morrow, et al., 1989; Davis and Bennett, 1990). Note that the distributions of Na^+, K^+ -ATPase, ankyrin and fodrin become restricted to the precise boundary of cell-cell contacts and are excluded from both the apical and basal membranes, demonstrating that the redistribution of this protein complex is dependent on the subcellular location of E-cadherin-mediated cell-cell contacts.

The increased retention time and accumulation of Na^+, K^+ -ATPase, and the coordinate redistribution of the membrane cytoskeleton at sites of cell-cell contact, are all a consequence of E-cadherin expression in RPE-J+EC cells. These results provide strong evidence to support a mechanism for regulating Na^+, K^+ -ATPase distribution that was indicated by earlier studies in MDCK cells and fibroblasts (Nelson and Hammerton, 1989; Wang et al., 1990; McNeill et al., 1990; Hammerton et al., 1991). Stabilization and accumulation of Na^+, K^+ -ATPase at sites of cell-cell contacts

in MDCK cells coincides temporally and spatially with the assembly of membrane cytoskeletal proteins ankyrin and fodrin (Nelson et al., 1990; Nelson and Veshnock, 1987; Hammerton et al., 1991), similar to our observations in RPE-J+EC cells. The distributions of Na^+, K^+ -ATPase and the membrane cytoskeleton are also reorganized in E-cadherin-transfected fibroblasts, but the mechanism for this reorganization was unclear (McNeill et al., 1990).

Our studies in RPE-J+EC cells also showed that targeted delivery of newly synthesized Na^+, K^+ -ATPase from the Golgi complex to the basal-lateral membrane is not required to generate a polarized distribution this protein. In MDCK cells, Na^+, K^+ -ATPase is sorted in the Golgi complex and delivered to the basal-lateral membrane in one clone (Gotardi and Caplan, 1993; Mays, R. W., K. Siemers, G. van Meer, B. Fritz, A. Lowe, and W. J. Nelson, manuscript submitted for publication), but it is not sorted in another clone (Hammerton et al., 1991; Siemers et al., 1993; Mays, R. W., K. Siemers, G. van Meer, B. Fritz, A. Lowe, and W. J. Nelson, manuscript submitted for publication); however, in both MDCK clones, Na^+, K^+ -ATPase has a significantly longer half-life in the basal-lateral membrane, than in the apical membrane (Mays, R. W., K. Siemers, G. van Meer, B. Fritz, A. Lowe, and W. J. Nelson, manuscript submitted for publication). Based on these studies, we propose that increased stabilization of Na^+, K^+ -ATPase in the membrane by retention in a complex with the membrane cytoskeleton is an important mechanism for regulating the distribution of this ubiquitous enzyme in different cell types.

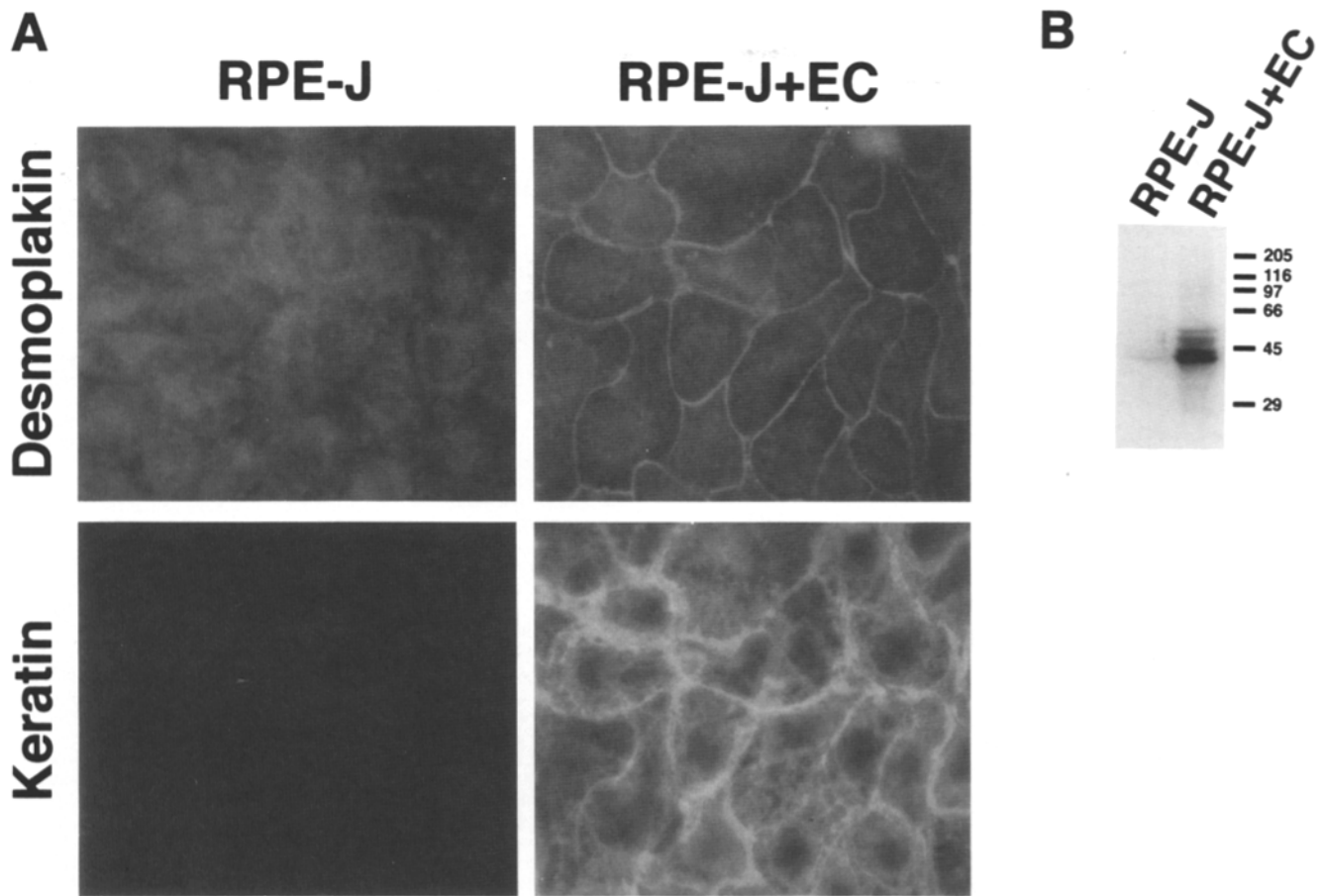


Figure 9 E-cadherin induces the accumulation and assembly of keratin filaments in retinal pigment epithelial cells. (A) Double label immunofluorescence microscopy of RPE-J (left panels) and RPE-J+EC (right panels) cells with rabbit polyclonal antibodies to desmoplakins I/II (top panels, rhodamine) and mouse monoclonal antibodies to keratin (bottom panels, FITC). Bar, 10 μ m. (B) Immunoblot analysis of total proteins from RPE-J (left lane) and RPE-J+EC (right lane) cells with a mixture of the antikeratin monoclonal antibodies AE1 and AE3.

Mechanisms Involved in Cadherin-mediated Induction of Polarized Epithelial Cell Phenotypes

Changes in RPE cell organization by expression of distinct cadherins (discussed above) clearly involve remodeling of constitutively expressed proteins. However, our studies also indicate that expression of E-cadherin induces changes in epithelial cell phenotype that requires cadherin-specific differentiation signals. E-cadherin-induced changes in epithelial cell differentiation that were observed in this study were not caused by clonal variation produced during the transfection procedure; E-cadherin-induced differentiation events (Na^+ , K^+ -ATPase redistribution, ankyrin isoform expression and accumulation, and desmosome assembly) were replicated independently in several clones (data not shown). Using RPE cells, we compared patterns of expression of specific proteins normally found in E-cadherin-expressing epithelial cells and showed that accumulation of these proteins was induced by E-cadherin expression. The membrane cytoskeletal proteins, ankyrins-1/2, were synthesized and accumulated in E-cadherin-transfected RPE-J cells. These ankyrin isoforms are expressed in many cell types (Bennett, 1990) in which Na^+ , K^+ -ATPase is localized to the basal-lateral plasma membrane. Ankyrins-1/2 are not expressed in

the choroid plexus epithelium (Marrs, J. A., unpublished observations), an epithelium, like the RPE, in which E-cadherin is not expressed and Na^+ , K^+ -ATPase is restricted to the apical plasma membrane (Marrs et al., 1993).

In addition, we showed that E-cadherin specifically induces the formation of desmosomes and the assembly of keratin filaments, two other features of E-cadherin-expressing epithelia (Schwarz et al., 1990). Note that rat RPE cells in situ do not form desmosomes (Owaribe et al., 1988). We showed that desmoplakins I/II and plakoglobin were expressed in both RPE-J and RPE-J+EC cells, but mRNA and protein of the desmosomal cadherin, desmoglein, were not detectable in RPE-J. As a consequence of E-cadherin expression, however, desmoglein protein and mRNA accumulated in RPE-J+EC cells. These data suggest that E-cadherin expression activates the transcription of a key component of the desmosomal complex, resulting in the assembly of this junctional complex.

These results demonstrate that, in addition to mediating cell adhesion, E-cadherin directly regulates the differentiation of epithelial cell phenotypes. Our results indicate the involvement of two mechanisms. First, our results demonstrate directly the induction of ankyrin-1/2 protein synthesis and desmoglein mRNA synthesis after expression of E-cad-

herin in RPE cells; as noted, these proteins are specific for other epithelial cell types that express E-cadherin. It is possible that cell adhesion molecules activate intracellular signaling pathways (Romer et al., 1992; Walsh and Doherty, 1992; Doherty et al., 1991) that directly regulate de novo expression of specific genes.

Second, E-cadherin provides unique positional information on the plasma membrane that triggers assembly, by mass action, of membrane and membrane cytoskeleton protein complexes at sites of cadherin-mediated cell-cell contact. The polarized assembly of these supramolecular complexes leads to the remodeling of distributions of specific proteins that results in changes in cellular organization and function (see also Musil et al., 1990). We note that the E-cadherin-induced changes in the polarized distributions of Na^+, K^+ -ATPase, and potentially other ion transport proteins between RPE-J and RPE-J+EC cells, are likely to result in profound changes in vectorial transport function of these cells. We also found that E-cadherin expression refined the distribution of tight junctions to the apico-lateral membrane boundary, similar to that in other simple epithelia.

The present study demonstrates directly that cadherins provide specific epithelial differentiation cues indicating a broader function for cadherin cell adhesion molecules than had been shown before. The results from our experiments support the concept that regulated expression of different cadherin family members during embryogenesis, in addition to sorting cells into distinct populations, may provide cadherin-specific signals at key stages of development that lead to the differentiation of specific cell types (Edelman, 1985; Takeichi, 1988).

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