Apical Polarization of N-CAM in Retinal Pigment Epithelium Is Dependent on Contact with the Neural Retina

Doris Gundersen, Sharon K. Powell, and Enrique Rodriguez-Boulan

Department of Cell Biology and Anatomy, Cornell University Medical College, New York, New York 10021

Abstract. The retinal pigment epithelium (RPE) is unique among epithelia in that its apical surface does not face a lumen, but, instead, is specialized for interaction with the neural retina. The molecules involved in the interaction of the RPE with the neural retina are not known. We show here that the neural cell adhesion molecule (N-CAM) is found both on the apical surface of RPE in situ and on the outer segments of photoreceptors, fulfilling an important requisite for an adhesion role between both structures. Strikingly, culture of RPE results in rapid redistribution of N-CAM to the basolateral surface. This is not due to an isoform shift, since the N-CAM expressed by cultured cells (140 kD) is the same as that expressed by RPE in vivo. Rather, the reversed polarity of N-CAM appears to result from the disruption of the contact between the RPE and the photoreceptors of the neural retina. We suggest that N-CAM in RPE and photoreceptors participate in these interactions.

THE retinal pigment epithelium (RPE)[1] is a highly specialized derivative of the neuroectoderm with multiple roles in the maintenance of normal ocular function (50). Unlike other epithelia, the apical surface of RPE is not free: its intimate association with the neural retina is a key element of the function of this epithelium. This association starts early in development, when invagination of the optic cup brings the still undifferentiated neural retina in close proximity to the primordial RPE monolayer. In the differentiated adult eye, microvilli and lamellipodia in the apical surface of RPE interact directly with photoreceptor outer segments and indirectly, via the interphotoreceptor matrix (IPM) (50). It is generally thought that adhesion molecules, both calcium-dependent and independent, participate in the maintenance of this tight association (14); however the molecules involved have not been identified.

The calcium-independent neural cell adhesion molecule (N-CAM) plays fundamental roles in the development of the central nervous system (11). N-CAM participates in homophilic binding between neural cells and is involved in neurite fasciculation (40) and nerve cell migration (25). During eye development, N-CAM is expressed at high levels early in recently induced neuroepithelia and, after optic cup formation, in all retinal layers (30). N-CAM antibodies inhibit the normal morphogenesis of the neural retina, implying a key role of N-CAM in the recognition and organization of a multilayered retina (5, 20). N-CAM can also modulate adhesion via calcium-dependent cadherins (41); it is known that retinal-RPE adhesion is reduced in media lacking calcium and magnesium, a hallmark of cadherin function (45). However, of known cadherins, only low levels of N-cadherin are present in RPE (22) and along with RPE-100, a more abundant integrin present in RPE (8, 9), it is concentrated at the lateral cell membranes in vivo (7), which makes these molecules unlikely candidates to mediate adhesion between RPE and neural retina.

Recent work has shown that epithelial cells generate a polarized distribution of their surface molecules by vectorial targeting of vesicles from the Golgi apparatus (39, 43), transcytosis (29), or selective recycling (35). For example, in the case of N-CAM, which occurs in multiple isoforms generated by alternative splicing of its m-RNA (2, 11), the GPI-anchored isoform (120 kD) is targeted apically whereas the transmembrane isoforms (140 and 180 kD) are targeted basolaterally in transfected epithelial MDCK cells (36). An alternative mechanism to achieve and maintain the basolateral distribution of the Na,K-ATPase was recently described: nonpolarized delivery to the cell surface followed by binding to a domain-specific ankyrin-fodrin membrane cytoskeleton (19). Stabilization of the ankyrin-fodrin cytoskeleton at the basolateral surface appears to be induced by homophilic interactions between E-cadherin liver cell adhesion molecule (L-CAM) expressed by neighboring cells of the epithelial monolayer (28).

Unlike most other epithelial cells, RPE cells in situ localize Na,K-ATPase, fodrin, and ankyrin predominantly to the apical surface (18, 44) and do not express E-cadherin. The reversed polarity of these three proteins might be a consequence of the expression of apical adhesion systems that

Please address all correspondence to E. Rodriguez-Boulan, Department of Cell Biology and Anatomy, Cornell University Medical College, 1300 York Avenue, NY 10021.

1. *Abbreviations used in this paper*: BSS, Balanced Salt Solution; D-MEM, Dulbecco's MEM; IPM, interphotoreceptor matrix; LSCM, Laser scanning confocal microscopy; NCAM, neural cell adhesion molecule; PSA, polysialic acid; ROS, rod outer segments; RPE, retinal pigment epithelium.

stabilize them on the apical surface of RPE (similar to their stabilization on basolateral membranes via E-cadherin in other epithelia). We show here that RPE cells in situ express the 140-kD isoform of N-CAM on the apical surface, and that the distribution of N-CAM reverts to basolateral when the RPE cells are cultured free from interactions with the neural retina. Since N-CAM is also shown to be present in photoreceptor outer segments, these results suggest that stabilization by homophilic contact with a different cell type expressing the same adhesion molecule may play an important role in the maintenance of epithelial polarity. These results also suggest that N-CAM may be an important molecule in the adhesion between neural retina and RPE.

Materials and Methods

Antibodies

Antibodies to E-cadherin were a generous gift from Dr. Rolf Kemler (Max Planck Institut für Immunbiology, Germany) and Dr. Barry Gumbiner (UCSF). N-CAM antibodies were kindly provided by Dr. Melitta Schachner (Department of Neurobiology, University of Heidelberg), Dr. Christo Goridis (Centre Nationale de la Recherche Scientifique de Marseille-Luminy, France), and Drs. Bruce Cunningham and Gerald Edelman (The Rockefeller University). All N-CAM antibodies were generated against the ectodomain common to the 120-, 140-, and 180-kD forms of N-CAM, and recognize all three forms equally.

Cell Isolation and Culture

The procedure used to generate primary RPE cultures is essentially as previously described (27). Eyes are enucleated from 7-d old Long Evans rats, rinsed 2–3× with a Balanced Salt Solution (BSS), incubated 40 min in an enzyme solution containing 10^5 U/ml collagenase, 50 U/ml testicular hyaluronidase, pH 7.0, followed by 50 min in 0.1% trypsin in BSS. The eyes are then opened by a circumferential incision below the ora serrata, the retina is lifted off, and the single layer of RPE cells is peeled from both the retina and the choroid. After rinsing and a gentle trypsinization (0.1%), the cells are plated in Dulbecco's MEM (D-MEM) containing 10% FCS. The following day the medium is changed to D-MEM containing 2% FCS. To produce explant cultures, RPE sheets were not trypsinized after removal from the eye but were directly plated on a coverslip.

MDCK cell lines transfected with either the 120-, 140-, or 180-kD isoforms of N-CAM (36) were maintained in D-MEM containing 10% FCS (GIBCO BRL, Gaithersburg, MD). After dissociation with trypsin-EDTA, the cells were plated at high density on the appropriate substrates and the medium was changed every 2–3 d. Experiments were performed ~1 wk after plating.

Isolation of Rod Outer Segments

Eyes were enucleated from 21-d Long Evans rats and the retinas harvested into 3 ml rod outer segments (ROS) isolation buffer (1.15 M sucrose, 65 mM NaCl, 5 mM Tris Base, 2 mM MgCl pH 7.4) (33). The tube was shaken vigorously for 1 min, and then centrifuged for 5 min (4,500 rpm) to pellet the large pieces. 2 vol of TBS was added to the supernatant, and then centrifuged 10 min (4,500 rpm) to pellet the ROS. The pellet was resuspended in 1.10 M sucrose and layered on a discontinuous sucrose gradient (bottom to top: 1.15, 1.13, 1.11; 4 ml each), and then centrifuged at 4°C for 30 min at 27 K. The ROS were collected and processed for immunoprecipitation.

Semithin Frozen Sections and Transmission Electron Microscopy

0.5 micron frozen sections were processed for immunofluorescence as described (47). Samples were photographed with a Leitz epifluorescence microscope. Cells grown on filters for 2 wk were fixed in 2% glutaraldehyde and processed for transmission EM (37). Sections were stained with uranyl acetate and lead citrate, and examined in a JEOL 100 CX electron microscope.

Immunoprecipitation

Confluent monolayers on filters were rinsed four times in DMEM without methionine/cysteine, and then labeled with the same medium containing 1 mCi Trans ^{35}S-label (ICN Biomedicals, Inc., Costa Mesa, CA) (1 Ci = 37GBq) per ml for 5 h. The filters were rinsed five times in PBS, the cells were solubilized, and N-CAM was immunoprecipitated (23). Metabolically labeled samples were subjected to SDS-PAGE (21) under reducing conditions. After fixation (40% methanol/10% acetic acid for 30–60 min), gels were rehydrated in distilled water, and then impregnated with sodium salicylate (1 M solution for 30 min). Dried gels were autoradiographed on Kodak XAR-5 film at −70°C for varying lengths of time.

Biotinylation of Surface Proteins

Selective biotin labeling of the apical and basolateral surface was performed essentially as previously described (42). Sulfo-NHS-biotin was stored at −20°C in DMSO and diluted to a final concentration of 0.5 mg/ml in ice cold PBS containing 1 mM Ca and 0.1 mM Mg (PBS-C/M) and used immediately. To visualize the polarized distribution of N-CAM, filters were excised from the chamber with a scalpel and extracted, immunoprecipitated with N-CAM antibody, electrophoresed (21), and transferred to nitrocellulose according to the method described by Towbin (48) in a Transblot apparatus (Bio Rad Labs., Hercules, CA) at constant voltage (60 V) for 14–16 h. Nitrocellulose sheets were incubated with ^{125}I-streptavidin, labeled with the chloramine T procedure (42) to a specific activity of 2–5 μCi/μg. Blots were dried and exposed for varying lengths of time at −70°C on Kodak XAR-5 film.

Confocal Microscopy

Laser scanning confocal microscopy (LSCM) was performed with a Phoibos 1000 U attached to a Nikon microscope (Sarastro, Molecular Dynamics, Sunnyvale, CA). The cell monolayer was sectioned in either a horizontal (x-y) or vertical (x-z) plane. Images generated by a Personal Iris graphics workstation (Silicon Graphics, Mountain View, CA) and Sarastro software were photographed from the screen using a Minolta ×500 camera and a 200-mm objective.

Results

RPE Expresses N-CAM but Not E-cadherin

We initially set out to determine whether E-cadherin is expressed by RPE, perhaps with an opposite orientation to that observed in kidney cells. Fig. 1 *A* shows the characteristic U-shaped staining pattern of E-cadherin along the basolateral membranes of 7-d old rat kidney tubules. Rat RPE cells, either freshly isolated from the eye as sheets or after primary culture, exhibited no specific E-cadherin immunofluorescence staining on either the apical or the basolateral surfaces after staining with the same polyclonal E-cadherin antibody (Fig. 1 *B*). A different E-cadherin antibody (see Materials and Methods) gave the same negative result.

Since RPE cells are derived from the optic cup region of the neural tube, we examined these cells for the expression of the neuronal cell adhesion molecule N-CAM. As shown in Fig. 1 *C*, N-CAM clearly stains the recently isolated RPE monolayers. The staining is confined to the apical surface and colocalizes with both Na-K,ATPase and aminopeptidase N (not shown). Two other N-CAM antibodies, polyclonal and monoclonal, were examined and they also stained the apical membrane.

N-CAM in Cultured RPE

When isolated RPE monolayers are placed under primary culture, their apical membrane microvilli project freely into

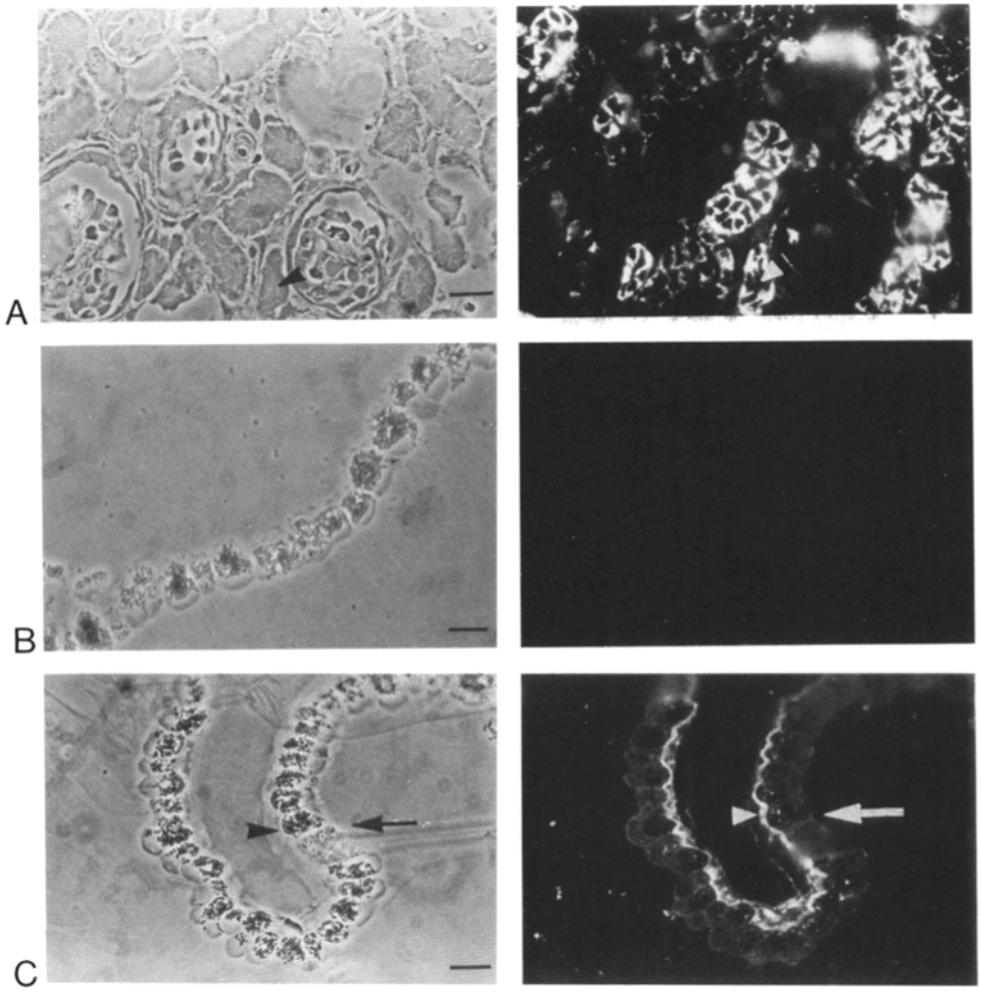


Figure 1. RPE does not express E-cadherin. (*A*) Immunofluorescence localization of E-cadherin in a 0.5 micron frozen section of kidney isolated from a 7-d old rat. Notice the staining along the basal and lateral membranes in the kidney tubules. In the more lightly stained samples, the lack of staining of the apical surface is apparent (*arrowhead*). (*B*) Immunofluorescence localization of E-cadherin in a 0.5-μm frozen section of a freshly isolated sheet of RPE cells. There is no E-cadherin staining on either the apical or the basolateral surfaces. (*C*) Immunofluorescence localization of N-CAM in freshly isolated monolayers of RPE. Note the prominent apical staining along the monolayer (*arrowhead*) while the basal portion of the cell is unstained (*arrow*). The apical surface can be distinguished by the presence of pigment granules in the phase micrograph. Bar, 10 μm.

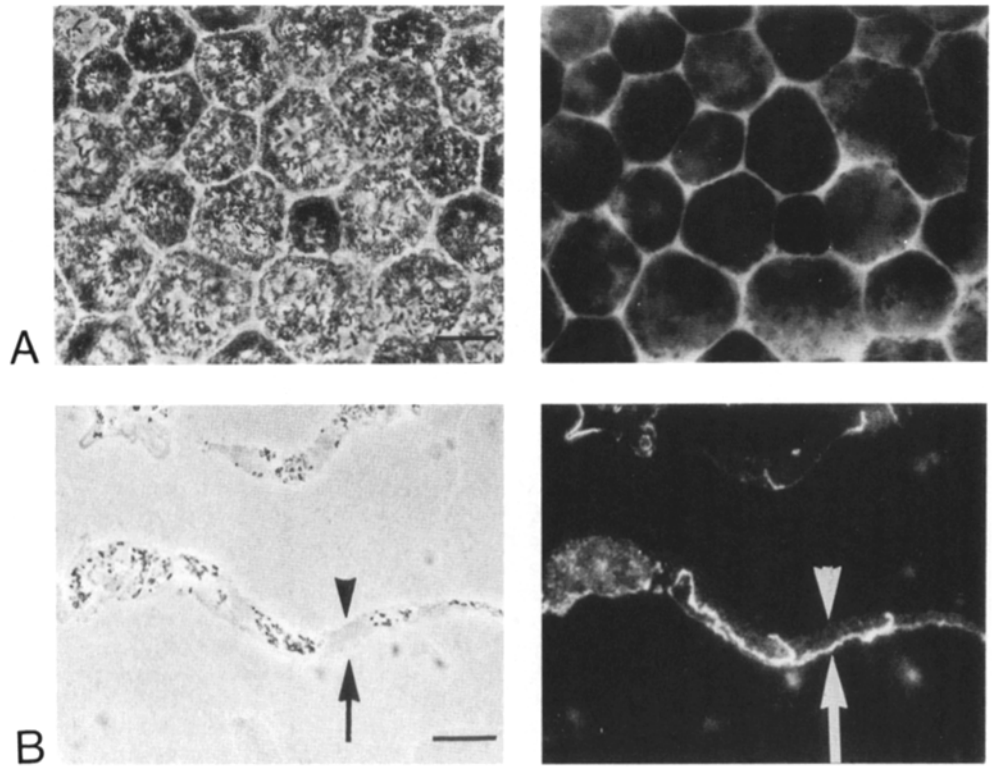
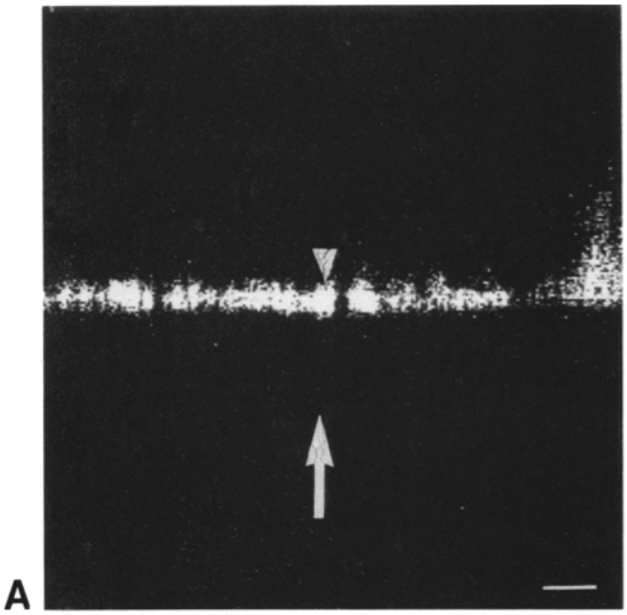
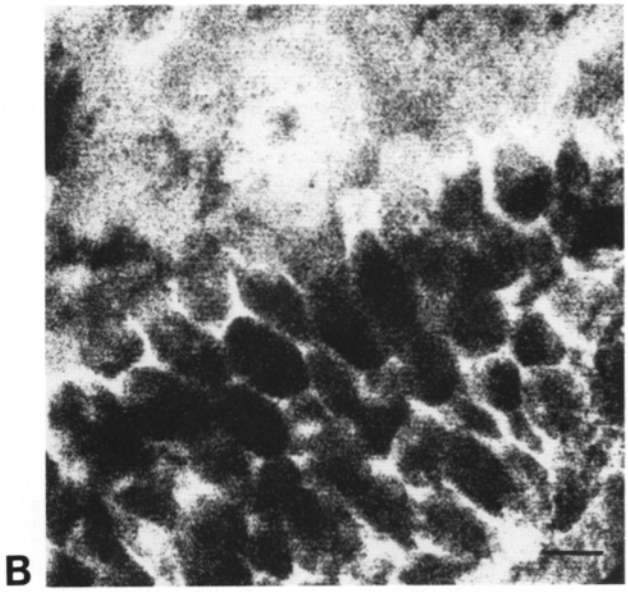
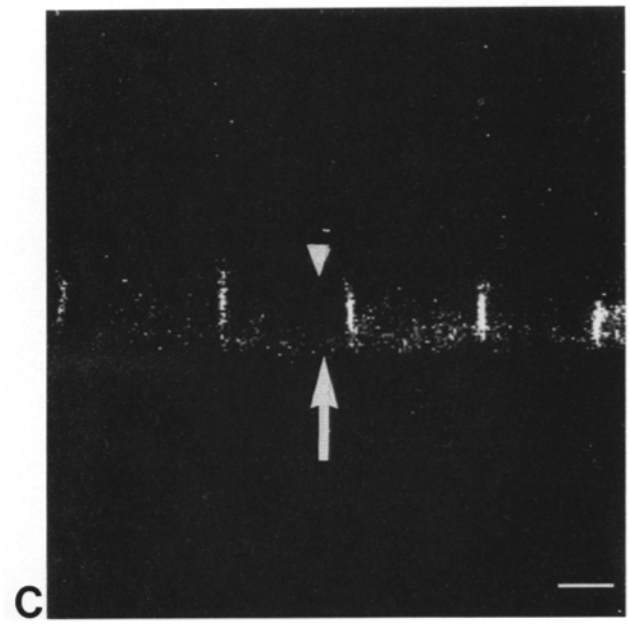


Figure 2. N-CAM localization in cultured RPE. (*A*) Immunofluorescence localization of N-CAM in cells growing out of an RPE explant. RPE monolayers were isolated and plated directly without trypsinization to break up cell aggregates. Note ringlike fluorescence of N-CAM indicating basolateral staining on these cells which have grown out of the explant. (*B*) Immunofluorescence localization of N-CAM in frozen cross sections of cultured RPE monolayers. Staining is along the basal and lateral surfaces which can be distinguished under phase optics. The apical surface in these cultured monolayers is free of N-CAM staining. These RPE cells have undergone cell division after plating and no longer interact with the photoreceptors on their apical surface. Bar, 10 μm.







the media and are no longer associated with an adjacent ocular layer. Strikingly, N-CAM is no longer associated with the apical microvilli but becomes redistributed to the basolateral surface. An example of this reversal of polarity of N-CAM in cultured cells is shown in Fig. 2. A monolayer which had grown out from a primary explant was permeabilized with saponin to ensure access of antibody to the basolateral surface and stained with an antibody to N-CAM (Fig. 2 *A*). In this experiment, the explant cells were not trypsinized to a single cell suspension after isolation; rather, they were allowed to divide and to grow out of the boundaries of the primary explant. A ringlike fluorescence is seen along the circumference of the cells, typical of a basolateral localization. A similar result was obtained when the isolated RPE sheets were trypsinized to single cells, plated, and grown on collagen gels for 7–10 d (Fig. 2 *B*). In a cross section of these monolayers, N-CAM staining is also restricted to the basolateral surface.

Confocal Microscopy

LSCM was used to further analyze the reversal of polarity of N-CAM in cultured RPE cells. Fig. 3 shows en face (*B*) and cross sectional (*A* and *C*) views of the border between RPE primary explants and proliferating RPE cells growing away from the explant. When these monolayers are permeabilized and stained with N-CAM antibodies two very different staining patterns are seen. In the en face LSCM view (Fig. 3 *B*), the primary explant RPE cells exhibit a punctate staining pattern characteristic of apical localization while the cells growing out from the explant have a ringlike fluorescence staining pattern typical of basolateral localization. This is confirmed by the LSCM cross sectional views, where the primary explant RPE cells show a single apical fluorescent line (Fig. 3 *A*) while the proliferating RPE cells display strong lateral fluorescence (Fig. 3 *C*). These experiments indicate that the reversed polarity of N-CAM is a consequence of in vitro cell division and remodeling of membrane proteins under culture conditions.

RPE Express the 140-kD Form of N-CAM

N-CAM is expressed as three major membrane bound iso-

Figure 3. LSCM localization of N-CAM in an RPE explant and in dividing cells growing away from the explant. An RPE explant which was not trypsinized to single cells was cultured for several days, fixed, and processed for immunofluorescence with N-CAM antibodies. The samples were visualized by LSCM. (*A*) Vertical section (x,z) taken from the explant cell region (upper region of micrograph in *B*). Note the single line of fluorescence at the top of the cells (*arrowhead*), indicative of apical localization. (*B*) En face (x,y) view of the RPE explant (*B*, upper region of micrograph) and the dividing cells growing from the explant (*B*, lower region of micrograph), showing different N-CAM distributions. Note the punctate apical staining pattern in the explant cells and the ringlike fluorescence typical of basolateral staining in the cells that grew away from the explant. (*C*) Vertical section taken from the area of cells growing away from the explant (lower region of micrograph in *B*). A characteristic basolateral U-shaped fluorescence can be seen with more intense staining at adjoining lateral cell membranes. Bar, 10 μm.

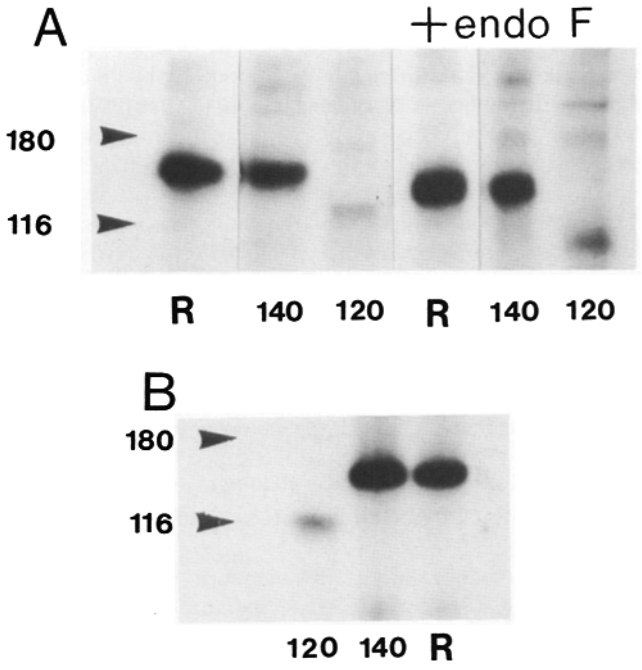


Figure 4. N-CAM 140 is expressed in freshly isolated and in cultured RPE cells. (*A*) Immunoprecipitation of N-CAM from freshly isolated, metabolically labeled RPE monolayers. RPE cells were isolated from 3-wk-old rats and were metabolically labeled immediately after isolation. Control lanes are MDCK cell lines which have been transfected with either the 120- or 140-kD isoforms of N-CAM, labeled, and immunoprecipitated under the same conditions as the RPE. In RPE monolayers, freshly isolated from 3-wk-old rats (*R*), N-CAM 140 kD is the predominant isoform expressed. In younger animals N-CAM is known to be heavily sialylated; therefore all samples were also treated with Endo F. The electrophoretic mobility of N-CAM expressed by RPE is identical to transfected N-CAM 140 kD. (*B*) Immunoprecipitation of metabolically labeled cultured RPE monolayers. In this experiment, the RPE were cultured for several days after isolation before labeling and immunoprecipitation. Control lanes are the same as in *A*. N-CAM 140 kD is also the major isoform expressed in cultured RPE monolayers (*R*). Labeled extracts were also treated with Endo F and showed identical results (not shown).

forms, 180, 140, or 120 kD (2, 11). Previous work from our laboratory demonstrated that the 120-kD GPI-anchored isoform is targeted to the apical surface whereas the transmembrane forms (140 and 180 kD) are targeted to the basolateral membrane (36). To determine whether the change of N-CAM localization in cultured RPE cells was due to the expression of a different N-CAM isoform, both freshly isolated RPE sheets and cultured RPE monolayers were metabolically labeled, and N-CAM immunoprecipitated with a polyclonal antibody recognizing all major isoforms of this molecule. Fig. 4 *A* shows the labeled immunoprecipitate from apically located N-CAM (in vivo cells). The control lanes (designated *120* and *140*) are immunoprecipitates of MDCK cell lines transfected with either the 140-kD or 120-kD form of N-CAM. Since N-CAM can be heavily sialylated, especially in younger animals, all samples were also treated with endoglycosidase F to reveal the unglycosylated core protein. N-CAM isolated from RPE cells in vivo clearly migrates with the 140-kD form in both glycosylated and deglycosylated lanes. An identical immunoprecipitation experiment using the trypsinized cells grown in vitro for 7 d produced the same result (Fig. 4 *B*).

Selective Surface Labeling of N-CAM Expressed In Vivo

In a parallel experiment, newly isolated RPE monolayers were cell surface biotinylated, immunoprecipitated, blotted, and probed with ^{125}I streptavidin. As seen in Fig. 5 *A*, only the 140-kD band is detected. This result rules out the possibility that a change in N-CAM isoform occurred during the overnight metabolic labeling period in the experiment discussed above. From these experiments it can be concluded that N-CAM 140 kD is the major isoform expressed in rat RPE monolayers both in vivo and in vitro. The relocalization of N-CAM is not due to expression of different N-CAM isoforms in newly isolated sheets and cultured cells, but to altered growth conditions of RPE cells in vivo versus in vitro.

To demonstrate that the 140-kD N-CAM expressed on the apical surface of RPE sheets was not derived from adjacent photoreceptors remaining interdigitated with the RPE microvilli, freshly isolated RPE sheets were processed for transmission EM. Outer segments of photoreceptors have a characteristic lamellar structure and can be easily distinguished from the microvilli of the RPE. Approximately 100 cells were examined and, as can be clearly seen in Fig. 6, there are no fragments of photoreceptor outer segments seen along the apical surface of the isolated RPE sheet.

N-CAM in Photoreceptor Outer Segments

The most dramatic difference between the environment of in situ RPE and the cultured monolayers is the presence of the ROS and IPM in close contact with the apical surface of the RPE in vivo. Homophilic interactions between N-CAM of the photoreceptors and RPE might explain N-CAM localization along the apical surface in vivo. In the absence of photoreceptors, N-CAM may be redistributed to lateral surfaces in the in vitro monolayer after cell division. Immunofluorescence staining of the outer portion of the neural retina with the adjacent RPE monolayer shows abundant N-CAM staining in the outer segments of the rods and cones of the retina (Fig. 7 *A*, *arrowhead*), demarcated by the outer limiting membrane (Fig. 7 *A*, *asterisk*). The RPE monolayer is visible in the phase micrograph. Basal and lateral surfaces are free of staining while extensive staining is seen in the region where the apical microvilli intertwine with the outer segments. This fluorescence staining extending from the RPE apical surface to the outer limiting membrane can not be due solely to the RPE's apical microvilli since these do not reach the outer limiting membrane, especially in younger animals (50), and therefore is most likely to be due to N-CAM in the outer segment of the rods and cones of the neural retina. To further demonstrate this point, a different preparation is shown in Fig. 7 *B*, in which the RPE monolayer has been peeled away from the retina, leaving the photoreceptors intact. N-CAM staining appears to localize specifically to the ROS (*arrowheads*), even in the absence of the RPE.

Photoreceptor Outer Segments Contain 120 kD N-CAM

Since the ROS are tightly interdigitated with the apical microvilli of the RPE, it was possible that the N-CAM stain-

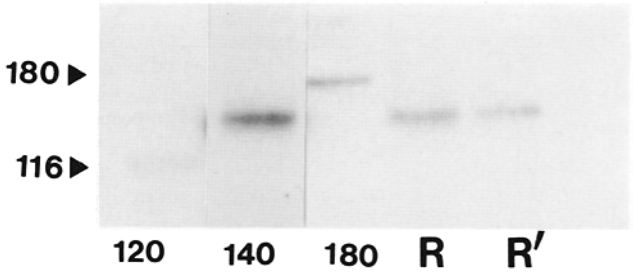


Figure 5. N-CAM isoform expressed on the surface of freshly isolated RPE monolayers. The surfaces of newly isolated monolayers were biotinylated, precipitated, blotted, and probed with labeled streptavidin. Control lanes on the left are transfected MDCK cells expressing the 120-, 140-, or 180-kD isoforms of N-CAM. The second RPE lane (*R'*) was mock metabolically labeled overnight followed by a surface immunoprecipitation the following day. An equivalent amount of N-CAM 140 kD is still present on the surface of the RPE monolayers after 24 h of mock labeling. This eliminates the possibility that the N-CAM isoform synthesized after metabolic labeling is different than that present on the apical surface of freshly isolated RPE monolayers.

ing shown in the photoreceptors in Fig. 7 was due to fragments of RPE which have been internalized. To confirm the presence of N-CAM on the surface of the photoreceptors, ROS were isolated from the retina, biotinylated, immunoprecipitated, and blotted with ^{125}I-streptavidin. A single band migrating at 120 kD was observed (Fig. 8). Since experiments on both explants and primary cultures demonstrate that RPE express the 140-kD form of N-CAM, the 120-kD form must originate from either the photoreceptors or some other cell type in the neural retina. Taken with the immunolocalization data shown in Fig. 7, these results suggest that N-CAM–N-CAM homophilic interactions between the RPE and ROS are mediating the change in localization between explant and primary cultures of RPE. However, since the photoreceptors are not easily propagated in culture, we cannot demonstrate unequivocally that N-CAM is actually synthesized by the photoreceptors.

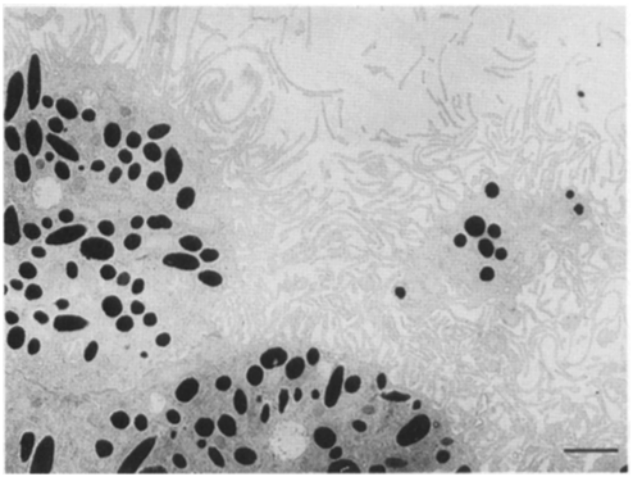


Figure 6. Recently isolated RPE is not contaminated with photoreceptor outer segments. Electron micrograph of a freshly isolated RPE monolayer. Microvilli of these isolated sheets of cells are free of contaminating photoreceptor membranes. Bar, 1 μm.

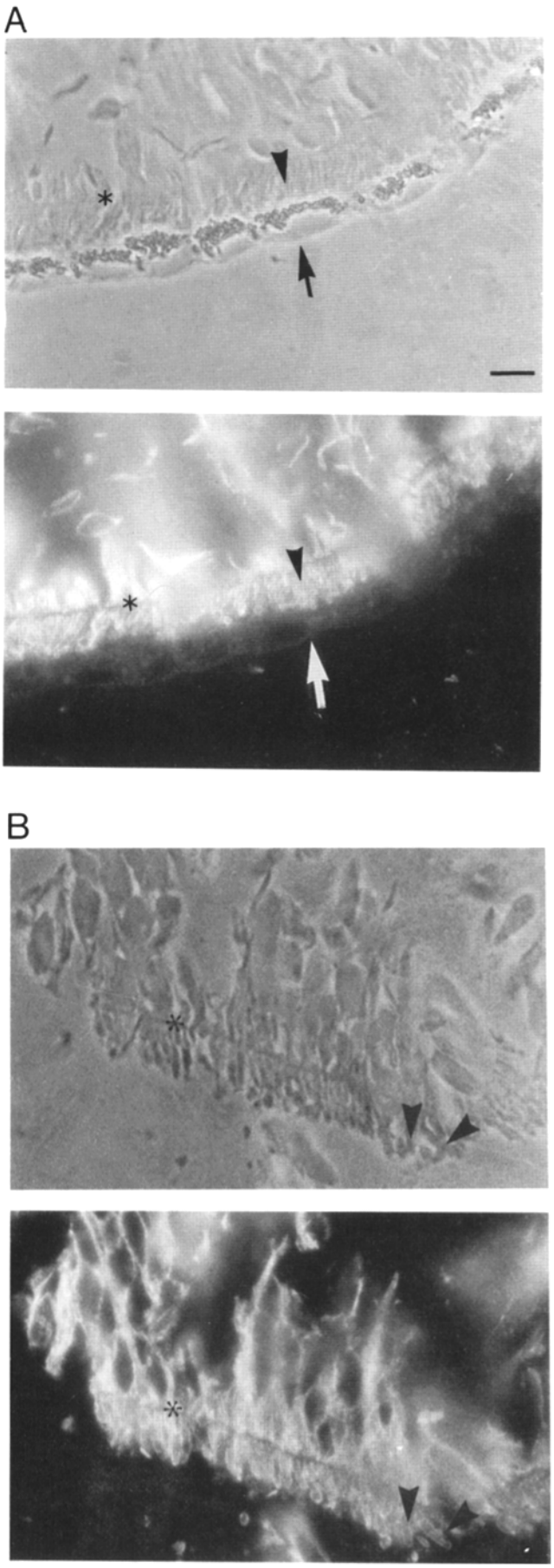
Discussion

A key event in the differentiation of epithelia is the expression of a characteristic set of adhesion molecules (39). Many developing epithelia express a complicated choreography of cell adhesion molecules; although suspected to play important morphogenetic roles, the functional role of this variation is essentially unknown (15). In kidney tubules, for example, the uninduced nephrogenic mesenchyme destined to become epithelia expresses N-CAM; upon epithelial induction, N-CAM and E-cadherin liver cell adhesion molecule (L-CAM) are coexpressed for a short time but, ultimately, only E-cadherin remains (46). Indeed, most adult epithelia express only E-cadherin (46). Recent work with MDCK cells has suggested that E-cadherin participates in the establishment of the polarized epithelial phenotype by stabilizing a complex of ankyrin, fodrin, Na,K-ATPase, and presumably other unidentified basolateral proteins in the lateral membrane (31).

Antibodies that brightly stain E-cadherin in kidney failed to detect it in RPE. Surprisingly, we found that a different cell adhesion molecule, N-CAM, was expressed on the apical surface of RPE in situ. The lack of E-cadherin expression and the fact that the apical surface does not face a lumen but closely interacts with the photoreceptors and the IPM are major differences between RPE and other transporting epithelia. It may be suggested that both factors play a role in the reversed (apical) distribution of Na,K-ATPase and the associated membrane cytoskeleton. However, our previous immunocytochemical observations indicated that unlike N-CAM, Na,K-ATPase is not rapidly redistributed from the apical to the basolateral surface when the cells are placed in culture (18). More recent biochemical results with primary human RPE and with a rat RPE cell line indicate that there is a considerable loss of polarity of Na,K-ATPase upon culturing of RPE, apparently due to unpolarized delivery to the cell surface and/or lack of selective retention at the apical cell surface (29*a*). The persistent apical distribution of Na,K-ATPase might be due to a slow turnover rate since the enzyme is in an insoluble complex with both ankyrin and fodrin. Further studies will be necessary to evaluate whether the apical distribution of N-CAM and Na,K-ATPase are related or whether other cell adhesion molecules are involved.

We show in this report that RPE expresses the 140-kD isoform of N-CAM on the apical surface. The apical localization of this N-CAM isoform is unusual in several regards. First, cell adhesion molecules are normally not apical; they are usually expressed on the basal membrane of epithelial cells to interact with either the substrate or on the lateral membrane to interact with other cells of the epithelium. Second, in transfected MDCK cells an N-CAM isoform is expressed on the apical surface. This is, however, the 120-kD isoform; the 140-kD N-CAM is targeted directly from the Golgi apparatus to the basolateral membrane of MDCK cells, where it is concentrated (36). Third, we show that N-CAM undergoes a striking redistribution to the basolateral membrane when the cells are placed under culture conditions. This change in localization does not entail the expression of a different isoform since both in vivo and in vitro RPE express the same N-CAM isoform (140 kD).

All of the above results support the hypothesis that the api-



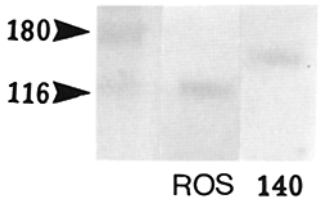


Figure 8. Photoreceptor outer segments express the 120-kD (GPI-anchored) N-CAM isoform. The surface of newly isolated photoreceptor outer segments were biotinylated, immunoprecipitated with anti-N-CAM, blotted, and probed with ^{125}I-streptavidin. The control lane is transfected MDCK cells expressing the 140-kD isoform of N-CAM. The photoreceptor outer segments of the neural retina express only the 120-kD isoform of N-CAM.

cal localization of N-CAM may result from direct or indirect interaction of RPE with the neural retina. We show that the 120-kD form of N-CAM, distinct from the 140-kD form expressed in RPE, is also found in the neural retina, most probably in the ROS which are tightly associated with the apical microvilli of the RPE. This result indicates that homophilic N-CAM binding may occur between the RPE and the neural retina; it is possible that other adhesion molecules are also involved in this interaction. The functional demonstration of N-CAM in such an interaction will require the development of RPE/neural retina coculturing systems which we are currently carrying out in our laboratory. A role of N-CAM in mediating interactions at sites of cell–cell contact, both between the same cell type and in interactions involving heterologous cell types has previously been demonstrated (4, 10, 13, 34).

The expression of the 120-kD GPI-anchored isoform of N-CAM in the photoreceptor outer segments may allow for the modulation of its surface expression by a phospholipase. To our knowledge, this is the first description of a CAM that fulfills the requirements for a molecule involved in the adhesion between RPE and neural retina. Both calcium-dependent and calcium-independent mechanisms appear to participate in RPE-neural retina adhesion. The presence of a beta-1 integrin subunit on the apical and lateral surfaces of RPE has been reported (1) but a candidate molecule with which it might interact in the neural retina or the IPM has not been identified.

Our results partially contradict previous work on the localization of N-CAM in RPE. Previous immunocytochemical results detected N-CAM throughout the entire chick retina from embryonic to adult stages (12) although it was not clear in the whole eye micrographs whether the RPE itself expressed N-CAM. More recent results using immunoblot on frog and bovine adult RPE suggested that N-CAM may be absent from both the RPE monolayer and the retinal outer segments (16). Another study detected N-CAM in rats only

Figure 7. Immunolocalization of N-CAM in photoreceptor outer segments. Semithin (0.5 micron) frozen sections of the neural retina along with the attached RPE monolayer (*A*) after detachment of RPE (*B*) were stained with N-CAM. (*A*) The basal surface of the RPE monolayer is negative (*arrow*) while the apical surface and outer segments are positive (*arrowhead*). The outer limiting membrane of the retina (*asterisk*) is visible and therefore the staining can not be due solely to the apical microvilli of the RPE since they do not extend to the outer limiting membrane, particularly in younger animals. (*B*) After detachment of RPE, the photoreceptor outer segments remain brightly stained. Arrowheads point at well defined outer segments. Bar, 10 μm.

before embryonic stage E17, although cultures from adult RPE were described to "reexpress" N-CAM on the lateral membranes (30). Using three different approaches, i.e., immunofluorescence, immunoprecipitation, and metabolic and surface labeling, we have demonstrated N-CAM in both RPE and photoreceptor outer segments in 7-21-d old rats. The difference with previous negative results may be attributed to the use of different antibodies or culture conditions. We cannot exclude that N-CAM be only expressed in younger animals; however, once N-CAM is expressed in tissues of neuroectoderm origin (such as neural retina) its expression tends to persist, although at reduced levels (15).

The apical localization of 140 kD N-CAM in RPE in vivo, opposite to that observed in cultured RPE and in transfected MDCK cells, may be due to reversed (apical) targeting, to apical stabilization, or to a combination of both mechanisms. Tissue-specific differences in protein distribution and targeting have been observed for a variety of proteins in different epithelial cells (39). In MDCK cells, direct targeting seems to be the predominant route to both the apical and basolateral surfaces (17, 24, 38). In the hepatocyte, a direct secretory route to the apical surface is likely to be absent; apical proteins are delivered first to the basolateral surface then rerouted apically via transcytotic vesicles (3). In yet another variation, both vectorial and transcytotic pathways are used by Caco-2 cells (24, 26). There is considerable evidence that these targeting phenotypes are not stable but, rather, are considerably influenced by extracellular cues. For example, MDCK cells (19) and the thyroid cell line FRT (51) establish their particular targeting phenotype gradually after confluency. Furthermore, MDCK cells (49) and thyroid cells (6, 32) reverse their surface polarity in response to the addition of collagen to the apical surface; extracellular cues may similarly result in apical targeting or stabilization of N-CAM in RPE.

Recently, domain-specific stabilization has been suggested as an alternative mechanism to account for the polarized distribution of membrane proteins in epithelial cells (31, 38). In MDCK cells, Na,K-ATPase appears to be randomly delivered to both surfaces, but its half life is considerably shorter on the apical domain, resulting in preferential accumulation on the basolateral surface (19). In the developing eye, when the optic cup invaginates to form the neural retina and the RPE epithelium, the cells of the future RPE and rods and cones are in close proximity to each other. Since N-CAM is expressed by all cells in the developing retina, stabilization between N-CAM expressed on the apical surface of RPE and the rods and cones of the retina may occur via a homophilic binding mechanism similar to the stabilization of E-cadherin on the basolateral membrane of MDCK cells. However, the results shown here are novel in that the homophilic interactions are between two different cell types, implying an important role for such interactions in the development of the cytoarchitecture of this tissue. Adhesion via N-CAM provides interesting possibilities for regulation since it has been shown that it can be modified by its degree of sialylation. Early in development neural cells express N-CAM with a high polysialic acid (PSA) content; such N-CAM has highly reduced adhesive properties (41). The N-CAM isoforms that we detected in RPE and neural retina appear to be of the adult type (highly adhesive) since they do not display the low and diffuse electrophoretic mobility characteristic of high PSA content. Low PSA content would certainly enhance the ability of the outer segments of photoreceptors and apical microvilli of the RPE monolayer to interact via N-CAM's homophilic binding mechanism.

The complex relationship between the RPE monolayer and the neural retina is key for the maintenance of normal retinal function. A number of human retinal dystrophies appear to involve a defect in the attachment of neural retina to RPE (50). Identification of cell adhesion molecules in RPE and photoreceptors is an essential first step in the analysis of the mechanisms that participate in this interaction. Furthermore, RPE represents an interesting paradigm for the study of the mechanisms responsible for cell polarity. Recently, our laboratory has generated an RPE cell line which exhibits many of the features of the primary culture system used for these studies (29*a*). Manipulation of the growth conditions of this cell line should facilitate the identification of the specific extracellular cues that regulate the polarized phenotype of epithelial cells.

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