Immortalization of polarized rat retinal pigment epithelium

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SUMMARY

Rat retinal pigment epithelial (RPE) cells were immortalized by infection with a temperature-sensitive tsA SV40 virus and following cloning and selection for epithelial properties the polarized RPE-J cell line was obtained. At the permissive temperature of 33˚C, RPE-J cells behave as an immortalized cell line. When RPE-J cells are grown on nitrocellulose filters coated with a thin layer of Matrigel in the presence of 10⁻⁸ M retinoic acid for 6 days at 33˚C and then switched for 33-36 hours to the non-permissive temperature of 40˚C, they acquire a differentiated polarized RPE phenotype. Under these growth conditions, RPE-J cells exhibit circumferential staining for the tight-junction protein ZO-1 and acquire a transepithelial resistance of 350 ohms cm². Morphologically, RPE-J cells exhibit a characteristic RPE morphology with extensive apical microvilli as well as numerous dense bodies including premelanosomes and varied multilamellar structures. Ruthenium red labeling revealed the frequent basal localization of the tight junction. The cells were identified to be of rat RPE origin by their expression of the rat RPE marker RET-PE2 and their ability to phagocytose latex beads. While RPE-J cells are capable of sorting influenza and vesicular stomatitis virus to the apical and basal surfaces, respectively, the Na,K-ATPase is not polarized and the neural cell adhesion molecule, N-CAM, is localized exclusively to the lateral surface. In vivo the apical surface of RPE interacts with the adjacent neural retina and the Na,K-ATPase and N-CAM are both apical; the altered polarity of these two proteins in RPE-J cells may be a consequence of the absence of apical interaction with the neural retina in culture. Previous studies of RPE have been restricted to the use of primary cultures and the RPE-J cell line should prove an excellent model system for the study of the mechanisms determining the characteristic polarity and functions of the retinal pigment epithelium.

Key words: RPE, epithelial polarity, SV40

INTRODUCTION

During the development of the organism cellular differentiation generates highly specialized cell types which form complex intercellular interactions enabling organ function. This is particularly true for epithelial cells which, while maintaining characteristics of epithelia, acquire distinctive properties particular to the organ in which they are found (Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989; Hanzel et al., 1991; Mostov et al., 1992). One of the most highly complex and distinct epithelia is the retinal pigment epithelium (RPE). The RPE is a monolayer of polarized epithelial cells sandwiched between the neural retina and choroid layers of the eye. The apical microvilli are interdigitated between the rods and cones of the retina while the basal surface rests on a basement membrane (Bruch’s membrane) like other transporting epithelia. The numerous functions of the RPE monolayer include phagocytosis of outer rod segments (Young, 1967; Young and Bok, 1969), storage of vitamin A (Dowling, 1960), protection of rods and cones from UV damage (Burnside and Laties, 1979) and the selective transport of ions and metabolites from the choriocapillaris towards the retina (Steinberg and Miller, 1979). A unique feature of RPE is the reverse apical polarity of the Na,K-ATPase (Steinberg and Miller, 1979; Bok, 1982; Caldwell and McLaughlin, 1984; Gundersen et al., 1991) compared to its basolateral localization in most other transporting epithelia (KYTE, 1976; Eustis and Hootman, 1981; Rodriguez-Boulan and Nelson, 1989).

The study of the molecular basis of these specialized functions is restricted in vivo by limited access to the cells within the organism. In vitro study of RPE-specific properties is restricted due to the necessity of using primary cultures, which are difficult to acquire and do not consistently retain the properties of interest. The study of immortalized cells in tissue culture, while enabling molecular approaches and yielding information on cellular function, is inherently limited in the study of properties characte-
tic of differentiated cells, since cells which can proliferate in culture do not necessarily mimic those which exist in vivo (i.e. they are transformed).

A novel approach to the immortalization of differentiated cells is the use of temperature-sensitive oncogenes which can be inactivated at specific non-permissive temperatures (Chou, 1985, 1989). Temperature-sensitive A mutants of polyoma and SV40 viruses encode a large T antigen which is transforming at the permissive temperature of 33°C but inactivated at the non-permissive temperature of 39-40°C (Fried, 1965; Tegtmeyer, 1975). Primary cell lines established with the temperature-sensitive SV40 large T antigen grow continuously at the permissive temperature but exhibit growth arrest at the non-permissive temperature (Ras-soulzadegan et al., 1983; Jat and Sharp, 1989). Immortalization of differentiated cells with temperature-sensitive viruses, both SV40 and Rous sarcoma, has resulted in the generation of cell lines which can be maintained indefinitely in culture at the permissive temperature and which acquire differentiated properties upon switching to the non-permissive temperature (Holtzer et al., 1975; Pacifi ci et al., 1977; Boettiger et al., 1977; Chou, 1978, 1983; Frederiksen et al., 1988; Zaret et al., 1988; Li et al., 1989; Plouzek and Chou, 1991; Volberg et al., 1991). We have transformed primary rat RPE cultures with a temperature-sensitive SV40 T antigen (Chou, 1985) and demonstrate here an RPE-derived cell line exhibiting a highly differentiated polarized phenotype in culture.

MATERIALS AND METHODS

Cells, antibodies and materials

Primary cultures of rat RPE cells were obtained from 7-day-old Long-Evans rats as previously described (Mayerson et al., 1985; Gundersen et al., 1991) and cultured in a 35 mm dish in Dulbecco’s modified essential medium (DMEM) supplemented with 10% heat-inactivated (30 min 56°C) fetal calf serum (FCS, CEL-Lect Gold, ICN-Flow, Costa Mesa, CA), glutamine and non-essential amino acids (Gibco, Gaithersburg, MD) for 2 days and then maintained in the same medium containing only 4% FCS (CMEM). MDCK II cells were grown in DMEM supplemented with 5% fetal bovine serum, glutamine, non-essential amino acids (Gibco). Primary cultures of rat RPE and MDCK cells were maintained in an air-5% CO₂ incubator at constant humidity at 37°C. Rat anti-ZO-1 monoclonal antibody (mAb) was obtained from Dr. Daniel Goodenough (Harvard Medical School), the rat RPE-specific mouse mAb RET-PE2 from Dr. Jim Neill (Yale University), mouse anti-Na,K-ATPase mAb from Dr. Michael Caplan (Yale University), and polyclonal anti-N-CAM from Drs. Bruce Cunningham and Gerald Edelman (Rockefeller University). Influenza and vesicular stomatitis (VSV) virus stocks were maintained as previously described (Rodriguez-Boulan, 1983). Affinity-purified polyclonal antibody was used to detect VSV G protein and a monoclonal antibody (H15-C5-1r1) to detect influenza hemagglutinin (HA) (Dr. W. Gerhard, Wistar Institute, Philadelphia, PA). FITC, rhodamine- and Texas Red-conjugated secondary antibodies were purchased from Jackson Laboratories (West Grove, PA). All chemicals were from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

SV40 infection of primary rat RPE cultures

Rat RPE cells were infected with the tsA temperature-sensitive SV40 virus essentially as previously described (Chou, 1985). Infection with SV40 tsA and all subsequent manipulations of the infected cells were performed at 33°C in an air-5% CO₂ incubator at constant humidity. Two-day cultures of primary rat RPE were rinsed with CMEM and incubated in CMEM for 4 hours at 33°C with approximately 10⁶ p.f.u. of SV40 tsA virus (kindly provided by Dr. Janice Chou, NIH), after which the virus fluid was removed and replaced with fresh medium. Following the viral infection, the cells were allowed to proliferate and then harvested with 0.1% trypsin/2 mM EDTA and replated in a larger dish. This was repeated twice and the surviving cells (passage 3), assumed to be transformed as the primaries cannot be passaged, were cloned by limiting dilution. Clones were selected for appearance of an epithelioid morphology and tested for expression of circumferential ZO-1 staining (Stevenson et al., 1986). The clone exhibiting the most extensive ZO-1 staining was recloned twice and a number of epithelioid clones exhibiting complete ZO-1 staining were obtained. One of these, RPE-J, was used for all further studies.

RPE-J cell culture conditions

RPE-J cells were maintained at the permissive temperature of 33°C in DMEM supplemented with 4% FCS (Flow CEL-Lect Gold), glutamine and non-essential amino acids. At 33°C the cells behaved as though transformed and could be passaged indefinitely. Continued growth and selection at 33°C apparently resulted in the selection of an inherently temperature-sensitive mutant cell line such that the cells do not grow at 37°C and must be cultured at 33°C. To maintain reproducibility, cells past passage 20 were not used for experiments and early passage cells thawed to renew the culture.

To obtain a differentiated RPE phenotype, RPE-J cells were grown on Millipore HA nitrocellulose filters (Millipore, Bedford, MA) coated with a thin layer of Matrigel (Collaborative Research, Bedford, MA) diluted 1:4 in DMEM and applied rapidly and allowed to dry three times in succession in the cold (Rizzolo, 1990). All-trans-retinoic acid (Sigma, St. Louis, MI) was dissolved in DMSO at a concentration of 10⁻¹⁰ M and stored in aliquots at −70°C. Medium containing 10⁻⁶ M retinoic acid was prepared by serial dilution and was replaced every two days. Cells were plated at a density of 300,000-350,000 cells/cm² and then grown for 6-7 days at 33°C in the presence of 10⁻⁶ M retinoic acid and then switched to the non-permissive temperature of 40°C for 33 hours in the presence of retinoic acid in the dark. For some immunofluorescence experiments, cells were plated on coverslips under the same conditions. Transepithelial resistance of RPE-J cells grown on filters was measured using a MilliCell-ERS ohmmeter (Millipore). The resistance measured from a control Matrigel-coated filter without cells was subtracted from all values measured.

Influenza and VSV viral infections

RPE-J cells and primary RPE cells were infected with influenza virus and vesicular stomatitis virus (VSV) as previously described for MDCK cells (Rodriguez-Boulan and Sabatini, 1978; Rodriguez-Boulan, 1983). Briefly, after the switch to 40°C, RPE-J cells grown on nitrocellulose filters as described above were washed three times with DMEM containing 0.2% BSA and 10 mM HEPES, pH 7.3, and then inoculated in the same solution for 1 hour at 37°C with wild-type influenza (WSN strain) and vesicular stomatitis (Indiana strain) viruses at a multiplicity of infection (m.o.i.) of 10-14 and 5-8 plaque forming units, respectively. After one hour the filters were washed three times with CMEM and then incubated for 14 (influenza) or 7 (VSV) hours at 33°C. Influenza HA and VSV G protein were detected by immunofluorescence labeling and confocal microscopy as described elsewhere.
Primary rat RPE monolayers grown on round 12 mm coverslips were inoculated with temperature-sensitive strains of either VSV (ts045) or influenza (ts61) at a multiplicity of infection of 10. The inoculum was removed and replaced after 1 hour of incubation at 37°C. Cells were first incubated for several hours at the non-permissive temperature, 39.5°C, to allow accumulation of the viral glycoproteins in the endoplasmic reticulum and then transferred to the permissive temperature, 32°C, to allow synchronous transport to the surface. After 2 hours at the permissive temperature the coverslips were fixed in 2% paraformaldehyde and processed for indirect immunofluorescence, as described elsewhere.

Electron microscopy

RPE-J cells grown on nitrocellulose filters were processed for electron microscopy as follows. RPE-J cultures were washed with PBS/CM, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, and then washed with the cacodylate buffer. The cells were post-fixed with 1% osmium tetroxide in the same buffer. To localize tight junctions, 0.2% ruthenium red was added to only the apical buffer during the post fixation. Following thorough washing with buffer, the samples were dehydrated through a graded ethanol series and then embedded in LX-112 resin (Ladd Research Industries, Burlington, VT) and polymerized at 70°C. Ultrathin (60-70 nm) sections were cut using a diamond knife (Diatome, USA). The samples which were not labeled with ruthenium red were stained en bloc with uranyl acetate (3% in 50% ethanol for 60 minutes) during the ethanol dehydration series and with lead citrate after sectioning. Sections were examined at 80 kV in a JEOL-100 CXII electron microscope.

Phagocytosis

MDCK cells grown at 37°C on glass coverslips or RPE-J cells grown at 33°C on Matrigel-coated coverslips or nitrocellulose filters were incubated with FITC-conjugated 1 μm latex beads (Polysciences, Warrington, PA) at a concentration of 25 μg/ml for 4 hours. Incubations at 4°C were performed using medium without bicarbonate and buffered with 20 mM HEPEs, pH 7.3. The cells were then washed 2 × 2 min and 4 × 5 min with PBS/CM. Cells on coverslips were fixed with 3% paraformaldehyde, mounted on slides with gelvatol and viewed by epifluorescence microscopy. Cells on filters were processed for electron microscopy.

Immunofluorescence

RPE-J cells plated on Matrigel-coated coverslips were fixed by the rapid addition of precooled (−80°C) methanol and rinsed in PBS/CM. Following viral infection, primary cultures of RPE cells on coverslips were incubated with 2% paraformaldehyde, rinsed with PBS/CM and permeabilized with 0.1% saponin in PBS/CM. The coverslips were then incubated with the indicated primary antibody followed by the corresponding rhodamine-conjugated secondary antibody. The fluorescently labeled cells were visualized in a Leica Orthomat microscope and photographed with Kodak TMAX film.

RPE-J cells grown on Matrigel-coated nitrocellulose filters were immunofluorescently labeled as follows. The filters were rinsed three times with PBS/CM and fixed with 1.5% paraformaldehyde in PBS/CM for 15 minutes. The filters were then rinsed with PBS/CM over half an hour with numerous changes of buffer and then incubated overnight in a blocking solution consisting of PBS/CM supplemented with 0.075% saponin, 0.2% gelatin, and 0.2% BSA in PBS/CM overnight. All subsequent incubations and washings were done in this solution. Filter sections were inverted on the primary antibody for two hours, washed 5 × 10 minutes, incubated with FITC-conjugated secondary antibodies for two hours and then washed for a minimum of 8 × 15 minutes. The cells were postfixed with 1.5% paraformaldehyde, washed with PBS/CM and then incubated for 20 minutes at 37°C with RNase (Boehringer-Mannheim, Indianapolis, IN) prior to incubation for 15 minutes with propidium iodide (Molecular Probes, Eugene, OR) to reveal the cell nuclei (Bomsel et al., 1989). The labeled cells were visualized in a dual channel laser scanning confocal microscope (Sarastro, Molecular Dynamics, Sunnyvale, CA) with the FITC signal appearing as green and the propidium iodide as red. Photographs of the screen were taken with a Screenstar camera using Kodak Ektachrome 100 film.

RESULTS

Immortalization of RPE

To acquire a polarized in vitro cell model for retinal pigment epithelia, we have generated an immortalized cell line from primary cultures of rat RPE. Cells which can proliferate in culture are necessarily transformed and consequently exhibit a loss of their differentiated phenotype relative to cells in vivo. As a result, most cell lines of epithelial origin are not polarized. To obtain a differentiated, polarized RPE cell line we transformed primary rat RPE cells with a temperature-sensitive T antigen which is functionally expressed at the permissive temperature of 33°C but is inactivated at the non-permissive temperature of 40°C.

Since the primary rat RPE cultures have a very low passing efficiency, SV40 tsA-transformed RPE cells were originally selected by their ability to survive three rounds of trypsinization and passing. The surviving cells were cloned through a number of rounds by limiting dilution and cell clones were selected for an epithelioid morphology in light microscopy and expression of circumferential staining with antibody against the tight junction protein, ZO-1 (Stevenson et al., 1986). This resulted in the isolation of a clone, RPE-J, which was used for all subsequent studies. When cultured on a thin coat of Matrigel for 6 days at 33°C and then switched to 40°C for 33-36 hours in the presence of 10 μM retinoic acid, a differentiation agent and inhibitor of cell growth (Lotan, 1980; Roberts and Sporn, 1984), RPE-J cells exhibit complete rings of ZO-1 staining (Fig. 1a). The cells were identified to be of rat RPE origin by their staining with the RET-PE2 (Neill and Barnstable, 1990) monoclonal antibody, which specifically recognizes rat RPE (Fig. 1b). RPE-J cells are SV40 transformed as nuclear T antigen is detectable in the nucleus of every cell by immunofluorescence labeling (Fig. 1c). RPE-J cells cultured on Matrigel-coated nitrocellulose filters in the presence of retinoic acid slowly acquire a transepithelial resistance of ~350 ohms cm² after 6 days of culture at 33°C, which is maintained following the temperature switch to 40°C (Fig. 2). RPE-J cells grown under these defined conditions therefore establish an epithelial monolayer with functional tight junctions.

Morphology of RPE-J cells by electron microscopy

Electron microscopy of RPE-J cells cultured on Matrigel-coated nitrocellulose filters reveals that although the cells are SV40 T antigen-transformed the conditions of growth
combine to induce the cells to acquire a typical RPE morphology including extensive microvilli and numerous dense bodies (Fig. 3). Some of these could be identified morphologically as premelanosomes (Fig. 3C, arrowhead) and others exhibited varied multilamellar structures (Fig. 3D,E).

**Fig. 1.** Immunofluorescent labeling of RPE-J cells for ZO-1, RET-PE2 and SV40 T antigen. RPE-J cells plated on Matrigel-coated coverslips for 6 days at 33°C and then switched for 33 hours to 40°C were fixed with cold (~80°C) methanol and labeled with antibodies to the tight junction marker ZO-1 (a), the rat RPE specific antibody RET-PE2 (b) and antibodies to SV40 T antigen (c) followed by the appropriate rhodamine-conjugated secondary antibody. Bar, 0.5 µm.

**Fig. 2.** Transepithelial resistance of RPE-J cells. RPE-J cells were grown on Matrigel-coated nitrocellulose filters in the presence of retinoic acid for 6 days at 33°C and then switched to 40°C. The transepithelial resistance was measured every day and the resistance from a control filter with no cells was subtracted from the measured values. The switch to 40°C (at 6 days) is indicated with an arrow. RPE-J cells acquire a transepithelial resistance of approximately 350 ohms cm² after 6 days in culture at 33°C and maintain it following the temperature switch.

In vivo RPE exhibit basal infoldings (Zinn and Benjamin-Henkind, 1979); while such basal activity was not observed, RPE-J cells frequently extended basal pseudopods into the subcellular Matrigel basement membrane and supporting nitrocellulose filter (Fig. 3A). RPE-J cells do not form a columnar epithelial monolayer and cells frequently extend pseudopodia over their neighbours (Fig. 3A,F). The addition of retinoic acid to cell cultures at 33°C induces the cells to grow as a single cell monolayer (best seen in the confocal images of Figs 7 and 8), as previously described for primary cultures of human RPE (Campochiaro et al., 1991).

The formation of an impermeant cell monolayer by RPE-J cells was confirmed by the restricted access of ruthenium red to opposing sides of the cell monolayer. Ruthenium red added apically labels the apical and lateral membranes, but not the basal cell surface, indicating that the tight junction in the cells is located at the basal pole of the cells (Fig. 3F). While the majority of experiments revealed the basal localization of the tight junction, apical tight junctions and mixed apical and basal tight junctions were also observed.

**RPE-J cells are phagocytic**

A distinguishing characteristic of RPE cells is their phagocytic ability, which in vivo serves to remove outer rod segments from the apical domain (Young, 1967; Young and Bok, 1969; Ishikawa and Yamada, 1970). To determine whether RPE-J cells have retained phagocytic ability cells
were presented with fluorescent 1 μm latex beads at 33°C and phagocytosis was followed by both immunofluorescence and electron microscopy. RPE-J cells grown on Matrigel-coated coverslips in the presence of FITC-latex beads for 4 hours at 33°C reveal a distinctive relocalization of the beads to the perinuclear region of the cell (Fig. 4A), a redistribution previously shown for phagocytosed particles in RPE (Mayerson and Hall, 1986). In MDCK cells, Fig. 3. Electron microscopy of RPE-J cells. RPE-J cells grown on Matrigel-coated nitrocellulose filters in the presence of retinoic acid for 6 days at 33°C and switched to 40°C for 33 hours were fixed and processed for transmission electron microscopy. A cross-section of RPE-J cells reveals numerous apical microvilli, assorted dark bodies and basal projections into the Matrigel-coated filter (a). Magnifications reveal the extensive apical microvilli (b) and the morphology of dense bodies including a premelanosome (c, arrowhead) as well as various multilamellar structures (d,e). Ruthenium red added to the apical membrane labels the apical and lateral cell surfaces but not the basal cell surface suggesting the presence of basal tight junctions (f). Bar, 2 μm (a,f), 1 μm (b), 0.2 μm (c,d,e).
which have no known phagocytic ability, the beads are distributed in a random pattern reflecting binding to the cell surface but no entry into the cell and exhibit decreased binding relative to RPE-J cells (Fig. 4C). Latex beads added to RPE-J cells at 4˚C exhibit the random distribution of MDCK cells at both 4˚C and 37˚C, indicating that the perinuclear distribution of beads in RPE-J cells at 33˚C is the result of active phagocytosis (Fig. 4B,D).

Latex beads phagocytosed by RPE-J cells were localized to intracellular membrane-bound compartments by electron microscopy. After 4 hours at 33˚C latex beads could be seen both within the cells and on the cell surface (Fig. 5). A few beads could be observed adherent to the apical cell surface of MDCK cells but none were found intracellularly by electron microscopy (not shown). RPE-J cells extend cell surface projections which engulf and phagocytose latex beads to phagosomes as described previously for RPE phagocytosis of both latex beads and rod outer segments (ROS) (Hollyfield, 1976; McLaughlin et al., 1983; Mayerson and Hall, 1986). The ability of RPE-J cells to phagocytose latex beads demonstrates that this cell line has retained a highly differentiated property of that epithelium.

Polarized distribution of viral proteins in RPE-J cells

Viral infection of various types of epithelial cells has characteristically demonstrated polarized delivery of viral envelope glycoproteins to distinct membrane surface domains (Rodriguez-Boulan and Sabatini, 1978; Misek et al., 1984; Matlin and Simons, 1984). To ascertain whether primary rat RPE cultures and RPE-J cells exhibit functional polarity, they were infected with influenza and vesicular stomatitis virus (VSV), which bud in a polar fashion, apically and basolaterally, respectively, in MDCK cells. Following infection of primary cultures of rat RPE cells, influenza HA exhibits a characteristic apical distribution and VSV G protein exhibits a basolateral distribution by immunofluorescence labeling (Fig. 6), indicating that primary cultures of rat RPE sort viral glycoproteins with the same polarity as other epithelial cells as has been previously described for primary human and bovine cultures of RPE (Bok et al., 1992). Similarly, in RPE-J cells influenza hemagglutinin (HA) is detected apically and VSV G protein basally (Fig. 7). The apical localization of HA and basal localization of G protein demonstrate that RPE-J cells are capable of polarized protein sorting within the trans-Golgi network (TGN).
and that the sorting mechanisms and targeting signals for VSV and influenza virus in RPE-J cells are equivalent to those in other epithelial cell lines derived from kidney (MDCK), intestine (CaCo-2) and thyroid (FRT) (Rodriguez-Boulan and Sabatini, 1978; Rindler et al., 1985; Zurzolo et al., 1992b).

**Polarity of endogenous Na,K-ATPase and N-CAM in RPE-J cells**

The polarized delivery of viral glycoproteins in RPE-J cells led us to examine the polarity of two endogenous proteins, Na,K-ATPase (the sodium pump) and N-CAM (the neural cell adhesion molecule). The Na,K-ATPase is not polarized and can be detected on the apical, basal and lateral surfaces of the cells (Fig. 8a,c). The lack of Na,K-ATPase polarity in RPE-J cells is consistent with studies of primary RPE cultures which have divided in culture and which do not express polarized apical Na,K-ATPase (Rizzolo, 1990, 1991). Another endogenous RPE-J protein, N-CAM (Cunningham et al., 1987), is localized to the lateral surface (Fig. 8b,d). The ability of RPE-J cells to target viral proteins in a polarized fashion to the apical and basal domains, the lack of polarity of the Na,K-ATPase and the lateral polarity of N-CAM support the idea that mechanisms other than directed delivery to surface domains, such as domain-specific stabilization via intercellular adhesive interactions, may also govern the polarity of certain proteins in RPE and other polarized epithelia.

**DISCUSSION**

RPE exhibits, along with the epithelium of the choroid plexus (Matsuzawa and Anderson, 1971; Ernst et al., 1986), a reversed apical polarity of the Na,K-ATPase which interacts and colocalizes with an apical fodrin-ankyrin cytoskeleton in primary RPE cell explants (Gundersen et al., 1991). This is in contrast to the basolateral polarity of the Na,K-ATPase in epithelia of other organs such as kidney (Kyte, 1976; Ernst and Hootman, 1981). Application of domain-specific targeting techniques, which have proved extremely fruitful in determining the surface biogenetic pathways of plasma membrane proteins in established epithelial cell lines such as kidney, MDCK, or intes-

**Fig. 5.** Electron microscopy of RPE-J cell phagocytosis. RPE-J cells grown on Matrigel-coated nitrocellulose filters at 33˚C were incubated in the presence of fluorescent-conjugated 1 µm diameter latex beads for 4 hours at 33˚C and then washed, fixed and processed for transmission electron microscopy. Beads were visualized as translucent spheres and could be seen both attached to the apical cell surface and within the cells (a). Phagocytosis was associated with the progressive extension of lamellopodia around the bead leading to its complete envelopment (b,c,d). Following phagocytosis the beads were localized to membrane-bound intracellular organelles exhibiting multilamellar membrane regions typical of phagosomes (e). Bar, 0.5 µm (b-e, same magnification).
tine, CaCo-2, require a confluent polar “tight” monolayer which prevents the diffusion of soluble labeling reagents from one side of the monolayer to the other (Hanzel et al., 1991). The study of RPE polarity to date has been restricted due to the necessity of working with primary cultures. To generate an RPE cell line which could be easily used to study the molecular mechanisms of the RPE polar phenotype, primary rat RPE cells were infected with the tsA SV40 virus expressing a temperature-sensitive T antigen (Chou, 1989). Under defined growth conditions RPE-J cells form a tight cell monolayer with a transepithelial resistance of 350-400 ohms cm\(^2\) and exhibit complete circumferential expression of the tight-junction protein, ZO-1. RPE-J cells were demonstrated to be of RPE origin by their expression of the rat RPE marker, RET-PE2 (Neill and Barnstable, 1990), their morphology by electron microscopy and by their distinctive phagocytic ability. RPE-J cells exhibit extensive apical microvilli which, in vivo, are interdigitated with photoreceptors in the interphotoreceptor matrix (Burnside and Laties, 1979). RPE cells characteristically contain electron-dense particles such as melanin granules, myeloid bodies, phagosomes and lipofuscin granules (Marshall and Ansell, 1971; Feeney, 1978; Streeten, 1961). Dense melanin granules are not found in RPE-J cells, as found for primary cultures of RPE, which shed the pigment granules as they divide (Albert and Buyukmichi, 1979); however, some structures morphologically similar to premelanosomes were observed (Fig. 3). Similarly, while primary cultures of rat RPE do exhibit lipofuscin granules, none are seen in the RPE-J cell line. The absence of both melanin and lipofuscin granules in RPE-J cells is not surprising, considering the multiple passaging of the cells in culture and the growth of individual cultures for no longer than 8 days. The presence of numerous multilamellar structures, with and without an enclosing membrane, suggests that an active phagolysosomal system is expressed in RPE-J cells.

Phagocytosis of rod outer segments by the apical RPE cell surface is an essential function which clears the retina of excess rod outer segments (Young, 1967; Young and Bok, 1969; Ishikawa and Yamada, 1970). Previous studies have shown that RPE phagocytosis exhibits specificity for rod outer segments and is receptor mediated (McLaughlin et al., 1983; Mayerson and Hall, 1986). Latex beads have classically been used to demonstrate phagocytic ability in a variety of cell types and studies of ligand-coated beads have proved highly useful in determining the specificity of phagocytosis in RPE and other phagocytes (Hollyfield, 1976; McLaughlin et al., 1983; Silverstein et al., 1977). While latex beads are a non-specific phagocytic ligand, the ability of RPE-J cells to extend cellular projections and ingest the beads to characteristic phagosomes demonstrates that they have retained phagocytic ability. This is in contrast to the kidney-derived epithelial cell line, MDCK.
Immortalization of RPE which is unable to phagocytose latex beads. The RPE-J cell line should facilitate studies of the specificity and mechanisms of RPE phagocytosis in vitro.

Interestingly, ruthenium red labeling of RPE-J cells plated on Matrigel reveals the frequent basal localization of the tight junction. Junctional complexes in RPE are atypical compared to other epithelia in that tight junctions, adherent junctions and gap junctions are intermixed; the tight junction is not always located at the apical pole of the cell and frequently overlaps with adherent junctions and gap junctions (Crawford, 1980; Hudspeth and Yee, 1973). In vivo, RPE from some species exhibit apical tight junctions (monkey, rat, dog) while others exhibit junctions at or towards the basal surface (frog, chick, fish, rabbit) (Kuwabara, 1979). The basis for the differential localization of tight junctions in RPE is not clear. That RPE-J cells, derived from rat RPE, which have apical tight junctions in vivo, can exhibit basal junctions in vitro is indicative of the variability of tight junction location in RPE.

Acquisition of a differentiated RPE phenotype by RPE-J cells is dependent on the culture conditions under which the cells are grown. RPE cells acquire a polarized phenotype when grown on Matrigel, with retinoic acid in the culture medium and a temperature switch from 33˚C to 40˚C for 33 hours. The individual contribution of these different factors to RPE-J cell differentiation is not clear. Temperature inactivation of the temperature-sensitive T antigen is achieved with a 48 hour incubation at the non-permissive temperature (Chou, 1985), yet optimal RPE-J polarity is obtained with a 33 hour incubation at 40˚C after which T antigen is still detectable in the cell nuclei (Fig. 1C). Retinoic acid regulates the growth and differentiation of epithelial cells and various preneoplastic and neoplastic cell lines in culture (Lotan, 1980; Roberts and Sporn, 1984). In cultures of RPE-J cells, retinoic acid may serve to prevent overgrowth of the SV40-transformed cells at the permissive temperature of 33˚C such that, while cells frequently extend cellular extensions over their neighbors (see Fig. 3), they grow as a single cell monolayer; this is best seen in the single layer of propidium iodide-labeled nuclei in confocal optical cross-sections (Figs 7 and 8). Cells grown on collagen type IV generate transepithelial resistance but do

Fig. 7. Polarized targeting of influenza and VSV in RPE-J cells. RPE-J cells grown on Matrigel-coated nitrocellulose filters in the presence of retinoic acid for 6 days at 33˚C and switched to 40˚C for 33 hours were infected with either influenza virus or VSV (see Materials and methods) and then fixed and immunofluorescently labeled for influenza hemagluttinin (HA) (a,c) or VSV G (b,d) protein. The fluorescently labeled cells were visualized in a Sarastro confocal laser scanning microscope, with the fluorescein antibody labeling appearing in green and the nuclei stained with propidium iodide, appearing red. A horizontal cross-section (XY-scan) through a fluorescein-labeled area revealed a punctate labeling pattern for both HA (a) and G protein (b). Vertical sections (Z-scans) showed HA to be localized to the apical surface (c) while G protein was found on the basal surface (d). RPE-J cells can therefore sort and target viral glycoproteins in a polar fashion. Bar, 5 µm.
not acquire the distinctive RPE morphology of cells cultured on Matrigel (not shown). While the extracellular matrix coating serves an adhesive function, particularly on glass coverslips to which the cells do not adhere, components of Matrigel may also serve to induce cellular differentiation. Laminin induces the transdifferentiation of primary cultures of tadpole RPE to neurons (Reh et al., 1987) and laminin present in Matrigel may be responsible for the differentiated polarized phenotype of RPE-J cells in culture. During development, polarity of RPE is regulated both temporally and spatially, suggesting that extracellular cues may influence cellular polarity (Rizzolo and Heiges, 1991). Culture of chick RPE on isolated Bruch’s membrane was shown to induce a polarized distribution of beta1-integrin, demonstrating a role for the basement membrane and its components in the polarization of RPE in vitro (Rizzolo, 1991).

RPE-J cells have retained the ability to sort viral proteins; influenza HA is sorted to the apical surface while VSV-G is sorted basally in RPE-J cells. The polarity of influenza virus and VSV budding in RPE is therefore the same as was previously found for other epithelial cell lines such as MDCK, CaCo-2 and FRT (Rodriguez-Boulan and Sabatini, 1978; Rindler et al., 1985; Zurzolo et al., 1992b), indicating that the apical/basal sorting mechanisms for these proteins in the trans-Golgi network (TGN) of RPE-J cells are similar to those of other epithelial cells. Similarly, infection of primary cultures of rat RPE reveals apical assembly of influenza virus and basolateral assembly of VSV, as previously shown for human and bovine RPE (Bok et al., 1992). The absence of both HA and G protein from the lateral membrane of RPE-J cells is curious. The lateral distribution of VSV G protein in primary RPE cultures suggests that its basal localization in RPE-J cells is not an intrinsic property of RPE cells but perhaps a consequence of the cell culture conditions and in vitro differentiation of the cells; which basolateral targeting pathway represents the in vivo situation is not clear. In other epithelial cell lines, the basal and lateral membranes form a continuous basolateral surface which is separated from the apical surface by the tight junction (Rodriguez-Boulan and Nelson, 1989). The basal restriction of VSV could not be attributed to the basal tight junction; experiments in which RPE-J cells were virally infected and labeled with ruthenium red in parallel revealed that the apical or basal location of the tight junction had no influence on the apical and basal polarity of HA and G protein or on their restriction from the lateral cell surface. In MDCK cells plated subconfluently, which do not form intercellular contacts or tight junctions, influenza virus buds only from the free surface and not from
the substratum-attached surface (Rodriguez-Boulan et al., 1983). The tight junction, while clearly important to the formation of an impermeant epithelial monolayer, may not be required for the restriction of proteins to a particular surface domain. Such an observation has significant implications for the mechanisms which define cell surface domains in polarized cells which lack tight junctions, such as neurons.

RPE differs from most other epithelia in that it expresses the Na,K-ATPase on the apical surface and does not express the epithelia specific cell adhesion molecule E-cadherin but rather the neural cell adhesion molecule, N-CAM (Steinberg and Miller, 1979; Bok, 1982; Caldwell and McLaughlin, 1984; Gundersen et al., 1991, 1992;Neill and Barnstable, 1990). While RPE-J cells target influenza virus to the apical and VSV to the basal surface, optical cross-sections of fluorescently labeled RPE-J cells reveal the localization of the Na,K-ATPase to the apical, lateral and basal surfaces of the cells and N-CAM exclusively to the lateral cell surface. The determination of protein polarity in epithelia may not be solely a consequence of polarized sorting and targeting from the TGN but also due to protein stabilization in distinct cell surface domains by intercellular interactions.

In kidney-derived MDCK cells, the basolateral polarity of the Na,K-ATPase is stabilized by its interaction with the submembrane ankyrin-fodrin cytoskeleton which is induced in the basolateral domain of MDCK cells by its interaction with E-cadherin, the epithelial cell-cell adhesion molecule (Nelson et al., 1990; Hammerton et al., 1991). RPE cells do not express E-cadherin and the apical localization of the Na,K-ATPase colocalizes with an apical ankyrin-fodrin submembrane cytoskeleton (Gundersen et al., 1991, 1992). The lack of polarity of the Na,K-ATPase in RPE-J cells is similar to that previously found for cultures of primary chick RPE cells, even after growth on Bruch’s membrane which induces the repolarization of beta1-integrin (Rizzolo, 1990, 1991). However, in contrast to chick RPE which do not exhibit polarized targeting of VSV, RPE-J cells are functionally polar, based on their ability to target influenza virus apically and VSV basally. The lack of polarity of the Na,K-ATPase suggests the existence of a mechanism stabilizing the ankyrin-fodrin submembrane cytoskeleton to the apical surface of RPE cells in vivo. In contrast to most other epithelia whose apical surface is free facing the lumen, the apical surface of RPE is in intimate contact with adjacent photoreceptors in vivo. Expression of an alternate cell adhesion molecule which mediates the interaction between RPE and photoreceptors may anchor the ankyrin-fodrin cytoskeleton in the apical domain of RPE and be the basis for the apical polarity of the Na,K-ATPase in vivo.

Rat RPE cells have been reported to express the larger transmembrane forms of N-CAM, both the 140 and 180 kDa isoforms (Neill and Barnstable, 1990) or only the 140 kDa form (Gundersen et al., 1992). In transfected MDCK cells both the 140 kDa and 180 kDa isoforms are targeted directly to the basolateral cell surface (Powell et al., 1991). Expression of N-CAM on the lateral surface of RPE-J cells may reflect its stabilization in that surface domain by intercellular N-CAM to N-CAM interactions. Primary cultures of RPE exhibit a striking redistribution of N-CAM from the apical surface in vivo to the lateral surface in vitro (Gundersen et al., 1992). The polarity of N-CAM in RPE might be determined by its stabilization due to homophilic N-CAM to N-CAM interactions, either apically with N-CAM in the neural retina in vivo or laterally with adjacent RPE cells within a cell monolayer in vitro. The intercellular interaction of the apical surface of RPE cells with photoreceptors and the interphotoreceptor matrix in vivo may provide multiple adhesive interactions stabilizing proteins to the apical surface domain of RPE. Domain-specific stabilization may be a widespread mechanism for maintaining protein polarity in RPE and other epithelial cell types.

The availability of a differentiated RPE cell line will allow the application of the extensively used transfection and targeting assays, which have defined the epithelial targeting pathways in the polarized epithelial cell lines, MDCK (kidney), CaCo-2 (intestine) and FRT (thyroid) for both endogenous and transfected proteins (Hanzel et al., 1991; Le Bivic et al., 1990a,b; Nabi et al., 1991; Zurzolo et al., 1992a,b). Both similarities and differences exist between the sorting mechanisms and protein targeting pathways of these polarized epithelial cell lines of different tissue origins. The selective use of mechanisms of protein polarization might lead to the cell type-specific protein polarity necessary for the function of different epithelial cells, such as the apical polarity of the Na,K-ATPase in RPE. The availability of an epithelial cell line lacking E-cadherin will provide a means to study, via transfection of E-cadherin into RPE-J cells, the role of E-cadherin in the generation of the epithelial phenotype as well as on the mechanisms determining the polarity of the Na,K-ATPase. Expression of E-cadherin in RPE-J cells by transfection will enable investigation of the role E-cadherin plays in generation of the epithelial phenotype of epithelial of other tissue origin and particularly its influence on the polarity of the Na,K-ATPase. The ability to generate a differentiated polarized RPE phenotype from an immortalized SV40 transformed cell line should provide an excellent in vitro model system to study the mechanisms underlying the characteristic polarity and functions of RPE.

We are especially grateful to Dr. Janice Chou for the kind gift of the tsA SV40 virus and to Drs. Jim Neill and Colin Barnstable for the gift of the RET-PE2 RPE marker. We thank Drs. Larry Rizzolo, Mario Wolosin and Reuben Lotan for their helpful suggestions and Dr. Peter MacLeish for his critical review of the manuscript. The excellent photographic reproductions were the work of Ms. Lori van Houten and Ms. Joy Hornung. This work was supported by NIH grants EY08538 and GM34107. I. R. Nabi was supported by a postdoctoral fellowship from the Medical Research Council of Canada.

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(Received 25 June 1992 - Accepted 28 September 1992)