Basement membrane stimulates the polarized distribution of integrins but not the Na,K-ATPase in the retinal pigment epithelium

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The basement membrane stimulates the differentiation and polarity of simple transporting epithelia. We demonstrated for the retinal pigment epithelium (RPE) of chicken embryos that polarity develops gradually. Although the RPE and an immature basement membrane are established on embryonic day 4 (E4), the distribution of the Na,K-ATPase and a family of basement membrane receptors containing the β_1 subunit of integrin is nonpolarized. The percentage of polarized cells increases gradually until cells in all regions of the epithelium are polarized on E11. During this time, the basement membrane increases in size and complexity to form Bruch's membrane. To study the ability of the basement membrane to stimulate the polarized distribution of the β_1 integrins or the Na,K-ATPase, RPE was harvested from E7, E9, or E14 embryos and cultured on Bruch's membrane isolated (in association with the choroid) from E14 embryos. As a control, the RPE was plated on the side of the choroid lacking a Bruch's membrane. The distribution of the β_1 integrins and the Na,K-ATPase was determined by indirect immunofluorescence. Bruch's membrane stimulated the polarized distribution of the β_1 integrins regardless of the developmental age of the RPE even though E7 RPE is nonpolarized *in vivo*. To examine the role of individual matrix components, RPE was plated on matrix-coated filters. The polarized distribution of the β_1 integrins was stimulated by laminin, collagen IV, and Matrigel but not by fibronectin. Interestingly, laminin and collagen IV are present in the basement membrane on E4 when RPE is not polarized *in vivo*. Under no circumstances was the distribution of the Na,K-ATPase polarized. These data indicate that the basement membrane influences the distribution of a subset of plasma membrane proteins but that other factors are required for full polarity.

Introduction

The retinal pigment epithelium (RPE)¹ plays a central role in the physiology of the eye. The RPE lies at the interface between the choroid and the neural retina where it forms the blood-retinal barrier, recycles retinoids used by the photoreceptors during the visual cycle, and phagocytoses the shed tips of photoreceptors during the turnover of photoreceptor outer segments (Zinn and Marmor, 1979). As in other transporting epithelia, the plasma membrane of RPE is differentiated into basolateral (facing the choroid) and apical (facing the neural retina) membranes. Each membrane has a different protein composition that reflects its unique functions (Bok, 1982; Simons and Fuller, 1985; Philp and Nachmias, 1987; Mircheff *et al.*, 1990). Unlike other transporting epithelia, the apical membrane of RPE is intimately associated with another cell layer, and it is in this membrane that the Na,K-ATPase is located (Bok, 1982; Gundersen *et al.*, 1991). During disease or injury, the RPE responds to changes in its environment by dissociating from the monolayer to form macrophage or fibroblasts (Machemer and Laqua, 1975; Pollack and Korte, 1990). By manipulating the environment of chick embryonic RPE *in situ* or in culture, RPE will transdifferentiate to form the various cells found in lens or the neural retina (Stroeva and Mitashov, 1983; Itoh and Eguchi, 1986; Park and Hollenberg, 1989).

The extracellular matrix influences the differentiation and polarity of many epithelia (Stoker *et al.*, 1990). In mouse, the transition of mesenchyme into kidney tubules requires the polarized deposition of the laminin A chain. Concomitantly, a laminin receptor must be expressed by the epithelium. Antibodies to laminin or its receptor inhibit the transition (Klein *et al.*, 1988; Sorokin *et al.*, 1990). Similarly in chicken, the appearance of a RPE monolayer

¹ Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; E, embryonic day; PBS, phosphate-buffered saline; RPE, retinal pigment epithelium.

on embryonic day 4 (E4) correlates with the basal deposition of a basement membrane containing fibronectin, laminin, and collagen IV (Turksen *et al.*, 1985). Despite the morphological appearance of polarity on E4, the distribution of the Na,K-ATPase and the β_1 -integrin family remains nonpolarized until later in development (Rizzolo and Heiges, 1991). During this time, the basement membrane (Bruch's membrane) increases in thickness and complexity forming collagenous and elastic fiber layers sandwiched between two basal lamina: one associated with the RPE and the other with the choriocapillaris (François *et al.*, 1963; Zinn and Marmor, 1979). Conceivably, changes in Bruch's membrane during development affect the development of polarity in the RPE.

In principle, the distribution of the Na,K-ATPase and β_1 -integrin family could be influenced by the basement membrane. There might be a direct effect on the β_1 integrins because they bind to components of the basement membrane such as laminin, fibronectin, and collagen IV (Albelda and Buck, 1990). However, these components are present on E4, which suggests other factors are involved. The distribution of the Na,K-ATPase might be affected indirectly via interactions of the basement membrane with the cytoskeleton. In other epithelia, the Na,K-ATPase is anchored in the basolateral membranes by the cortical cytoskeleton (Nelson and Hammerton, 1989; Rodriguez-Boulan and Nelson, 1989). In RPE, the polarity of the cortical cytoskeleton is reversed, and therefore, likely still affects the distribution of the Na,K-ATPase (Gundersen *et al.*, 1991). Conceivably, Bruch's membrane might induce the polarity of the cytoskeleton that in turn would redistribute the Na,K-ATPase. An analogous redistribution of the Na,K-ATPase was observed when uvomorulin was expressed in fibroblasts. Uvomorulin redistributed the cytoskeleton, and concomitantly the Na,K-ATPase, along regions of cell-cell contact (McNeill *et al.*, 1990).

Studies of Bruch's membrane *in vivo* are complicated by interactions of the RPE with the neural retina and choriocapillaris (Coulombre and Coulombre, 1965; Korte *et al.*, 1989). In the current study, we take advantage of improved culture conditions to study the effects of Bruch's membrane in primary cell culture. Earlier studies with chick RPE in primary or secondary culture employed culture media containing fetal calf serum (Hayashi *et al.*, 1978; Turksen *et al.*, 1983, 1984; Vielkind and Crawford, 1988; Philp *et al.*, 1990). Under these conditions, the plating effi-

ciency was low, and initially, the cells appeared undifferentiated. As the colonies grew, cells in the center assumed an epithelioid morphology, whereas those at the periphery remained undifferentiated (Hayashi *et al.*, 1978; Turksen *et al.*, 1983). The observation that the plating efficiency diminished with increasing age of the embryonic donor led to the speculation that only immature, mitotic cells survived in culture (Vielkind and Crawford, 1988). We have developed a serum-free medium in which the plating efficiency is high even for older embryonic cells. The RPE rapidly assumed an epithelioid morphology that formed a barrier to the diffusion of inulin and a low but measurable transepithelial electrical resistance. Nonetheless, the distribution of several plasma membrane markers remained nonpolarized (Rizzolo, 1990).

To test the roles of the basement membrane and the developmental age of the RPE on the development of polarity, we cultured RPE from E7, E9, and E14 on matrix-coated filters or on Bruch's membrane isolated from E14 embryos. Bruch's membrane and basement membrane components stimulated the polarization of β_1 integrins regardless of the age of the RPE. These substrates failed to polarize the distribution of the Na,K-ATPase. These data indicate the basement membrane stimulates the polarized distribution of only a subset of RPE proteins.

Results

Culture of RPE on Bruch's membrane

Bruch's membrane is the native basement membrane of RPE. It is a trilaminar structure comprised of a thin basal lamina adjacent to the RPE, a thicker discontinuous lamina adjacent to the choriocapillaris, and intervening collagenous and elastic layers. Although increasing in thickness until E19 (François *et al.*, 1963), by E14 the developing Bruch's membrane has formed these features (Figure 1a). At this age, Bruch's membrane in association with the choroid was readily isolated as a thin, vascular sheet devoid of RPE (Figure 1b). The absence of RPE was readily verified under the dissecting microscope because these cells appear black due to the high content of melanin granules. On isolation, capillaries in the choroid collapsed about the entrapped blood cells and Bruch's membrane became thick and undulating, likely due to the contraction of elastic fibers in the collagenous layer. This preparation was adhered to a nitrocellulose filter with either the choroid side or the Bruch's membrane side adjacent to filter.

Figure 1. Electron micrographs of E14 Bruch's membrane in situ and in culture. Eye cups were fixed immediately (a) or the neural retina and sclera were removed and the remaining choroid with Bruch's membrane was incubated in serum-free media before fixation (b and c) and prepared for electron microscopy. In each panel, the choroid is on the bottom and the epithelial aspect of Bruch's membrane faces the top. (a) In situ, Bruch's membrane (demarcated by the large and small arrow heads) is bounded by a large broad capillary filled with red blood cells (RBC) on the bottom and an RPE monolayer laden with dark melanin granules (M) on the top. Bruch's membrane contains a thin, continuous basal lamina (small arrowhead) that underlies the RPE and a thicker, discontinuous basal lamina (large arrowhead) that underlies the capillary endothelium (arrow). A fibrillar matrix is observed between the two basal lamina. The tips of developing photoreceptors (P) are at the top. (b) After 1 h in culture, both Bruch's membrane and endothelial cells appear swollen and undulating. The lumen of the capillaries have shrunk about the entrapped red blood cells. (c) After 1 wk in culture, both basal lamina of Bruch's membrane are still evident. Fibroblasts have proliferated in the choroid, and multilayers of fibroblasts embedded in a matrix have overgrown the epithelial side of Bruch's membrane. Bar, 2 μ m.

After 1 wk in culture, vessels containing red blood cells were still evident, but fibroblasts proliferated both in the choroid and as multilayers that spread along the bare filter and on top of Bruch's membrane (Figure 1c). The proliferating fibroblasts appeared to secrete

fibrillar type collagens, as the cells were always embedded in a fibrillar matrix. Despite the overgrowth of fibroblasts, the general morphology of Bruch's membrane remained intact during the course of the experiments.

Figure 2. Electron micrographs of E7 RPE plated on Bruch's membrane or on the opposing side of the choroid. Dissociated RPE cells isolated from E7 embryos were plated on Bruch's membrane (a and b) or choroid (c and d) isolated from E14 embryos. The tissues were fixed after a 1-wk incubation and prepared for electron microscopy. In each panel, the basal membrane of the RPE faces down. Arrowheads mark the epithelial (small arrowhead) and endothelial (large arrowhead) aspects of Bruch's membrane. (a) Monolayers formed on Bruch's membrane exhibited apical microvilli, apically disposed junctional complexes (arrows) on the lateral membranes, and infoldings of the basal and lateral membranes. (b) A higher power view of the basal pole of the RPE resting on the thin basal lamina of Bruch's membrane (small arrowhead). The region of the small arrowhead is enlarged in the inset. (c) A monolayer similar to that shown in (a) was obtained when RPE was plated on the opposing side of the choroid. (d) A higher power view of the basal pole of the RPE. Note the absence of a basal lamina. F, fibroblast; Bars, a and c, 5 μ m and b and d, 1 μ m; inset, 0.5 μ m.

Dissociated RPE cells harvested from E7, E9, or E14 embryos were plated on either Bruch's membrane or the opposite side of the choroid. In parallel, cells were plated at the same density on Matrigel-coated filters. After 1 d, monolayers formed on the filters that were visually confluent with a transepithelial electrical resistance of 20–25 Ω/cm^2 . Cultures were maintained for 1 wk to allow the cells to adapt to culture conditions. During this time, the resistance rose to a maximum value of 25–35 Ω/cm^2 . The low resistance was typical of chick embryonic RPE (Rizzolo,

1990). After 1 wk in culture, the RPE plated on Bruch's membrane was readily distinguished by its melanin content and epithelioid morphology. As shown for E7-derived cells (Figure 2a), the RPE prevented fibroblasts from growing onto its apical surface or between the reformed monolayer and Bruch's membrane. Microvilli, apically disposed junctional complexes, and basal and lateral infoldings were evident, similar to E7 RPE plated on Matrigel-coated filters (Rizzolo, 1990). The thin basal lamina of Bruch's membrane was closely applied to the basal

Figure 3. Localization of β_1 integrins and Na,K-ATPase in E7 RPE plated on Bruch's membrane (a and c and d and e) or choroid (b). E7 RPE was cultured as described in Figure 2 and processed for cryosectioning and immunofluorescence with anti- β_1 integrins (a-c) or anti-Na,K-ATPase (d) monoclonal antibodies. Downward white arrowheads precisely mark the apical membranes; upward white arrowheads mark the basal membranes. (a) When RPE was plated directly on Bruch's membrane, β_1 integrins were observed only in the basal and lateral membranes of most cells (see Table 1). The black arrowhead marks an oblique lateral membrane. (b) When RPE was plated on the choroidal surface the distribution of β_1 integrins was nonpolarized on most cells. (c) Bruch's membrane/choroid was pretreated with NH_4OH for 1 h before plating RPE on Bruch's membrane. In $\sim 50\%$ of the cells, the distribution of β_1 integrins was basolateral as in the cells shown here. (d) When RPE was plated directly on Bruch's membrane, the distribution of the Na,K-ATPase in all cells was nonpolarized or slightly enriched in the basolateral membranes. (e) The bright-field micrograph corresponding to (d) illustrates the RPE monolayer laden with black melanin granules resting on a layer of Bruch's membrane/choroid. Bar, 10 μm .

membrane of the RPE (Figure 2b). Although similar monolayers formed on the choroid, a basal lamina was not evident (Figure 2, c and d). Although RPE of any age formed monolayers on Bruch's membrane, only E7 RPE adhered to the choroid.

Bruch's membrane induces polarity of the β_1 subunit of integrin, but not the Na,K-ATPase

After 1 wk in culture, the distribution of the β_1 subunit of integrin and the Na,K-ATPase was determined by indirect immunofluorescence. The β_1 subunit of integrin was identified using the monoclonal antibodies JG22 (Greve and Gottlieb, 1982) or CSAT (Horwitz *et al.*, 1985). Both antibodies yielded the same results. The distribution of the Na,K-ATPase was determined using a monoclonal antibody, Ab24, directed against the β subunit of the Na,K-ATPase from chicken muscle (Fambrough and Bayne, 1983; Tamkun and Fambrough, 1986). Ab24 recognizes isoforms of the Na,K-ATPase found in nerve, muscle, and kidney but not isoforms found in fibroblasts or capillary endothelium.

The data represent the average of at least three experiments in which a minimum of 500 cell profiles was scored. When cultured directly on Bruch's membrane, the β_1 integrins were greatly enriched in the basal and lateral membranes of $\sim 70\%$ of E7 RPE cells compared with 20% when cells were cultured on the choroid (Figure 3, a and b and Table 1). Thus, E14-derived Bruch's membrane stimulated a polarity of E7 RPE that is not observed in vivo. To test whether Bruch's membrane alone was sufficient or if ongoing protein synthesis was required in the choroid, the Bruch's membrane/choroid preparation was pretreated with ammonium hydroxide. The pretreated preparation stimulated polarity in $\sim 45\%$ of the cells (Figure 3c and Table 1). Although this is significantly more than choroid alone, it is less than the native preparation. Although the choroid may stimulate polarity, it is also possible that the pretreatment extracted or denatured an important component of Bruch's membrane. In contrast to the β_1 integrins, the distribution of the Na,K-ATPase was always nonpolarized or slightly enriched in the basolateral membranes (Figure 3d).

Table 1. Percentage of RPE cells with a basolateral distribution of the β_1 subunit of integrin^a

Matrix	Percentage of polarized cells (standard deviation)		
	E7	E9	E14
Bruch's membrane	69.2 (8.2)	79.8 (5.3)	79.7 (11.6)
Choroid	21.8 (13.7)	ND	0 ^b
Bruch's membrane (NH ₄ OH)	46.1 (3.9)	ND	ND

^a RPE was harvested from embryos of the indicated age, and dissociated cells were plated on Bruch's membrane/choroid isolated from E14 embryos. Cells were plated either directly on Bruch's membrane or on the opposing side of the choroid. In some experiments, the Bruch's membrane/choroid was treated with NH₄OH to kill choroidal cells before plating RPE cells. Cryosections were analyzed by immunofluorescence to determine the percentage of cells with a predominantly basolateral distribution. Data represent the average of at least three experiments in which >500 cell profiles were examined.

^b RPE monolayers could not be established on the choroid.

In vivo, the percentage of RPE polarized with respect to β_1 integrins and Na,K-ATPase increases by E9 and approaches 100% by E11 (Rizzolo and Heiges, 1991). To determine whether cells from E9 or E14 are more readily polarized in culture, the experiments were repeated using dissociated RPE from E9 and E14. Except for the inability of E14 RPE cells to adhere to the choroid, results similar to E7 were obtained (Figure 4 and Table 1). In both cases, the β_1 subunit of integrin was polarized in ~80% of the cells (Table 1). Given the size of the standard deviation, this represents little, if any, improvement over E7 RPE. These data indicate that E7 RPE is capable of responding to a stimulus in mature Bruch's membrane, suggesting that on E7 in vivo this stimulus either is inhibited

or lacking. Unable to isolate a basement membrane from E7 embryos, we could not test whether RPE of different developmental ages would be polarized on an immature basement membrane. Under no circumstances was the distribution of the Na,K-ATPase polarized (Figure 4).

Polarity of RPE maintained on filters

In the previous experiments, a small fraction of RPE plated on choroid had a polarized distribution of β_1 integrins. To test whether the choroid stimulated polarity or whether RPE could develop polarity on its own, dissociated RPE was plated on uncoated filters. The cells from E7, E9, or E14 embryos formed large multicellular aggregates that were weakly adherent to the filters. Unlike other epithelia that form cysts with lumens, the cells formed multilayers of closely packed cells. Only cells derived from E7 embryos spread from the clumps along the bare filter to form monolayers with a typical epitheloid morphology (Figure 5b). Only cells in the monolayers were scored for polarity. Similar to RPE plated on choroid, ~25% of the RPE was polarized with respect to integrin; as shown in Figure 5a, the β_1 subunit of integrin was stained in the apical membrane of most cells. No cells were polarized with respect to the Na,K-ATPase (data not shown).

To determine which factors might stimulate the polarized distribution of β_1 integrins, filters were coated with a variety of matrix components. All of the components tested promoted the attachment, spreading, and proliferation of E7–14 RPE cells to form a confluent monolayer, but these properties depended on how the matrix was applied. When Matrigel, a mouse-tumor basement membrane extract (Kleinman *et al.*, 1986), was applied as a thick gel, the RPE ag-

Figure 4. Localization of β_1 integrins and Na,K-ATPase in E9 (a and b) or E14 (c and d) RPE plated on Bruch's membrane. RPE was cultured on Bruch's membrane as described for E7 RPE in Figure 2 and processed for cryosectioning and immunofluorescence with anti- β_1 integrins (a and c) or anti-Na,K-ATPase (b and d) monoclonal antibodies. Downward arrowheads, apical membranes; upward arrowheads, basal membranes. (a and c) The distribution of β_1 integrins was basolateral in most cells for both E9 and E14 RPE (see Table 1). (b and d) By contrast, the distribution of the Na,K-ATPase was nonpolarized in all cells from each developmental age. Bar, 10 μ m.

Figure 5. Localization of β_1 integrins in E7 RPE plated on uncoated filters. Dissociated RPE was plated on millicell-CM filters at a density of 9.0×10^5 cells/cm². Initially the cells formed multilayered aggregates that were sparsely distributed about the filter. Over the next several weeks cells spread onto bare areas of the filters to form monolayers. Filters were then processed for electron microscopy or cryosectioning and staining with anti- β_1 -integrin monoclonal antibody. (a) The distribution of β_1 integrins in monolayer regions was nonpolarized. Upward arrowhead, basal membrane; downward arrow head, apical membrane; Bar, 10 μ m. (b) Electron micrograph of a monolayer region exhibiting a typical epithelial morphology with microvilli and apically disposed junctional complexes (arrows) on the lateral membranes. Bar, 2 μ m.

gregated into tubular arrays. But when applied as a thin coating (Rizzolo, 1990) or dried onto the filter, RPE of all ages tested formed differentiated monolayers with a transepithelial electrical resistance. When laminin was applied, only high concentrations of laminin (30 μ g/cm² vs. 3 μ g/cm²) promoted cell attachment and spreading. For each matrix tested, the most consistent results were obtained when the matrix was dried on to the filter at room temperature.

Matrigel was the most effective in stimulating the polarized distribution of the β_1 integrins. In contrast to E14-derived Bruch's membrane, virtually all of the cells exhibited a basal polarity (Figure 6). Little or no lateral staining was observed. Although laminin, collagen IV, and fibronectin are major components of Matrigel and might trap β_1 integrins in the basal membrane, they are also present in the basement membrane in vivo on E7 when the β_1 integrins are not polarized. However, although fibronectin was unable to stimulate polarity, a basal and lateral distribution was observed when cells were plated on laminin or collagen IV. Although

a majority of the cells on collagen IV were polarized, virtually all of the cells on laminin were polarized. As reported earlier for Matrigel (Rizzolo, 1990), no matrix coating could polarize the distribution of the Na,K-ATPase regardless of the developmental age of the RPE (data not shown).

Discussion

We have begun to dissect the development of epithelial polarity to understand the role of the basement membrane. Although various laboratories have demonstrated the importance of the basement membrane in the differentiation of epithelia, we observed that different effects can be resolved temporally during embryogenesis (Rizzolo and Heiges, 1991). The current study examined the effects of developmental age and matrix. Regardless of the age when the cells were harvested, when the cells were cultured on choroid or on bare or fibronectin-coated filters, only a small percentage of RPE cells became polarized with respect to β_1 integrins. However, polarity of β_1 integrins was stimulated by Bruch's membrane or other matrix coatings even if the RPE was harvested from E7 embryos when the distribution of β_1 integrins are nonpolarized. Nevertheless, these substrates failed to induce the polarity of the Na,K-ATPase. Despite its importance in the initial formation of the RPE and subsequent role in further stimulating polarity, the basement membrane is insufficient to induce full polarity in chick RPE.

Several studies suggest the basement membrane induces the initial formation of an epithelial monolayer. Turksen *et al.* (1985) observed that the establishment of an RPE monolayer in chick embryos on E4 correlated with the basal appearance of laminin, collagen IV, and fibronectin. A similar correlation was observed in vitro. When grown on plastic in fetal calf serum-containing medium, chick embryonic RPE was initially undifferentiated. As colonies grew larger, a matrix containing various basement membrane components was deposited on the plastic, and in the center of the colony where the matrix was thickest, the cells assumed a cobblestone appearance that reflected the formation of junctional complexes around the apical end of the lateral membranes. Additionally, the cytoskeleton of the central cells assumed an in vivo-like structure (Hayashi *et al.*, 1978; Turksen *et al.*, 1983, 1984). Ekblom and colleagues observed a similar correlation in the transition of mesenchyme into kidney tubules both in vivo and in organ culture. In this case,

Figure 6. Localization of β_1 integrins in RPE plated on matrix-coated filters. E7 (a–d), E9 (e), or E14 (f) RPE was plated on Millicell-CM filters coated with various matrixes as described in Materials and methods. After 1 wk in culture, cells were prepared for cryosectioning and immunofluorescence using anti- β_1 -integrin monoclonal antibody. β_1 integrins were found only in the basal membranes of E7 RPE plated on Matrigel (a) but on the basal and lateral membranes of E7 RPE plated on laminin (b). When E7 RPE was plated on collagen IV (c), β_1 integrins were found in the basolateral membranes of most cells. When E7 RPE was plated on fibronectin (d), the distribution of β_1 integrins was nonpolarized. As for E7 RPE, when E9 (e) or E14 (f) RPE was plated on laminin, the β_1 integrins were found on basolateral membranes. Downward arrowheads, apical membrane; upward arrowheads, basal membrane; Bar, 10 μ m.

a requirement was demonstrated for the A chain of laminin and its receptor as antibodies to either protein inhibited the transition (Klein *et al.*, 1988; Sorokin *et al.*, 1990).

When applied to filters, Matrigel, laminin, collagen IV, and fibronectin all promoted the attachment and spreading of RPE. In their absence, only E7 cells would spread slowly along the filter and form an epithelial monolayer. This ability may be related to ability of this age cell to adhere to the choroid (Figure 2). Presumably, RPE on bare filters synthesized laminin, collagen IV, and fibronectin (Turksen *et al.*, 1984), and the slow rate of spreading reflected the time needed to produce a matrix. Although this presumptive matrix allowed the RPE to spread on the filter, it was inefficient in stimulating polarity as only 25% of the cells had a basolateral distribution of β_1 integrins—comparable with that of E7 RPE plated on the choroid.

After the initial formation of a RPE monolayer, Bruch's membrane continues to mature and membrane polarity of the RPE develops gradually. Two patterns of development were observed for the Na,K-ATPase and β_1 -integrin family (Rizzolo and Heiges, 1991). 1) A polarized distribution of the Na,K-ATPase was established in the central region (posterior pole) of the RPE by E7 but not until E11 in the periphery. This pattern parallels the development of the neural retina. Possibly, the distribution of the Na,K-ATPase is affected by interactions of the apical membrane with the neural retina or intervening subretinal matrix. Consistent with this hypoth-

esis, the current study demonstrated that neither Bruch's membrane nor matrix-coated filters induced a polarized distribution. 2) By contrast, the polarization of β_1 integrins proceeded at equal rates in the central and peripheral regions. The β_1 integrins were located in the apical and basolateral membranes on E7. On E9, cells with a basolateral polarity were intermixed with nonpolarized cells in the epithelium, whereas all cells were polarized on E11. By E16, the β_1 integrins were detected only in the basal membranes, as observed by Philp and Nachmias (1987) in E18 embryos. In this case, Bruch's membrane must play a role, because in the current study, E14-derived Bruch's membrane induced polarity in immature cells that are nonpolarized *in vivo*.

The observation *in vivo* that the distribution of β_1 integrins becomes polarized 5–7 d after the appearance of laminin and collagen IV and fibronectin in the basement membrane, together with the result that E14-derived Bruch's membrane could polarize E7-derived RPE, suggests that these matrix components alone should be unable to stimulate polarity. Surprisingly, only fibronectin was ineffective. Although there may be an inhibitory factor early in development, these artificial matrix coatings may misrepresent the role of these matrix components *in vivo*. There are multiple isoforms and post-translational modifications of these components, and it is unclear which of these are present in Bruch's membrane. In addition to their chemical properties, the three-dimensional

structure of the matrix, the concentration of its components, and its physical properties have been shown to affect epithelial or endothelial properties (Kubota *et al.*, 1988; Opas and Dziak, 1988; Ingber and Folkman, 1989; Opas, 1989). Furthermore, impurities in the matrix preparations could account for the activity. These considerations illustrate the value of using the natural basement membrane to investigate events in development.

Our findings conflict with those of Philp *et al.* (1990), who observed a nonpolarized distribution of β_1 integrins when primary cultures of chick embryonic RPE were plated on Bruch's membrane or matrix-coated filters. These authors cultured cells in a medium containing fetal calf serum in which the plating efficiency is low. Such conditions may select a subset of cells, such as relatively immature mitotic cells (Vielkind and Crawford, 1988). Furthermore, the cells are initially undifferentiated and must re-differentiate in an unnatural environment. Conceivably, these immature cells are unable to respond to a stimulus from Bruch's membrane. Alternatively, fetal calf serum may contain an inhibitory factor. We do not favor this alternative as once a confluent monolayer is established in serum-free medium, medium supplemented with fetal calf serum can be substituted without affecting our results (Rizzolo, unpublished observations). To circumvent these problems, we developed a serum-free medium that in combination with Matrigel yielded a high plating efficiency and rapidly differentiating cells. Despite these improvements, the monolayers were not polarized with respect to the Na,K-ATPase or the budding of vesicular stomatitis virus (Rizzolo, 1990).

During the time that the β_1 integrins become polarized in vivo, the Bruch's membrane continues to develop; morphological changes are observed up to E19 (François *et al.*, 1963). There are two general mechanisms how the Bruch's membrane might affect the polarity of the β_1 integrins. Matrix components may trap β_1 integrins in the basal membrane or induce changes in the cortical cytoskeleton that in turn confines the β_1 integrins in the basolateral membrane via interactions with the cytoplasmic domain of integrin. Alternatively, localization may be specified by the α subunit of integrin. Because the β_1 subunit interacts with many different α subunits, the changes in polarity observed in vivo may reflect changes in α -subunit expression (Albelda and Buck, 1990). We are currently investigating this possibility.

Interestingly, the ability of Matrigel to polarize the distribution of β_1 integrins does not extend to all basolateral proteins. In all epithelia examined, the G protein of vesicular stomatitis virus and viral budding are confined to the basolateral membrane (Rodriguez-Boulan and Sabatini, 1978; Gottlieb *et al.*, 1986). Bok *et al.* (1990) observed the same phenomenon in human RPE. However, we reported that virus budding was nonpolarized when E7 RPE monolayers were established on Matrigel-coated filters (Rizzolo, 1990). This observation further illustrates that the effects of the basement membrane on polarity are limited to a subset of proteins.

Materials and methods

Cell culture

RPE was isolated from E7, E9, or E14 white leghorn chicken embryos essentially as described by Hayashi *et al.* (1978). Briefly, the anterior structures of the eye and vitreous were removed, the neural retina stripped away, and the remaining choroid and RPE incubated in phosphate-buffered saline (PBS) containing 0.54 mM EDTA at 37°C for 30 min (E7) or 60 min (E9 and E14). After stripping the RPE from the choroid, the cells were dissociated in 100 μ g/ml trypsin, 60 μ g/ml DNase, and 120 μ g/ml Collagenase in PBS, washed with Dulbecco's modified Eagle's medium (DMEM) containing 10% NuSerum IV (Collaborative Research, Bedford, MA), and incubated for 2 h at 37°C in a polycarbonate tube containing a serum-free medium. This medium was DMEM supplemented with 6.3 μ g/ml insulin, 6.3 μ g/ml transferrin, 6.3 μ g/ml selenious acid, 1.25 mg/ml bovine serum albumin, 5.3 μ g/ml oleic acid, 6.6 ng/ml progesterone, 18.0 ng/ml hydrocortisone, 50 μ g/ml bovine pituitary extract (P-Neurex, Upstate Biotechnologies, Lake Placid, NY), and antibiotics (Rizzolo, 1990). During the incubation, the cell suspension was occasionally drawn up and down through a pipette. This procedure yielded single cells that were then plated on E14-derived Bruch's membrane or matrix-coated Millicell-CM filters (Millipore, Bedford, MA) at a density of 4.5×10^5 cells/cm².

Bruch's membrane in association with the choroid was isolated from E14 embryos. After the removal of the RPE as described above, Bruch's membrane and choroid were peeled from the sclera starting at the periphery and working toward the pecten. A scalpel was used to free the tissue from the pecten, and the resultant sheet of tissue was laid on a 0.45- μ m nitrocellulose filter (Sartorius Filters, Hayward, CA) with either the choroid or the Bruch's membrane adhering to the filter. In this way, RPE could be plated as described above directly on Bruch's membrane or as a control on the scleral side of the choroid. In some experiments, the Bruch's membrane/choroid alone was incubated in serum free-media for up to 1 wk. To examine the effects of Bruch's membrane alone, the Bruch's membrane/choroid was incubated for 1 h at room temperature in 0.25 M NH₄OH. After washing the tissue three times with PBS, RPE was plated as described above.

Millicell-CM filters were coated with Matrigel, collagen IV (Collaborative Research), laminin, or fibronectin (Upstate Biotechnologies). RPE is sensitive to the thickness and concentration of Matrigel. Monolayers will not grow to the edge of the filter if the matrix forms a meniscus. Confluent monolayers were consistently obtained when Matrigel was diluted

1:4 with DMEM and applied at 4°C by wetting the filters, draining the solution, and air drying them overnight. Mouse laminin (200 µg/ml), mouse collagen IV (0.7 µg/ml), and human fibronectin (20 µg/ml) were applied by adding 100 µl to 12-mm filters and allowing the matrix to air dry overnight. In each case, when RPE was plated as described above, confluent monolayers with a typical transepithelial electrical resistance of 20–25 Ω/cm² (Rizzolo, 1990) were obtained after 1 d in culture.

Immunofluorescence and electron microscopy

Immunofluorescent staining was performed on frozen thin sections (6 µm) of RPE grown on filters or Bruch's membrane/choroid. Samples were fixed with 3.0% paraformaldehyde in PBS for 10 min at room temperature, quenched for 10 min in PBS containing 50 mM ammonium chloride, infused overnight at 4°C with 1.5 M sucrose, and embedded and cryosectioned in OCT Compound (Miles, Elkhart, IN). Sections were incubated with a monoclonal antibody to the β subunit of the Na,K-ATPase (Fambrough and Bayne, 1983) or to the β₁ subunit of integrin (either JG22 [Greve and Gottlieb, 1982] or CSAT [Horwitz *et al.*, 1985]) followed by biotinylated anti-mouse IgG and then Texas-Red conjugated streptavidin (Amersham, Arlington Heights, IL). Between incubations, the sections were washed six times with PBS containing 0.2% gelatin. The sections were analyzed by epifluorescence using a Zeiss Axiovert 35 microscope with a 100× plan-neofluor infinity corrected objective (Zeiss, Thornwood, NY) and photographed using Kodak T-Max 3200 film (Kodak, Rochester, NY). For electron microscopy, samples were fixed in 2.5% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.2, and post-fixed in 1% osmium tetroxide, 0.1 M sodium cacodylate, pH 7.2. En bloc staining with uranyl acetate was followed by embedding in epon-araldite. Sections were stained with uranyl acetate and lead citrate.

Measurement of the transepithelial resistance

Transepithelial electrical resistance was measured at 25°C in *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid-buffered DMEM using a Millicell-ERS (Millipore). Because embryonic RPE is inherently leaky and the resistance measured in culture is low (see Discussion in Rizzolo, 1990), the precision of the measurements was increased by the following precautions. An electrode holder was used to ensure the distance separating the electrodes was constant and the position of the apical electrode was centered on the filter. The vertical distance between the tips of the basal and apical electrodes was also held constant and was set with the aid of a reference filter. Because of the variation in filters, the resistance of each sample filter and the reference filter was measured before plating the cells on the sample filters. In each subsequent measurement, the vertical offset of the electrodes was adjusted so that the measured resistance of the reference filter equaled its original value. Measurements represent the average of 5–6 filters.

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