Purpose. To determine the relative expression of cell cycle-associated proteins in human corneal and limbal epithelium and corneal endothelium in situ, to correlate staining patterns of cell cycle-associated proteins with the known proliferative status of corneal and limbal epithelial cells, and to determine the relative proliferative status of corneal endothelial cells in situ by comparing their staining patterns with those of corneal and limbal epithelial cells.

Methods. Corneas from donors 6 weeks and 17, 27, 37, 53, 66, and 67 years of age were preserved in Optisol, received on ice within 24 to 36 hours of death, and immediately fresh frozen. Transverse 6-μm corneal sections were prepared for indirect immunofluorescence localization using commercial antibodies that specifically recognize the following cell cycle-associated proteins: cyclins D, E, A, and B1; protein kinases p33cdk2 and p34cdc2; and Ki67, a marker of actively cycling cells.

Results. All cells of the corneal and limbal epithelium and corneal endothelium stained positively for protein kinases, p33cdk2 and p34cdc2, and for cyclin B1. Staining patterns for cyclins D, E, and A and for Ki67 differed depending on the relative proliferative status of the cells. Terminally differentiated, noncycling corneal epithelial suprabasal cells did not stain significantly for cyclins D, E, or A, or for Ki67. Some corneal epithelial basal cells showed nuclear staining, particularly for cyclin D and Ki67, indicating the presence of actively cycling cells in this regenerative cell layer. In peripheral corneal epithelium, the relative number of basal cells with positive cytoplasmic staining for cyclins D, E, and A increased with proximity to the limbus. Within this region, an occasional nucleus stained positively for Ki67. Limbal basal cells, which contain a slow-cycling stem cell population, stained positively for cyclins D, E, and A within the cytoplasm. Nuclear staining for cyclin D and Ki67 was observed in a few basal cells. Occasional cells within the suprabasal layers of the limbus stained positively for Ki67. The corneal endothelium, considered a nonrenewing population, exhibited staining patterns similar to those of limbal basal cells, except that in no specimen was Ki67 staining observed.

Conclusions. All corneal and limbal epithelial and corneal endothelial cells express protein kinases, p33cdk2 and p34cdc2, and cyclin B1. Relative staining patterns of the cell cycle-dependent proteins, cyclins D, E, and A, and of Ki67 acted as markers to distinguish terminally differentiated epithelial suprabasal cells that have exited the cell cycle, actively cycling epithelial basal cells, and slow-cycling limbal basal (stem) cells. Staining patterns of the corneal endothelium most closely corresponded to those of limbal basal cells, suggesting that endothelial cells are arrested in G1-phase and have not exited the cell cycle. Invest Ophthal Mol Vis Sci. 1996;37:645–655.

The cell cycle is driven by a family of closely related and highly regulated protein kinases, referred to as the "cyclin-dependent kinases." Kinases p33cdk2 and p34cdc2 are among the cyclin-dependent kinases whose synthesis is not cell-cycle dependent but which are only activated when bound by a specific cyclin.1 p33cdk2 is active primarily in G1-phase, whereas p34cdc2 is active mainly in G2- to M-phase of the cell cycle. Cyclins are regulatory proteins that undergo a cell cycle-dependent variation in concentration. Cyclins A and B were first identified in developing sea urchin2 and clam eggs3 as proteins that were synthesized, transported into the nucleus where they bound a specific protein kinase, and then rapidly degraded at specific stages of the cell cycle. Cyclin A is synthesized in late G1- to S-phase, whereas cyclin B synthesis peaks in G2-phase of the cycle.4 Cyclin A is degraded...
Within the limbal region of the epithelium is a slow-fusing cells and terminally differentiated cells similar to those found in the suprabasal layers of central corneal epithelium. Tissue culture studies of human corneal epithelial cells in situ, correlate staining patterns with those of corneal and limbal epithelial cells. Results of these studies showed that all corneal and limbal epithelial and corneal endothelial cells express protein kinases, p33cdc2 and p34cdc2, and cyclin B1. Relative staining patterns of the cell cycle-dependent proteins, cyclins D, E, and A, and Ki67 acted as markers to distinguish the cell cycle status of individual corneal and limbal epithelial and corneal endothelial cell populations.

**METHODS**

**Human Corneal Tissue**

Donor human corneas were obtained through National Disease Research Interchange (Philadelphia, PA). All corneas were preserved in Optisol (Chiron Vision, Irvine, CA) and were received on ice in the laboratory within 24 to 36 hours of death. Donor ages were 6 weeks, 17, 27, 37, 53, 66, and 67 years old. Criteria for exclusion of corneas for this study included history of endothelial dystrophy, presence of central guttae, low endothelial cell count, ocular inflammation or disease, diabetes, and glaucoma. On receipt in the laboratory, corneas were sectioned into quadrants, fresh frozen in Tissue-Tek OCT Compound (Miles, Elkhart, IN) and stored at −70°C until ready for cryostat sectioning.

**Immunocytochemical Localization**

The indirect immunocytochemical localization protocol was essentially that of Spurr and Gipson. Briefly, 6-μm transverse corneal sections were applied to formalin-fixed, gelatin-coated slides and air dried. All subsequent steps were carried out at room temperature. Slides were rinsed for 10 minutes in phosphate-buffered saline (PBS) and then incubated with 2% bovine serum albumin in phosphate-buffered saline to block nonspecific binding. Primary and secondary antibodies were prepared in the same blocking buffer. Primary antibody was applied to the tissue sections for vivo model for studies of mitotic regulation. Because the synthesis of specific cell cycle-associated proteins and their appearance in the nucleus is temporally regulated, the approach was taken in these studies to correlate specific cell cycle protein expression and location with relative cell cycle position in corneal cell populations of known proliferative capacity. Specifically, immunofluorescence localization was used to determine the relative expression of cell cycle-associated proteins in human corneal and limbal epithelium and corneal endothelium in situ, correlate staining patterns of cell cycle-associated proteins with the known proliferative status of corneal and limbal epithelial cells, and determine the relative proliferative status of corneal endothelial cells in situ by comparison of their staining patterns with those of corneal and limbal epithelial cells. Results of these studies showed that all corneal and limbal epithelial and corneal endothelial cells express protein kinases, p33cdc2 and p34cdc2, and cyclin B1. Relative staining patterns of the cell cycle-dependent proteins, cyclins D, E, and A, and Ki67 acted as markers to distinguish the cell cycle status of individual corneal and limbal epithelial and corneal endothelial cell populations.
1 hour in a moist chamber. Optimal antibody concentrations were determined empirically. After primary antibody incubation, sections were washed first with PBS and then with blocking buffer for 10 minutes. Fluorescein-conjugated anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG was used as secondary antibody, depending on the primary antibody source. Secondary antibody, diluted to 1:100, was applied to the sections for 1 hour in a moist chamber. Sections were then washed as above and mounted in para-phenylene-diamine-glycerol for visualization and photography using a Nikon UFX II photomicroscope (Donsanto, Natick, MA). Negative controls consisted of tissue sections incubated with secondary antibody alone and washed as above. Whenever purified antigen was available commercially, it was used to preadsorb its corresponding antibody. The preadsorbed antibody then acted as an additional negative control.

**Antibodies**

Rabbit polyclonal anti-human cyclin D, -cyclin E, -cyclin A, -p33cdk2, and -p34cdc2 were obtained from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal anti-human Ki67 antigen (clone MIB-1) was purchased from Zymed Laboratories (San Francisco, CA). Mouse monoclonal anti-recombinant cyclin B1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody specificity was confirmed by Western blots of sodium dodecyl sulfate extracts of SV-40 transformed human corneal endothelial cells (gift of Steven Wilson, MD, Cleveland Clinic Foundation, Cleveland, OH). All antibodies reacted with a single band of the correct molecular weight (data not shown).

**RESULTS**

Figure 1 gives a cross-sectional view of human cornea as a reference for the immunofluorescence studies. Moving from anterior to posterior, the cornea consists of a protective epithelial layer five to seven cells thick, an underlying basement membrane, an acellular collagenous layer termed Bowman’s membrane, a collagenous stroma interspersed with fibroblasts, a second thickened basement membrane termed Descemet’s membrane, and a single cell layered endothelium. Because cell density decreases with age, endothelial cells become enlarged and flattened, yielding an attenuated profile in corneal cross-sections. At the circumference of the cornea is the limbal region. The transition from corneal to limbal epithelium is demarcated by the absence of Bowman’s membrane in the limbus. Limbal epithelium contains more cell layers than the epithelium of central cornea and overlies a basement membrane and loose connective tissue containing blood vessels.

For the immunocytochemical localization studies, the relative staining intensity and subcellular localization for each antibody was determined in the central epithelium, peripheral epithelium (that portion of the epithelium proximal to the limbus that overlies Bowman’s membrane), limbal epithelium, and endothelium of corneas from 6-week-old and 17-, 27-, 37-, 53-, 66-, and 67-year-old donors. Table 1 summarizes the results. For the cell cycle-associated protein antigens studied, there were no observable age-related differences in either the relative intensity or localization pattern of antibody staining in any region of the cornea.

Tissue sections exposed to secondary antibody alone appeared similar, regardless of whether an antirabbit IgG or anti-mouse IgG was used. Sections exposed to antibody preadsorbed with its respective antigen followed by secondary antibody appeared similar to sections exposed to secondary antibody alone. There was a low level of nonspecific staining, particularly in the apical-most layers of the corneal and limbal...
epithelium. The corneal epithelial basal cell layer and several suprabasal cell layers, as well as the endothelial cell layer, were relatively unstained. Connective tissue underlying the limbal epithelium also tended to stain nonspecifically as seen in Figure 2C. Fresh frozen tissue sections, untreated and mounted for microscopy, revealed autofluorescence in the corneal epithelial basement membrane and in Descemet’s membrane overlying the endothelial monolayer. The anterior-most layer of Descemet’s membrane exhibited particularly strong autofluorescence. The autofluorescence observed in the two membranes did not significantly interfere with detection of positive antibody staining in either the epithelium or endothelium.

Figure 2 illustrates the staining patterns for p33cdk2, a protein kinase active in the G1-phase of the cell cycle. Positively stained nuclei were observed throughout the cell layers of central, peripheral, and limbal epithelium and in the endothelial monolayer. As seen in Figure 3A, the majority of basal and suprabasal cells in the central epithelium did not stain above background level for the early G1-phase cyclin D; however, positive cytoplasmic staining could be observed in an occasional basal cell. The relative number of basal cells with positive cytoplasmic staining for cyclin D increased in the corneal epithelium with increasing proximity to the limbus (Fig. 3B). In rare instances, a nucleus appeared positively stained for cyclin D in the basal cell layer of the central or peripheral epithelium (Fig. 3C). The cytoplasmic staining patterns in the central and peripheral epithelium, as seen in Figures 3A and 3B, suggest that, in some cases, basal cell bodies protrude into the suprabasal layer but remain associated with the basement membrane through somewhat elongated, stalk-like structures. Within the limbal epithelium, the cytoplasm of the vast majority of basal cells and of a relatively large number of suprabasal cells was stained positively for cyclin D (Figs. 3D, 3E); however, the relative intensity of cytoplasmic staining differed, particularly within the suprabasal cells. Positive nuclear staining for cyclin D was observed occasionally in limbal basal cells (Fig. 3E). Because of the thinness of the endothelium in most corneal specimens, it was difficult to determine unequivocally the subcellular localization of some proteins; however, examination of a number of specimens strongly suggested a cytoplasmic localization for cyclin D in corneal endothelial cells (Fig. 3F).

Little to no positive staining for the mid-to-late G1-phase cyclin E (Fig. 4A) was observed in either the basal or suprabasal layers of the central epithelium. The relative number of basal cells exhibiting positive cytoplasmic staining for cyclin E increased within the peripheral epithelium with increasing proximity to the limbus (Fig. 4B). Within the limbus, positive cytoplasmic staining was present in most, but not all, basal cells and in individual cells or small groups of cells in the suprabasal layers (Fig. 4C). In the endothelium, the staining pattern for cyclin E (Fig. 4D) suggested a nuclear localization.

Staining for cyclin A (Fig. 5), the late G1/S-phase cyclin, showed a level of staining intensity just above background level in the cytoplasm of the majority of cells within the central and peripheral epithelium, whereas some cells, particularly within the basal layer, appeared unstained (Fig. 5A). As was observed with cyclins D and E, the relative number of positively stained cells in the basal cell layer increased in the peripheral epithelium with increasing proximity to the limbus (Fig. 5B). The cytoplasm of the majority of limbal basal cells stained positively for cyclin A (Fig. 5C). The majority of limbal suprabasal cells stained

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**TABLE 1. Immunolocalization of Cell Cycle-Associated Proteins in Human Corneal Epithelium, Limbus, and Endothelium**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Proliferative Capacity</th>
<th>cdk2</th>
<th>Cyclin D</th>
<th>Cyclin E</th>
<th>Cyclin A</th>
<th>Ki67</th>
<th>cdc2</th>
<th>Cyclin B1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central epithelial basal cells</td>
<td>Rapid</td>
<td>N*</td>
<td>Majority+</td>
<td>Majority+</td>
<td>Majority §</td>
<td>Majority-</td>
<td>Few N</td>
<td>C</td>
</tr>
<tr>
<td>Central epithelial suprabasal cells</td>
<td>Nonproliferative</td>
<td>N</td>
<td>—</td>
<td>—</td>
<td>C§</td>
<td>—</td>
<td>—</td>
<td>C</td>
</tr>
<tr>
<td>Limbal basal cells</td>
<td>Slow-cycling</td>
<td>N</td>
<td>C</td>
<td>C</td>
<td>Majority C</td>
<td>Majority-</td>
<td>Rare N</td>
<td>Majority C§</td>
</tr>
<tr>
<td>Limbal suprabasal cells</td>
<td>Proliferative and nonproliferative</td>
<td>N</td>
<td>Few N</td>
<td>—/C</td>
<td>—/C</td>
<td>Majority C§</td>
<td>Few N</td>
<td>C</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>Nonproliferative</td>
<td>N</td>
<td>C</td>
<td>N</td>
<td>C</td>
<td>—</td>
<td>—</td>
<td>C</td>
</tr>
</tbody>
</table>

* Positive nuclear localization (N).
‡ No binding above background level.
§ Staining just above background level.

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Within the limbal epithelium, the cytoplasm of the vast majority of basal cells and of a relatively large number of suprabasal cells was stained positively for cyclin D (Figs. 3D, 3E); however, the relative intensity of cytoplasmic staining differed, particularly within the suprabasal cells. Positive nuclear staining for cyclin D was observed occasionally in limbal basal cells (Fig. 3E). Because of the thinness of the endothelium in most corneal specimens, it was difficult to determine unequivocally the subcellular localization of some proteins; however, examination of a number of specimens strongly suggested a cytoplasmic localization for cyclin D in corneal endothelial cells (Fig. 3F).
FIGURE 2. p33cdk2 is localized to the nucleus in all cells of central (A) and peripheral corneal epithelium (B) and limbal epithelium (C). Nuclei of corneal endothelial cells also stained positively (D). Arrow in (C) indicates nonspecific staining within the connective tissue underlying the limbal epithelium. Arrowhead in (D) indicates the autofluorescence of Descemet's membrane. Ep = epithelium; L = limbus; S = corneal stroma; En = endothelium. Magnification, ×400.

just above background. The cytoplasm of the attenuated endothelium stained positively for cyclin A (Fig. 5D).

Positive nuclear staining for Ki67 was observed in some cells within the central (Fig. 6A) and peripheral corneal epithelium (Fig. 6B) and within the limbal epithelium (Fig. 6C). Within the corneal epithelium, cells immediately adjacent to the basement membrane generally did not stain for Ki67, but the nuclei of some cells in the cell layer just above the basal layer were strongly positive. This location may reflect the position of basal cell bodies displaced into the suprabasal layer. There appeared to be no difference in the relative number of Ki67-positive basal cells in central versus peripheral epithelium. In the limbal epithelium, the majority of cells, including limbal basal cells, did not stain positively for Ki67; however, occasional positive nuclear staining was present in basal cells or in cells one to two layers above the basal layer. No positive staining for Ki67 was observed in the endothelium of any donor tissue examined (Fig. 6D), although the endothelial layer was present in all specimens as indicated by toluidine blue staining of representative corneal sections (data not shown).

Positive cytoplasmic staining for the G2/M-phase protein kinase, p34cdc2, was found in all layers of the corneal epithelium (Fig. 7A). There was no apparent difference in the relative staining intensity for this antigen in the basal and suprabasal cell layers. In limbal epithelium (Fig. 7B), the cytoplasm of basal cells appeared positively stained but with relatively less intensity than cells within the suprabasal layers. The cytoplasm of cells within the endothelial layer also showed positive, but low level, staining for p34cdc2 (Fig. 7C). Because of the attenuation of this cell layer, it was difficult to determine whether there may have been a low level of positive staining in the endothelial nuclei as well. Positive punctate nuclear staining for cyclin B1, the G2/M-phase cyclin, was observed in almost all cells and cell layers in the corneal and limbal epithelium and in the endothelial monolayer (Figs. 8A to 8C).

DISCUSSION

Together, results of these studies show that antibody staining and subcellular localization of cell cycle-asso-
FIGURE 3. Relative expression of cyclin D in central (A) and peripheral (B,C) corneal epithelium, limbal epithelium (D,E), and endothelium (F). Within the corneal epithelium, positive cytoplasmic staining increased in basal cells with proximity to the limbus. Note the positively stained stalk-like structures (arrows) in A and B, where basal cell bodies protrude into the suprabasal layer but remain attached to the underlying basement membrane. A nucleus, positively stained for cyclin D, is indicated by the arrow in C. In the limbal epithelium, basal cells and suprabasal cells proximal to the basal layer stained intensely for cyclin D, whereas only individual cells within the more anterior cell layers were stained intensely. Positively stained nuclei in limbal basal cells are indicated by the arrows in E. The cytoplasm of corneal endothelial cells stained intensely for cyclin D. Arrowhead in F indicates autofluorescence in the anterior portion of Descemet’s membrane. S = corneal stroma. Magnification, ×300.

Associated proteins can differentiate among cell populations with different proliferative status in intact tissue. As expected, both p33cdk2 and p34cdc2 protein kinases were expressed in all cell types because their synthesis is not cycle dependent. p33cdk2, which exhibits G1-phase activity, was located within the nucleus in both proliferative and nonproliferative cell populations, whereas p34cdc2, which exhibits predominantly G2- and M-phase activity, was detected within the cytoplasm of these populations. The cytoplasmic localization of p34cdc2 is consistent with the findings of Pines and Hunter in HeLa cells and human foreskin fibroblasts. In their immunocytochemical localization studies of synchronized cell populations, p34cdc2, as well as cyclin B1, were localized in the perinuclear region of the cytoplasm until just before nuclear envelope breakdown. A surprising finding was that cyclin B1, a regulator of p34cdc2 activity, showed punctate nuclear staining in all corneal tissues examined, regardless of proliferative state. Because synthesis of this cyclin is highly regulated during the cell cycle, positive punctate staining for cyclin B1 in the nuclei of cells throughout the cornea suggests that a non-cell-cyclodependent form of this protein may exist in these cells. In support of this idea is the finding that, in E6 embryonic chicken lens, cyclin B is located in the nuclei of postmitotic annular pad and fiber cells, suggesting that this kinase also may function in a noncell-cycle dependent manner.

Of the cell cycle-associated proteins studied, cyclins D, E, and A, and Ki67 produced differential staining patterns within corneal cells and helped distinguish among various proliferative states. The terminally differentiated, nonproliferative cells within the suprabasal layers of central epithelium did not stain positively for the G1-phase cyclins D or E, and only a very low level of cytoplasmic staining was observed for the late G1/S-phase cyclin A. No staining was observed for Ki67, the marker of actively cycling cells. A majority of basal cells within the central epithelium showed
similar staining patterns to those of suprabasal cells, indicating that, at any point in time, most basal cells within this cell layer are not actively proliferating. With the cell cycle-associated proteins studied, it was not possible to use relative protein expression or localization to distinguish between terminally differentiated suprabasal cells that have exited the cell cycle and basal cells in a quiescent, G0-like state, but capable of proliferation. The observation of positive nuclear staining, particularly for cyclin D and Ki67, in some basal cells within the corneal epithelium suggests that these cells represent a portion of the actively cycling population responsible for normal renewal of this tissue. The Ki67 staining pattern within the corneal epithelium is consistent with that observed in other epithelia. Basal cells in the proliferative zones of normal epidermis and in hair follicles show positive nuclear staining for Ki67, indicating the presence of actively cycling cells in these regions.25–27

A transition in cell cycle protein expression was observed in the basal cells of the peripheral epithelium suggestive of a transition in relative proliferative status in this region. With increasing proximity to the limbus, there was an increase in the number of cells that stained positively for cyclins D, E, or A. In peripheral epithelium, positive cytoplasmic staining was observed in clusters of basal cells and in cells whose cell bodies were located immediately above the basal layer but had connecting cytoplasmic “stalks” that made contact with the epithelial basement membrane, identifying them as partially displaced basal cells. This observation is similar to that of Lavker et al.,15 who observed 3H-thymidine incorporation in central corneal epithelial cells that were displaced partially into the suprabasal compartment but that retained attachment to the basement membrane by cytoplasmic stalks. As was seen in central epithelium, an occasional basal cell exhibited positive nuclear staining, particularly for cyclin D or Ki67, suggesting the presence of some actively cycling basal cells in this region.

The staining pattern for limbal basal cells clearly differed from that found in terminally differentiated suprabasal cells and from actively cycling basal epithelial cells. Unlike terminally differentiated suprabasal cells, the majority of limbal basal cells stained positively for cyclins D, E, and A; however, unlike actively cycling cells, most limbal basal cells exhibited cytoplasmic, rather than nuclear, staining for these cyclins and did not stain positively for Ki67. The heterogeneity of staining patterns in cells within the suprabasal layers of the limbus most likely reflects heterogeneity in the overall cell population in this region, which contains transient amplifying cells capable of rapid proliferation on appropriate stimulation and terminally differentiated cells that, like the suprabasal cells in central epithelium, have exited the cell cycle.

The staining patterns of the corneal endothelium were unexpected because these cells are considered to be nonproliferative and their staining patterns were expected to be similar to those of the terminally differentiated suprabasal cells in central epithelium. The positive staining for cyclins D, E, and A and the lack of positive staining for Ki67 was similar to that of limbal basal cells.
FIGURE 5. Cyclin A protein expression in central (A) and peripheral (B) corneal epithelium, limbal epithelium (C), and endothelium (D). The cytoplasm of most cells within the central and peripheral epithelium was stained only slightly above background levels. More intense staining was observed in basal cells with proximity to the limbus. Note the putative nuclear localization of cyclin A in the basal cell (arrow) in the peripheral epithelium (B). The cytoplasm of basal cells within the limbal epithelium was consistently positively stained. Suprabasal cells appeared to have a low level of positive staining. The cytoplasm of corneal endothelial cells stained positively for cyclin A. S = stroma. Magnifications, ×200 (A,B,D) and ×100 (C).

A surprising finding was the relatively large population of cells that express cyclins D, E, or A but that exhibit a cytoplasmic rather than a nuclear localization. This population was observed with increasing frequency in basal cells with proximity to the limbus, in the majority of limbal basal cells, and in corneal endothelial cells. In synchronized, serum-stimulated cultured cells, synthesis, nuclear appearance, and degradation of the cyclins is cycle dependent and relatively rapid. One explanation for the cytoplasmic localization of these cyclins is that in limbal basal cells and endothelial cells, cycle-dependent proteins may be synthesized and held in the cytoplasm awaiting a specific triggering mechanism to move them into the nucleus, thus permitting rapid proliferation in response to appropriate stimulation. The relative lack of cyclin protein expression in basal cells of the central epithelium suggests that they would take longer to enter the cycle on mitogenic stimulation because de novo synthesis of these proteins would be needed. Perhaps cytoplasmic localization of the G1-phase cyclins results from the nonsynchronous division of cells. In synchronized cultures of peripheral mononuclear blood leukocytes, Ki67 is synthesized and moves rapidly into the nucleus in mid-G1-phase; however, while cells begin to lose synchrony, Ki67 appears to be constantly present.12 This suggests that degradation of cell cycle proteins may not be as efficient or as rapid in nonsynchronized cells in situ or that, on completion of mitosis, cells may not reenter a G0-like quiescent phase before a new round of mitosis, resulting in the accumulation of cell cycle-dependent proteins in the cytoplasm. There also may be non-cell cycle-dependent functions for these proteins, which have yet to be defined.

Another interpretation for the gradual increase in the cytoplasmic expression of cyclins D, E, and A in basal cells from central to peripheral corneal epithelium is that, in humans, the proliferative rate differs, i.e., the relative number of population doublings per unit time may increase in basal cells with proximity to the limbus. This interpretation appears unlikely because 3H-thymidine pulse labeling of mouse and rab-

FIGURE 6. Ki67 protein expression was observed in the nuclei of a few basal cells within the central (A) and peripheral corneal epithelium (B). In limbal epithelium (C), an occasional basal cell showed positive nuclear staining (arrow, C). More positively stained nuclei were observed in the suprabasal layers of the limbus proximal to the basal layer than were seen in the basal cell layer. Note the lack of Ki67 staining in the corneal endothelial monolayer in (D). Ep = epithelium; L = limbus; En = endothelium; S = stroma. Magnification, ×330.
Figure 7. Intense cytoplasmic staining for p34cdc2 was observed in the cytoplasm of cells throughout the corneal epithelial layers (A) and suprabasal cells of the limbal epithelium (B). In limbal basal cells and endothelium (C), the cytoplasm of cells appeared less intensely stained. S = stroma. Magnification, ×400.

The bit cornea shows little, if any, difference in proliferative rate between limbal, peripheral, and central cornea. In addition, the relative number of basal cells with nuclear localization of Ki67, which acts as a marker for actively cycling cells, did not appear to differ significantly within central and peripheral cornea. An alternative explanation is that the increase in expression of cyclins D, E, and A in basal cells of peripheral cornea reflects a gradual change in the degree of differentiation in basal epithelial cells of the cornea and limbus. Within the limbal basal cell layer is a population of cells considered to be slow-cycling stem cells that exhibit a less differentiated phenotype than either limbal or corneal epithelial suprabasal cells or basal cells of central corneal epithelium. The observed transition in cyclin expression from limbal to central corneal epithelial basal cells is similar to the findings of Lauwereyns et al. They detected a transition zone in peripheral corneal epithelium that shares staining characteristics with limbal basal cells in their expression of α1β1-integrin, metallothionein, AE1, and transferrin receptor, and they suggested that these cells maintain some stem cell-like properties. If, indeed, cytoplasmic expression of the G1-phase cyclins reflects a difference in the relative degree of differentiation of the basal cell population, it also reflects documented differences in relative proliferative capacity between limbal and corneal epithelial basal cells, i.e., limbal basal cells are capable of significantly more

Figure 8. Punctate nuclear staining for cyclin B1 was observed in all cell layers of the corneal epithelium (A), limbal epithelium (B), and endothelium (C). S = stroma. Magnification, ×400.
population doublings than either transient amplifying cells or epithelial basal cells within central cornea.

Positive nuclear staining for Ki67 in corneal and limbal epithelial cells is consistent with the results of previous studies using 3H-thymidine to label corneal cells undergoing DNA synthesis. In situ immunocytochemical staining for Ki67 is similar to pulse-labeling with 3H-thymidine in that it reflects the proliferative status of cells at a particular point in time. Ki67 staining was observed in occasional basal cells within the central and peripheral corneal epithelium. This finding is consistent with the fact that individual cells within this layer undergo asynchronous cell division to replace cells lost from the epithelial surface. The relative lack of positive nuclear staining for Ki67 within limbal basal cells was expected and consistent with the slow-cycling nature of the stem cell population within this region. In previous studies, it was shown that limbal basal cells did not efficiently incorporate 3H-thymidine on pulse-labeling. In fact, even after exposure to 3H-thymidine for relatively long periods, it was difficult to label these cells efficiently. Because Ki67 expression normally is observed from the mid-G1 phase through the M-phase, it appears that the majority of limbal basal cells are in the G1-phase of the cycle at a point upstream from Ki67 synthesis. The similar expression and localization of the G1-phase cyclins and the lack of Ki67 staining in corneal endothelial cells suggests that they are arrested in early G1-phase.

In summary, we have used a novel approach to study corneal cell proliferation in vivo by correlating cell cycle-associated protein expression and proliferative status. Immunofluorescent localization of cell cycle-associated proteins in human corneal tissue sections showed that all cells express the noncycle-dependent protein kinases, p33cdk2 and p34cdc2, as well as a form of cyclin B1. The cell cycle-dependent proteins, cyclins D, E, A, and Ki67, acted as markers for identifying the proliferative status of cells within corneal cell populations. Lack of positive staining for these proteins indicated that epithelial suprabasal cells and a majority of corneal epithelial basal cells are not proliferating. Immunolocalization of the cell cycle proteins studied could not differentiate between terminally differentiated cells that have exited the cell cycle and quiescent cells that are capable of proliferation. Staining patterns for these proteins could distinguish actively cycling cells in the corneal and limbal epithelium. Both limbal basal cells and corneal endothelial cells appear to be arrested in the G1-phase of the cycle.

**Key Words**
cell proliferation, corneal endothelial cells, corneal epithelium, cyclins, Ki67

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**References**


