Regulation of a Rho-Associated Kinase Expression during the Corneal Epithelial Cell Cycle

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**PURPOSE.** It has been recognized that an increased expression of the Rho-associated kinase (ROCK-I), a downstream target of Rho (a Ras-related small guanosine triphosphatase [GTPase]), is associated with limbal-to-corneal epithelial transition. The purpose of the present study was to determine whether the expression of ROCK-I is regulated during the cell cycle of corneal epithelial cells.

**METHODS.** Rabbit corneal epithelial cells in culture were subjected to different culture conditions to enrich them in the G0, G1, and S phases of the cell cycle. Indirect immunofluorescence staining and western blot techniques were used for analyzing the changes in the relative intracellular concentrations of ROCK-I. Northern blot analysis of the isolated cellular RNA was performed to estimate the relative concentrations of ROCK-I mRNA.

**RESULTS.** Serum deprivation did not cause all the corneal epithelial cells in culture to be arrested in the G0 phase of the cell cycle. However, the cells could be arrested in G0 by treating them with culture medium supplemented with transforming growth factor (TGF)-β1. The relative concentration of ROCK-I in the G0-arrested cells was higher than in the corresponding control untreated cultures. G1-arrested cells were induced to enter G2, followed by the S phase of the cell cycle, by refeeding them with the medium devoid of TGF-β1. The total intracellular concentration of ROCK-I significantly decreased during the G1 phase of the cell cycle and increased again during the S phase. The decrease in intracellular ROCK-I during the G1 phase was confirmed by arresting the cells in G1 with isoleucine deprivation and thymidine-mimosine treatments. ROCK-I mRNA levels were also found to be decreased during the G1 phase of the cell cycle.

**CONCLUSIONS.** The levels of ROCK-I in the corneal epithelial cells were significantly lower in the G1 phase than those in the S and G0 phases of the cell cycle. Therefore, a Rho signaling pathway(s) involving ROCK-I may be regulated during the corneal epithelial cell cycle. The downregulation of ROCK-I during the G1 phase, at least in part, is due to the decreased levels of its mRNA. Based on these findings, ROCK-I may have a role in the progression of the cell cycle in the corneal epithelial cells as they migrate centripetally from the limbal to the corneal surface. (Invest Ophthalmol Vis Sci. 2001;42:933–940)

Corneal epithelium is a self-renewing tissue that is maintained by the centripetal migration of differentiated corneal epithelial cells derived from the stem cells located in the limbus. Differentiated corneal epithelial cells give rise to transient amplifying cells, which can undergo a limited number of cell divisions before following the pathway of terminal differentiation.1–3 Several phenotypic changes associated with corneal epithelial differentiation from the limbal epithelial cells have been identified.4–5 One of the phenotypic changes during limbal-to-corneal epithelial transition is an increased expression of a Rho-dependent kinase (ROCK-I), a downstream target of Rho.6–8 Rho, which activates ROCK, is a Ras-related family of small guanosine triphosphatases (GTPases) that function as molecular switches cycling between active GTP–bound forms to inactive guanosine diphosphate (GDP)–bound forms. Rho is well known for its involvement in the formation of actin stress fibers and focal adhesions.9–14 Other effects of Rho include regulation of gene expression, cellular proliferation, cellular transformation, and endocytic and exocytic pathways, which have been reviewed extensively.10–24 The activities of Rho appear to regulate diverse signaling pathways that are initiated by the activation of Rho-associated kinases and other effector proteins including rhohtein, citron, and p140mDi (for review see References 23,24). In the past few years, several isoforms of Rho-dependent kinases, including p160 ROCK,25,26 ROKα (ROCK-II),27,28 and ROKβ (ROCK-I)29 have been identified. They have been implicated in many different cellular processes, including cytoskeletal organization,30–31 regulation of morphology,32–35 adhesion and migration,36 cytokinesis,37 transcriptional activation of c-fos serum response element,38 insulin signaling,39 and cell growth and transformation.40–42 Differential expression of ROCK-I in the limbal and corneal epithelium suggests that the Rho signaling pathway involving ROCK-I may be important in regulating further differentiation (maturation) of corneal epithelial cells as they migrate from the limbal to the corneal surface.43,44 However, the functional significance of the increased expression of ROCK-I in corneal epithelial cells is currently not known.

In the present study, cultured corneal epithelial cells expressed ROCK-I; however, the levels of ROCK-I varied significantly in a nonsynchronously growing cell population consisting of cells in all different phases of the cell cycle. This finding suggests that ROCK-I expression may be regulated during the cell cycle of corneal epithelial cells. This is a report of the initial study to determine whether ROCK-I expression is regulated during different phases of the cell cycle in corneal epithelial cells.

**MATERIALS AND METHODS**

**Cell Culture**

Corneal epithelial primary cultures were derived from limbal explants from eyes of New Zealand White rabbits (Pel-Freez, Rogers, AK). Corneas with the adjacent limbus were excised from the eyes and used for growing the primary cultures (P0), in supplemental hormonal epithelial medium (SHEM).45 According to Ebaro et al.,46 cells in P0 were subcultured, using 0.25% trypsin-ENDTA (Gibco, Grand Island, NY), into 60-mm tissue culture dishes or four-well chamber tissue culture slides (Nalge–Nunc, Naperville, IL) at a density of 3 × 104 cells/cm2. After passage one (P1), cultures reached the desired confluence and were subjected to various treatments to arrest them in specific phases of the cell cycle. All the experiments were performed...
used for arresting keratinocytes in G0,44 consisted of first maintaining CA), supplemented with 0.1 ng/ml human epidermal growth factor, 5 serum-free keratinocyte basal medium (KBM; Clonetics, San Diego, the P1 cells in keratinocyte growth medium (KGM) consisting of

briefly with KGM, and new KGM was added. The medium was

cells to enter the cell cycle, the growth-arrested cells were washed

and the cultures were incubated for 48 to 52 hours. To restimulate the

medium was replaced with SHEM without thymidine for 10 hours. The medium was then replaced with SHEM supplemented with 400 μM mimosine, and the cultures were further incubated for 12 hours. To restimulate the cells, the medium was replaced with SHEM containing 10 μM deoxycytidine for 24 hours.

**Immunostaining**

Cultures grown in the chamber slides and treated as just described were rinsed three times with phosphate-buffered saline (PBS), fixed for 10 minutes with methanol that had been cooled to ~20°C, and then immunostained using an indirect immunofluorescence staining technique.8,9 The stained cells were viewed under a photomicroscope (BX60; Olympus, Tokyo, Japan) with fluorescence attachments and photographed with a digital camera. The digital images were processed with image analysis software (Photoshop; Adobe, San Jose, CA). The primary antibodies included, polyclonal goat anti-ROCK-I antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) against a peptide corresponding to amino acids 1318-1337 of ROCK-I, used at a 4 μg/ml concentration, and monoclonal anti-Ki-67 (a proliferative nuclear antigen) antibody (Zymed, South San Francisco, CA) used at the recommended dilution of 1:50. The secondary antibodies were fluorescein-isothiocyanate (FITC)-conjugated donkey anti-goat at 1:100 dilution and rabbit anti-mouse antibodies (ICN, Costa Mesa, CA) at 1:1800 dilution. Nonspecific staining was monitored by using 5 and 10 μg/ml of either goat or mouse IgG in place of ROCK-I or Ki-67, respectively. To determine the specificity of anti-ROCK-I antibody staining, the antibodies were preabsorbed with the peptide that was used as the immunogen before using it for the immunostaining of the cells. To estimate the percentage of cells in the S phase, the number of cells with Ki-67 nuclear staining were counted in three different fields containing 200 to 300 cells. For the double staining of Ki-67 and ROCK-I, the mixture of primary antibodies was used at the same concentrations, and the secondary antibodies consisted of a mixture of conjugated donkey anti-goat antibody (Alexa 488; Molecular Probes, Eugene, OR) at 1.2500 dilution and tetrarhodamine isothiocyanate (TRITC)-conjugated rabbit anti-mouse antibodies (Accurate Chemical and Scientific, Westbury, NY) at 1.200 dilution. The fluorescence images were collected using a confocal scanning laser system (Radiance 2000; Bio-Rad, Richmond, CA) attached to an inverted microscope (IX70; Olympus). The fluorescence images were constructed from sequential optical sections (0.5 μm) of the red and green fluorescence, through the entire thickness of the cells.

**Western Blot Analyses of ROCK-I**

Cultures grown in 60-mm dishes and treated as described earlier were extracted in RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM

![Figure 1](image1.png) **Figure 1.** Distribution of ROCK-I in corneal epithelial cells. Cultured corneal epithelial cells were fixed with methanol and immunoreacted with (A) goat anti-ROCK-I antibodies (K18) or (B) with K18 that was preabsorbed with the K18-peptide (the immunogen), followed by the secondary FITC-conjugated anti-goat antibody. Scale bar, 30 μm.

![Figure 2](image2.png) **Figure 2.** Double immunofluorescence staining of corneal epithelial cells for Ki-67 and ROCK-I. Corneal epithelial cells in culture were fixed with methanol and reacted with a mixture of goat anti-ROCK-I and mouse anti-Ki-67 antibody followed by a mixture of conjugated donkey anti-goat antibody (Alexa 488; Molecular Probes) and TRITC-conjugated rabbit anti-mouse antibodies. Immunostaining of ROCK-I (A) and Ki 67 (C) projected from the confocal microscopic images of the optical sections taken at every 0.5 μm through the entire thickness of the cells and staining of ROCK-I (B) projected from two consecutive optical sections taken through the planes of the nuclei. Scale bar, 30 μm.
monobasic sodium phosphate, and 150 mM NaCl (pH 7.4), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.03 trypsin inhibitor unit (TIU)/ml aprotinin (Sigma), 1 mM sodium orthovanadate, and 100 μg/ml phenylmethylsulfonyl fluoride [PMSF]) using the protocol recommended by the manufacturer (Santa Cruz Biotechnology). Briefly, the cells in the dishes were rinsed with cold PBS, and 0.2 ml of RIPA buffer was added per dish to lyse the cells. The cell lysate was scraped and collected, and the dishes were rinsed with an additional 0.1 ml RIPA buffer, which was mixed with the first lysate. The lysate was passed through a 21-gauge needle and then centrifuged at 10,000 g for 20 minutes at 4°C. The proteins in the supernatants were estimated using a micro BCA protein assay reagent (Pierce, Rockford, IL). Western blot analysis was performed to detect ROCK-I in the cell extracts, as described previously.8,9 Briefly, aliquots from each sample, containing 20 μg of protein, were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schull, Keene, NH). After treating the membranes with Blotto (Santa Cruz Biotechnology) to block the nonspecific binding sites, the blots were treated with anti-ROCK-I antibodies followed by peroxidase-conjugated anti-goat antibodies. The immunoreactive bands were detected using the chemiluminescence reagents (ECL reagent from Amersham, Arlington Heights, IL; or Super Signal West Femto reagent from Pierce), according to the manufacturers’ protocols. The relative differences in the chemiluminescence of the bands on the blots were measured by a phosphoimager (Bio-Rad) program to measure the intensities. Data are represented as mean ± SD.

**Northern Blot Analyses**

Total RNA was isolated from the corneal epithelial cells using a commercial extraction procedure (Trizol; Gibco) and subjected to Northern blot analysis.7 Equal aliquots of total RNA (20 μg) from different samples were electrophoresed through a 1% agarose-formamide gel, and the RNA bands were transferred to nitrocellulose membranes and hybridized with a cDNA probe (nucleotides corresponding to 847-1456 of ROCK-I), labeled with 32P using a random prime labeling kit (Pharmacia–Amersham, Piscataway, NJ). The RNA bands hybridized to the labeled probe and were visualized by exposing the blots to film (X-Omat; Kodak, Rochester, NY) with an intensifying screen at −80°C for a suitable length of time. The blot was stripped by a brief immersion in boiling water and rehybridized with 32P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) nucleotide probe. The intensities of the bands on the film were quantified by computer-assisted scanning densitometric analyses, and the densities of ROCK-I mRNA bands were normalized by comparing the densities of GAPDH mRNA bands.

**RESULTS**

A heterogeneous pattern of distribution of ROCK-I in the P1 culture of rabbit corneal epithelial cells was apparent from the immunofluorescence staining (Fig. 1A and 2A). The P1 cultures of corneal epithelium consisted of nonsynchronized populations of cells that were in different phases of the cell cycle. Twenty to 30% of the cells in confluent P1 cultures were in the S phase of the cell cycle, as was evident from the bright nuclear staining for Ki-67, a proliferative nuclear antigen, expressed during the S phase (Figs. 2C, 3B). To study whether there was any relationship between the proliferative state of the cells and ROCK-I expression, double immunostaining for ROCK-I and Ki-67 antigen was performed. The cells in the S phase (exhibiting Ki-67 nuclear staining) reacted more intensely for ROCK-I than other cells in the same cultures (Figs. 2C, 3B). However, some of the cells not in the S phase stained equally intensely for ROCK-I. Confocal microscopic analyses showed that ROCK-I staining was evident mostly in the cytoplasm of the cells. Negligible nuclear staining was seen in the optical sections at the plane of the nuclei (Fig. 2B).

To study the relative levels of ROCK-I expression during different phases of the cell cycle, corneal epithelial cells, arrested in different phases of the cell cycle, were analyzed by

**Figure 3.** Distribution of ROCK-I and Ki-67 (a proliferative nuclear antigen) in serum-starved corneal epithelial cells. (A, B) Corneal epithelial cells grown in the culture medium with serum; (C, D) equivalent cultures deprived of serum for 48 hours. The cultured cells were fixed with methanol and immunoreacted with (A, C) goat anti-ROCK-I antibody (K18) or (B, D) a mouse monoclonal antibody against Ki-67 followed by an FITC-conjugated anti-goat or rhodamine-conjugated anti-mouse IgG antibodies, respectively. Scale bar, 50 μm.
immunostaining and Western blot analyses of the cell extracts. Serum deprivation has been a widely used procedure to arrest cultured cells in G\textsubscript{0}/G\textsubscript{1}. However, when P1 cultures of rabbit corneal epithelial cells were serum deprived, not all the cells were arrested in G\textsubscript{0}/G\textsubscript{1}. Based on the nuclear staining for Ki-67, 25% to 30% of the cells were in the S phase after 48 hours of serum deprivation (Fig. 3D). The distribution of ROCK-I antigen in the serum-deprived cells did not differ significantly from that in the nonsynchronously growing corneal epithelial cells as seen in Figures 3C and 3A, respectively. TGF-β1 has been successfully used to arrest the growth of cultured keratinocytes in G\textsubscript{0}/G\textsubscript{1}, in a reversible manner. A similar approach was used to arrest P1 corneal epithelial cells in G\textsubscript{0}. Corneal epithelial cells were grown in KGM, and 17% to 26% of these cells were in the S phase (reacted with anti-Ki-67 antibody). The cells in KGM exhibited significantly different morphology and were larger in diameter than the cells grown in SHEM with serum.

The gross appearance of the ROCK-I staining pattern was also different in the cells grown in these two media (Figs. 1A, 4A). After P1 cultures were exposed to TGF-β1 for 52 hours, they showed development of surface protrusions. Only 0.5% to 1% of these cells reacted with anti-Ki-67 antibody (Fig. 4D), indicating that the majority of the cells were arrested in the G\textsubscript{0}/G\textsubscript{1} state. TGF-β1–treated cells showed significantly brighter staining for ROCK-I (Fig. 4C) than the nontreated, nonsynchronously growing control cells (Fig. 4A). To stimulate the TGF-β1–treated cells to progress to the G\textsubscript{1} phase of the cell cycle,
the culture medium was replaced with the KGM without TGF-β1. Cells, restimulated for 12 to 24 hours, did not show an increase in Ki-67–positive cells, indicating that the majority of the cells had not progressed beyond G1 (Fig. 4F). These cells exhibited a significantly weaker intensity of staining for ROCK-I (Fig. 4E) compared with the cells that were arrested in G0 (Fig. 4C). On further incubation of the cells for 72 to 96 hours, 60% to 70% of the cells began to express Ki-67 (Fig. 4I); however, the expression was not to the levels expressed in the proliferative cells in the control nontreated cultures (Fig. 4B), based on the intensities of nuclear staining. These cells that were restimulated for 72 to 96 hours expressed significantly higher levels of ROCK-I (Fig. 4H) than the cells in G1 (Fig. 4E). The decreased levels of ROCK-I in the cells that had entered G1 from G0 were confirmed by Western blot analyses of the cell lysates (Fig. 5I). Densitometric analyses, as summarized in Table 1, indicated that the relative intracellular levels of ROCK-I were increased by 20% ± 7% in the TGF-β1–treated cells (arrested in G0). Restimulation of TGF-β1–treated cells for 24 hours in TGF-β1–free KGM decreased the levels of ROCK-I by 65% ± 8%. Further incubation of the cells for 96 hours in KGM without TGF-β1 caused a 1.9-fold increase in the relative concentration of ROCK-I.

To confirm that the ROCK-I concentrations were reduced during the G1 phase of the cell cycle, cells arrested in the early G1 phase by isoleucine deprivation and in the G1/S phase by double thymidine and mimosine treatment were also analyzed. Although the former treatment reduced the number of Ki-67–positive cells from 20% to 30% to 2% to 5% (Figs. 6D, 6E), the latter treatment reduced the number of Ki-67–positive cells to 0.2% to 0.5% of the total cell population (Fig. 6F). The intensity of staining for ROCK-I was significantly less in isoleucine-deprived cells than that in the nontreated cells, and it was less in thymidine-mimosine–treated cells than isoleucine-deprived cells as shown in Figures 6A, 6B, and 6C. Western blot analyses of the cell lysates further confirmed that the concentration of ROCK-I in the isoleucine-deprived cells was reduced by 45% ± 7% (not shown) and thymidine-mimosine–treated cells by 65% ± 15% compared with the nonsynchronized, actively proliferating cells (Fig. 5I). When the thymidine-mimosine block was removed for 24 hours, the relative levels of ROCK-I increased by 30% ± 13%. The results of the changes in the relative levels of ROCK-I in corneal epithelial cells arrested in different phases of the cell cycle are summarized in Table 1.

To determine whether the decreased levels of ROCK-I protein during the G1 phase of the cell cycle were regulated by changes in the levels of mRNA, the relative levels of ROCK-I mRNA in the thymidine-mimosine–treated cultures and the nontreated cultures were compared by Northern blot analyses of total RNA extracted from these cells. Figure 7 shows the results from one representative experiment. The ROCK-I mRNA levels in the thymidine-mimosine–treated cultures were reduced to 18% ± 6% of the normal nontreated cultures. When the cells were stimulated by providing culture medium without thymidine and mimosine, the levels of ROCK-I mRNA were found to increase again to 50% ± 5% of the level of the proliferative cultures before their exposure to thymidine-mimosine.

**DISCUSSION**

To understand the mechanisms of corneal epithelial differentiation and maturation, it is important to know the significance of the phenotypic changes associated with them. We had previously reported that a significant increase in the expression of a Rho-associated kinase, ROCK-I, is one of the phenotypic changes that occurs after the limbal epithelial cells migrate over the corneal surface. The present study was performed to examine the involvement of ROCK-I in the process of corneal epithelial differentiation. ROCK-I is one of the several downstream targets of Rho, a Ras homologue of small GTPases. Rho has been known to be involved in the assembly of actin stress fibers and focal adhesions. In recent years, Rho has drawn a great deal of attention as a regulator of many other cellular processes, including cell cycle progression, transcription, nuclear signaling, endocytosis and exocytosis, and cellular transformation (for review, see References 10–24).
In addition to ROCK-I, several other downstream targets of Rho have been identified, which suggests that different Rho signaling pathways, involving distinct Rho targets, may regulate different cellular processes. ROCK-I and a closely related Rho-associated kinase, ROCK-II, have been implicated in several processes regulated by Rho (Fig. 8). The mechanism of the involvement of ROCK in many different processes is currently not known. ROCK-I has interesting structural features, including the kinase domain at its N terminus, a large coiled-coil domain in the middle, and a pleckstrin homology (PH) domain interrupted by a cysteine-rich zinc finger at its C terminus. The unique structure of ROCK-I probably enables it to interact with its downstream targets.

In addition to its unique structure, regulation of its expression, its intracellular translocation, and its posttranslational modifications may be some of the factors that are responsible for the regulation of ROCK-I activity in the cells. Whereas there is 90% homology between the catalytic domains of ROCK-I and ROCK-II, the coiled-coil domains and PH domains exhibit approximately 54% and 65% identities, respectively. The differential distribution of ROCK-I and not ROCK-II in the limbal and corneal epithelium (our unpublished observation) suggested that ROCK-I may have a more specific role in limbal-to-corneal epithelial transition. ROCK-I was expressed by the primary and passaged corneal epithelial cells in culture. However, these cultures, consisting of a nonsynchronized cell population, exhibited heterogeneity in the levels of intracellular ROCK-I. When the cells in different phases of the cell cycle were compared, the variations in the relative concentrations of ROCK-I suggested that its expression may be regulated during the cell cycle of corneal epithelial cells.

### TABLE 1. Relative Concentrations of ROCK-I in Corneal Epithelial Cells Arrested in Different Phases of the Cell Cycle

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell Cycle Phases</th>
<th>Ki-67 Positive Cells (%)</th>
<th>Relative ROCK-I Levels*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>All</td>
<td>20–30</td>
<td>1</td>
</tr>
<tr>
<td>Serum deprivation</td>
<td>All</td>
<td>25–30</td>
<td>1.1 ± 0.06</td>
</tr>
<tr>
<td>Control</td>
<td>All</td>
<td>22–27</td>
<td>1</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>G₀</td>
<td>0.5–1</td>
<td>1.2 ± 0.07</td>
</tr>
<tr>
<td>24-hour recovery</td>
<td>G₁</td>
<td>0.5–1</td>
<td>0.42 ± 0.09</td>
</tr>
<tr>
<td>72-hour recovery</td>
<td>S + G₁</td>
<td>60–70</td>
<td>0.8 ± 0.09</td>
</tr>
<tr>
<td>Control</td>
<td>All</td>
<td>20–30</td>
<td>1</td>
</tr>
<tr>
<td>Isoleucine deprived</td>
<td>G₁</td>
<td>2–5</td>
<td>0.55 ± 0.07</td>
</tr>
<tr>
<td>Thymidine-mimosine</td>
<td>G₁</td>
<td>0.2–0.5</td>
<td>0.35 ± 0.15</td>
</tr>
<tr>
<td>24-hour recovery</td>
<td>S + G₁</td>
<td>25–35</td>
<td>0.60 ± 0.13</td>
</tr>
</tbody>
</table>

*The relative levels of intracellular ROCK-I were determined by densitometric analysis of the Western blots of the cell-extracts.

In addition to ROCK-I, several other downstream targets of Rho have been identified, which suggests that different Rho signaling pathways, involving distinct Rho targets, may regulate different cellular processes. ROCK-I and a closely related Rho-associated kinase, ROCK-II, have been implicated in several processes regulated by Rho (Fig. 8). The mechanism of the involvement of ROCK in many different processes is currently not known. ROCK-I has interesting structural features, including the kinase domain at its N terminus, a large coiled-coil domain in the middle, and a pleckstrin homology (PH) domain interrupted by a cysteine-rich zinc finger at its C terminus. The unique structure of ROCK-I probably enables it to interact with its downstream targets.

In addition to its unique structure, regulation of its expression, its intracellular translocation, and its posttranslational modifications may be some of the factors that are responsible for the regulation of ROCK-I activity in the cells. Whereas there is 90% homology between the catalytic domains of ROCK-I and ROCK-II, the coiled-coil domains and PH domains exhibit approximately 54% and 65% identities, respectively. The differential distribution of ROCK-I and not ROCK-II in the limbal and corneal epithelium (our unpublished observation) suggested that ROCK-I may have a more specific role in limbal-to-corneal epithelial transition. ROCK-I was expressed by the primary and passaged corneal epithelial cells in culture. However, these cultures, consisting of a nonsynchronized cell population, exhibited heterogeneity in the levels of intracellular ROCK-I. When the cells in different phases of the cell cycle were compared, the variations in the relative concentrations of ROCK-I suggested that its expression may be regulated during the cell cycle of corneal epithelial cells.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Distribution of ROCK-I and Ki67 (a proliferative nuclear antigen) in the corneal epithelial cells arrested in the G₁ phase of the cell cycle. Cultured corneal epithelial cells were fixed with methanol and immunoreacted with (A, B, and C) goat anti-ROCK-I antibody (K18) or with (D, E, and F) the mouse monoclonal antibody against Ki67, followed by FITC-conjugated anti-goat and rhodamine-conjugated antimouse antibodies, respectively. (A, D) Control nontreated cells; (B, E) isoleucine-deprived cells; and (C, F) thymidine-mimosine-treated cells. Scale bar, 40 μm.
This hypothesis was tested in the present study by comparing the levels of ROCK-I in corneal epithelial cells arrested in different phases of the cell cycle using the procedures that have been previously used for other types of cells. In actively dividing corneal epithelial cultures, between 20% and 30% of the cells were in the S phase of the cell cycle. Serum deprivation, a widely used technique, did not cause the decrease of the relative percentage of the cells in the S phase. A similar observation has been reported for keratinocytes in culture.44 The serum deprivation did not affect the distribution pattern but showed a slight increase in the expression levels of ROCK-I. TGF-β1 induced the growth arrest of corneal epithelial cells in G₀, and the total level of ROCK-I in these cells was increased compared with the levels in the nonsynchronously growing cells. When the G₀-arrested cells were allowed to progress to the G₁ phase by withdrawing TGF-β1, the levels of ROCK-I in the cells decreased significantly.

As the cells progressed through G₁ and entered the S phase again, the levels of ROCK-I increased but not to the same level as that of the control cultures that were not treated with TGF-β1, perhaps due to the terminal differentiation of some of the cells in these cultures. Nonetheless, these findings indicate that ROCK-I expression was significantly decreased in corneal epithelial cells in the G₁ phase compared with the cells in the S and G₀ phases of the cell cycle. This was further confirmed by analyzing epithelial cells arrested in the G₁ phase using two additional techniques including, thymidine-iminosine treatment and isoleucine deprivation. The levels of mRNAs were also significantly decreased during the G₁ phase, indicating that the changes in the expression of ROCK-I during the cell cycle may be regulated, at least in part, by the regulation of the levels of mRNA encoding ROCK-I.

A reported study48 indicates that the limbal epithelial cells are arrested in the G₁ phase of the cell cycle. Therefore, low levels of ROCK-I in the limbal epithelial cells in vivo may be associated with the G₁ phase of the cell cycle. The increased expression of ROCK-I in corneal epithelial cells in vivo may be involved with the progression of the cell cycle. Although the present findings indicate that the Rho signaling pathway involving ROCK-I may be regulated during the epithelial cell cycle (Fig. 8), further investigation will be necessary to determine whether ROCK-I is involved in the regulation of the cell cycle.

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