



Growth factor induced activation of Rho and Rac GTPases and actin cytoskeletal reorganization in human lens epithelial cells

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Purpose: To determine the involvement of the Rho GTPases-mediated signaling pathway in growth factor-stimulated actomyosin cytoskeletal organization and focal adhesion formation in lens epithelial cells.

Methods: Serum starved human lens epithelial cells (SRA01/04) were treated with different growth factors including epidermal growth factor (EGF), basic-fibroblast growth factor (b-FGF), platelet derived growth factor (PDGF), transforming growth factor β (TGF- β), insulin-like growth factor 1 (IGF-1), lysophosphatidic acid (LPA), and thrombin. Growth factor stimulated activation of Rho and Rac GTPases were evaluated by GTP-loading pull-down assays. Changes in actin cytoskeletal organization and focal adhesions were determined by fluorescence staining using FITC-phalloidin and anti-vinculin antibody/rhodamine-conjugated secondary antibody, respectively. Fluorescence images were recorded using either confocal or fluorescence microscopy.

Results: Rho GTPase activity was significantly augmented in human lens epithelial cells treated with EGF, b-FGF, TGF- β , IGF-1, and LPA. Rac GTPase activation, in contrast, was significantly enhanced in response to only EGF or b-FGF. Serum starved human lens epithelial cells exhibited a strong increase in cortical actin stress fibers and integrin-mediated focal adhesions in response to b-FGF, PDGF, TGF- β , thrombin, and LPA. While EGF induced a striking increase in membrane ruffling and a marginal increase on focal adhesion formation, IGF-1 had no effect on either. Pretreatment of lens epithelial cells with C3-exoenzyme (an irreversible inhibitor of Rho-GTPase), lovastatin (an isoprenylation inhibitor), or the Rho kinase inhibitor Y-27632 abolished the ability of the different growth factors to elicit actin stress fiber and focal adhesion formation. EGF induced membrane ruffling, however, was not suppressed by Y-27632 and C3-exoenzyme.

Conclusions: These results demonstrate that different growth factors induce actin cytoskeleton reorganization and formation of cell-ECM interactions in lens epithelial cells and this response of growth factors appears to be mediated, at least in part, through the Rho/Rho kinase-mediated signaling pathway. The ability of growth factors to trigger activation of Rho and Rac GTPases along with actomyosin cytoskeletal reorganization and formation of focal adhesions might well play a crucial role in lens epithelial cell proliferation, migration, elongation and survival.

Lens epithelial cells at the annular zone of the anterior epithelium proliferate, migrate and progressively withdraw from the cell cycle and differentiate into fiber cells. Unlike epithelial cells, which are cuboidal in shape, lens fiber cells possess ribbon-like elongated morphological structures, which are several times the length of the epithelial cells. During fiber cell elongation and differentiation, the cytoskeleton and its associated proteins conceivably reorganize to generate a ribbon-like cell shape [1-11]. Further, crystallin gene expression is initiated and upregulated following these morphological changes [1-3]. Therefore, it is possible that growth factors and hormones, which are known to influence and regulate lens epithelial cell proliferation and differentiation, might exert their effects via pathways controlling cytoskeletal and morphological events associated with these cellular processes [1,2].

The actin cytoskeleton mediates a wide spectrum of cellular functions providing the structural framework upon which cell shape, cell migration, polarity, cytokinesis, adhesion com-

plex formation, and several other cellular events are dependent [12,13]. The cytoskeleton is regarded as one of the primary targets for growth factor action and mediates several cellular responses to extracellular cues [12-16]. Several investigators have debated and investigated the possible involvement of cytoskeletal reorganization in lens epithelial cell elongation and differentiation. However, at present, there is no concrete understanding of the intracellular mechanism(s) linking these events [1-11].

The Rho subfamily of small GTPases (Rho and Rac) are recognized to play a critical role in the regulation of actomyosin cytoskeletal organization, cell adhesion, and cell motility. They are also involved in transcriptional regulation, cell cycle progression, and cell survival [12-16]. We have previously profiled, the expression and distribution of small GTP binding proteins in the lens and demonstrated that inactivation of Rho GTPase in lens epithelial cells and in organ cultured lenses leads to altered cell morphology and cataractogenesis, respectively [17-20]. Here we hypothesize that Rho GTPases (Rho and Rac) play an important role in modulation of cytoskeletal dynamics in lens epithelial cells in response to growth factors and thereby influence lens epithelial cell migration, proliferation, and survival. Although a great deal of work has been

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done to implicate growth factors in mediating lens epithelial cell proliferation and differentiation [1-3,21,22], growth factor effects on actin cytoskeletal organization in lens cells has not been explored. In particular, participation of signaling pathways mediating such effects is not known. Therefore, in this study, we have investigated the effects of growth factors on Rho and Rac GTPase activation, actomyosin cytoskeletal organization, and formation of focal adhesions in human lens epithelial cells. The data reported here demonstrate that growth factors stimulate Rho and Rac GTPases activities and cause cytoskeletal reorganization via Rho and Rac GTPase-mediated signaling pathways.

METHODS

Materials: Transformed human lens epithelial cells (SRA01/04) characterized by Ibaraki, et al. [23] were used in this study. Human recombinant EGF, IGF-1, b-FGF, PDGF, TGF- β , and LPA were procured from Sigma-Aldrich (St. Louis, MO). Human thrombin was purchased from CalBiochem (San Diego, CA). Rho kinase inhibitor Y-27632 [(+)-R-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexanecarboxamide] and lovastatin were obtained from Welfide Corporation (Osaka, Japan) and Merck Pharmaceuticals (Rahway, NJ), respectively. C3-exoenzyme was purchased from Cytoskeleton, Inc. (Denver, CO). All the antibodies used were from Sigma-Aldrich. Assay kits used to determine activation of Rho and Rac GTPases were procured from Upstate Biotechnology (Lake Placid, NY).

Cell Cultures: Human lens epithelial cells (SRA01/04) were cultured at 37 °C under 5% CO₂ in Dulbecco's Modified Eagles Medium (Gibco BRL, Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum, penicillin (100 U/

ml), streptomycin (100 μ g/ml) and 20 μ g/ml gentamicin.

Rho/Rac activation assay: Human lens epithelial cells were grown to confluence in culture dishes in complete media containing 10% FBS. The serum content in the medium was dropped gradually from 10% to 5% within a time interval of 24 h, and later cells were maintained in 1% serum for 24 h. Cells were then treated with one of the following in the absence of serum; thrombin (1.0 U/ml), LPA (5 μ g/ml), or different growth factors (EGF, b-FGF, IGF-1, PDGF, or TGF- β at a concentration of 20 ng/ml) for a period of either 10 min or 1 h. Treated cells were washed with phosphate buffered saline (PBS) and lysed with Mg²⁺ lysis buffer (MLB) containing 10% glycerol, 10 μ g/ml each of leupeptin and aprotinin, and with the phosphatase inhibitors NaF (25 mM) and Na₂VO₃ (1 mM). Cell lysates were precleared at 14,000 RPM for 5 min at 4 °C and protein concentration was determined by the Bio-Rad protein assay. Lysate from each of the treatments was incubated either with Rhotekin-Rho binding domain-agarose slurry (25 μ g) or PAK-binding domain tagged agarose (10 μ g), by gently rocking at 4 °C for 1 h (as per the manufacturer's instructions). The agarose beads were then washed three times with MLB and suspended in 2X Laemmli sample buffer. The GTP bound form of Rho and Rac were detected by western blot analysis using monoclonal Rho antibody and polyclonal Rac antibody, respectively. Equal amounts of cell lysate protein from untreated cells were incubated either with GTP γ S or with GDP, which serve as positive and negative controls, respectively. Immunostained protein bands were quantified by densitometric analysis using NIH image software (National Institutes of Health, Bethesda, MD). Data obtained from three independent experiments were utilized for this analysis. Densitometric values (arbitrary units) were analyzed using the paired t-test to test differences between control and treated samples.

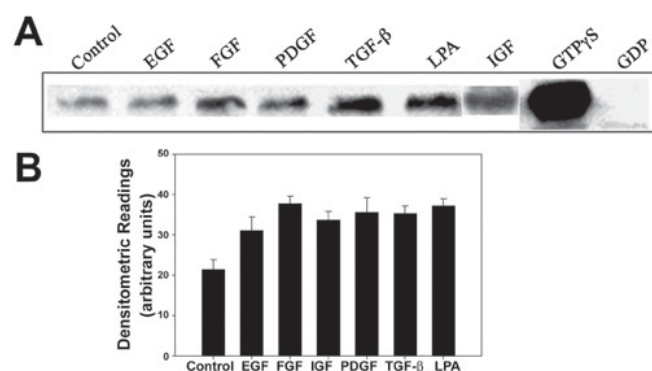


Figure 1. Growth factor induced activation of Rho GTPase in human lens epithelial cells. Serum starved human lens epithelial cells treated with different growth factors and LPA exhibited strong activation of Rho GTPase. **A:** A representative picture of western blotting of Rho GTPase activation induced by different growth factors. All the growth factors including LPA used in this study significantly induced activation of Rho GTPase (**B**). GTP γ S and GDP treatment served as positive and negative controls, respectively, for the pull-down assays. As expected, Rho GTPase was not pulled down in the presence of GDP, confirming the specificity of the assay for the active GTP-bound form of Rho GTPase. Densitometric values were mean of three independent experiments.

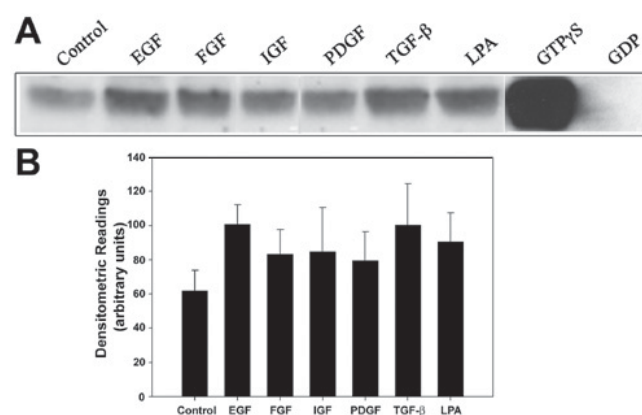


Figure 2. Rac GTPase activation induced by different growth factors in lens epithelial cells. Various growth factors tested in this study exert a definite activation of Rac GTPase in lens epithelial cells as shown in **B**, however with mean values of three experiments only EGF and FGF confirmed significant activation as compared to untreated cells. A representative western blot of Rac GTPase activation induced by different growth factors is shown in **A**. GTP γ S and GDP treatment served as positive and negative controls, respectively, for the pull-down assays as in the case of Rho GTPase activation assay.

Immunostaining and fluorescence microscopy: Cells were grown to semi-confluence on glass cover slips coated with 2% gelatin in complete media containing 10% FBS. Serum concentration was dropped to 1% and maintained for 24 h. Cells were then treated with different growth factors in the absence of serum as described above for 1 h and then fixed for cytoskeletal staining. In a second set of experiments, cells were pretreated with different inhibitors of Rho GTPase to assess the involvement of this small GTPase in growth factor-mediated cytoskeletal events in human lens epithelial cells. Following pretreatment with C3-exoenzyme (10 μ g/ml for 48 h), lovastatin (20 μ M for 18 h), or the Rho kinase inhibitor Y-27632 (5 μ M for 20 min), cells were treated with the appropriate growth factors for 1 h, after which they were fixed for cytoskeletal staining.

After the above treatments, cells were fixed in 3.7% formaldehyde in PBS (v/v) for 15 min and stored in serum containing buffer (10% FBS in PBS with 0.02% sodium azide) at 4 °C until further processing. For immunostaining, cells were initially washed in cytoskeletal buffer (10 mM MES [2-N-morpholino-(ethanesulfonic ether) N,N,N,N-tetra acetic acid], 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$, and 5 mM Glucose, pH 6.1) and then permeabilized in 0.1% Triton X-100 in PBS for 15 min, blocked in serum buffer (10% FBS in PBS with 0.02% sodium azide), and finally labeled for actin stress fibers and focal adhesions as described previously [19,24]. Briefly, cells were incubated with either rhodamine-phalloidin (500 ng/ml in serum buffer for 45 min) to stain for F-actin, or with primary antibody to vinculin (Sigma Aldrich) to detect focal adhesions. Secondary antibody conjugated to FITC

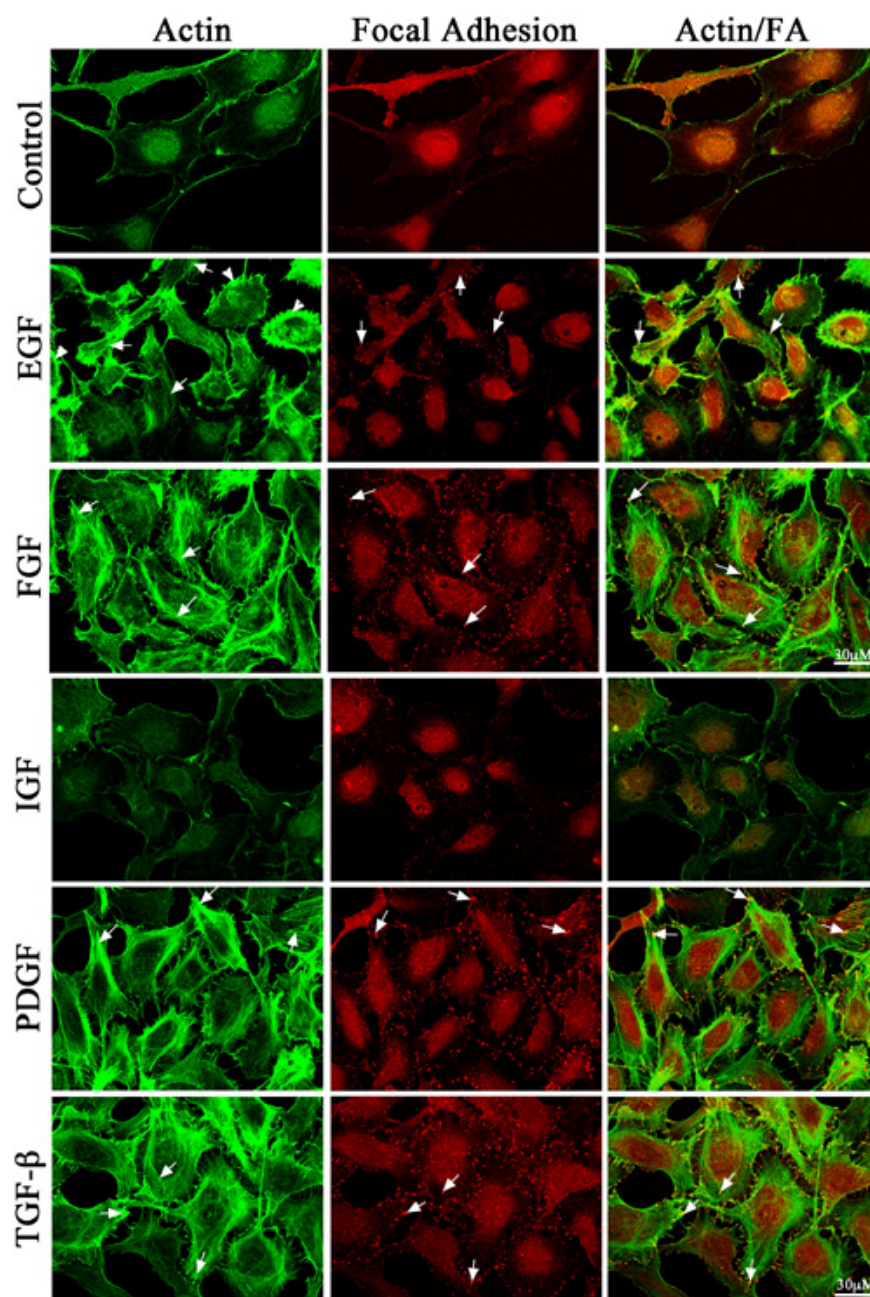


Figure 3. Growth factor-induced actomyosin reorganization and focal adhesion formation. Treatment of semi-confluent cultures of human lens epithelial cells maintained under serum deprivation with different growth factors (at a concentration of 20 ng/ml) led to reorganization of the cellular actin cytoskeleton. Addition of b-FGF, PDGF, and TGF- β induced very strong actin stress fiber formation (FITC-phalloidin staining indicated with arrows), which were localized predominantly to the cortical regions of the cell body indicated with arrows. EGF induced membrane ruffles (indicated with arrow heads) along with weak transverse actin stress fiber formation (indicated with arrows). Stimulation with b-FGF, PDGF, and TGF- β induced very intense focal adhesions, as shown by the arrows (middle panel), whereas EGF caused a marginal increase in focal adhesions compared to untreated controls. Interestingly, unlike other growth factors, addition of IGF-1 caused no obvious change in actin cytoskeletal organization or focal adhesion formation. Images in the third panel from left, depict the costaining of actin stress fibers (green) and focal adhesions (yellowish-orange indicated with arrows) induced by different growth factors. As shown by the arrows, increased actin stress fibers at the leading edges of cell membranes are associated with increased focal adhesion formation. In addition, cells treated with FGF, PDGF, and TGF- β undergo noticeable changes in cell shape, appearing rigid with increased cortical actin stress fiber formation, as compared to untreated cells. Bars indicate the magnification.

(fluorescein isothiocyanate) was then used in the final detection step for focal adhesions. Micrographs were captured using a Zeiss Axioplan-II fluorescence microscope.

Confocal microscopy: For double labeling actin stress fibers and focal adhesions, lens epithelial cells treated with growth factors were fixed, blocked and incubated with anti-vinculin antibody and subsequently probed with a rhodamine-conjugated anti-mouse secondary antibody along with phalloidin tagged FITC (fluorescein isothiocyanate isomer). Photographs were captured using a confocal microscope (Zeiss, LSM 410).

RESULTS

Pull down assays were conducted to study the effects of growth factors on activation of Rho and Rac GTPases in human lens epithelial cells. These assays utilized the Rho binding domain of Rhotekin and the Rac binding domain of PAK as affinity reagents to capture activated Rho GTPase and Rac GTPase, respectively. Treatment of lens epithelial cells with different growth factors for 1 h led to significant stimulation of Rho GTPase activation, yielding increases of 45% ($P < 0.005$) with EGF, 76% ($P < 0.008$) with b-FGF, 57% ($P < 0.05$) with IGF-1, 66% ($P < 0.002$) with PDGF, 65% ($P < 0.019$) with TGF- β , and 74% ($P < 0.003$) with LPA over corresponding control cells. Figure 1A depicts a representative western blot for the activation of Rho GTPase by different growth factors, while the histograms in Figure 1B show the mean value of densitometric readings obtained from 3 such independent experiments. EGF, TGF- β , and LPA elicited an early Rho GTPase activation (within 10 min of growth factor addition to cells, data not shown), which was sustained for up to 1 h post-addition. b-FGF and PDGF, in contrast, supported a mild activation of Rho GTPase at 10 min post-addition. In the case of Rac GTPase, all the growth factors evaluated in this study supported slight increases in activation at the early time point (10 min, data not shown), with EGF (63%), b-FGF (35%), IGF-1 (37%), PDGF (28%), TGF- β (62%) and LPA (42%) stimulated cells exhibiting a definite increase in Rac activity over the serum starved control cells at the 1 h time point (Figure 2). The noted increases in Rac activation were found to be statistically significant only in the case of EGF ($P < 0.0025$) and b-FGF ($P < 0.005$), based on the mean values from three independent experiments.

Confocal microscopic analysis was performed to localize actin stress fibers and focal adhesions in lens epithelial cells by double labeling for F-actin and vinculin with FITC-labelled phalloidin and Rhodamine-conjugated secondary antibody for vinculin, respectively. While human lens epithelial cells, maintained in 1% serum for 24 h, exhibited no prominent actin stress fibers or focal adhesions (Figure 3, control), treatment with b-FGF, PDGF, and TGF- β evoked strong increases in formation of actin stress fibers and focal adhesions (Figure 3), while IGF-1 had very little effect on cytoskeletal organization in human lens epithelial cells. Unlike in the case of TGF- β , PDGF and b-FGF stimulation with EGF elicited formation of weak transverse actin stress fibers but pronounced strong membrane ruffling with increased focal adhesions (ar-

rowheads indicate membrane ruffles in Figure 3). As can be seen in Figure 3 (third panel from left) with double labeling for actin (FITC staining) and vinculin (rhodamine staining), an association was revealed between the increased actin stress fibers at cortical regions with increased focal adhesion formation (bright yellow-orange focal points indicated with arrows) in cells treated with b-FGF, PDGF, and TGF- β . Furthermore, lens cells with increased actin stress fibers and focal adhesions induced by b-FGF, PDGF and TGF- β exhibit changes in cell morphology (increased cortical rigidity) as compared to control cells, indicating that growth factors influence cell shape in lens epithelial cells through reorganization of the actin cytoskeleton (Figure 3).

Both LPA and thrombin are known to signal through Rho GTPase-mediated pathways to effect cytoskeletal reorganization [12,13,25,26]. LPA in particular, is an important bioactive

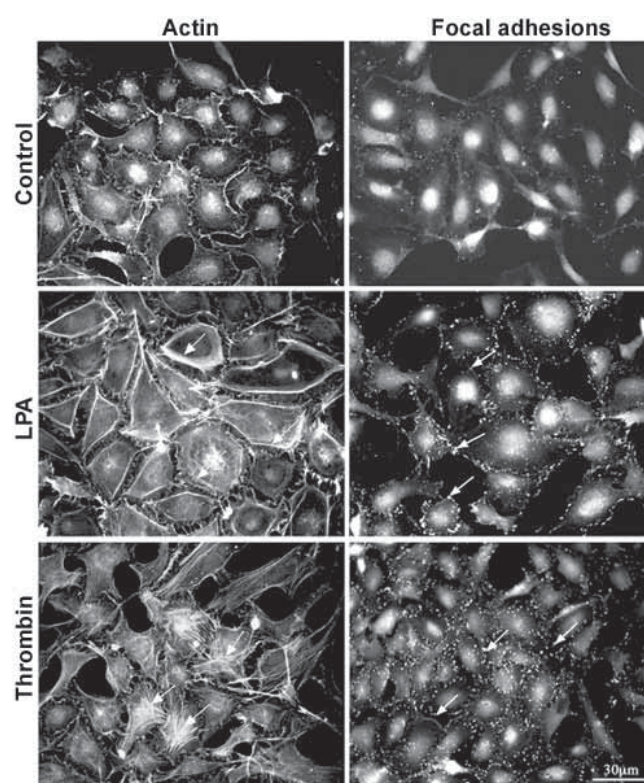
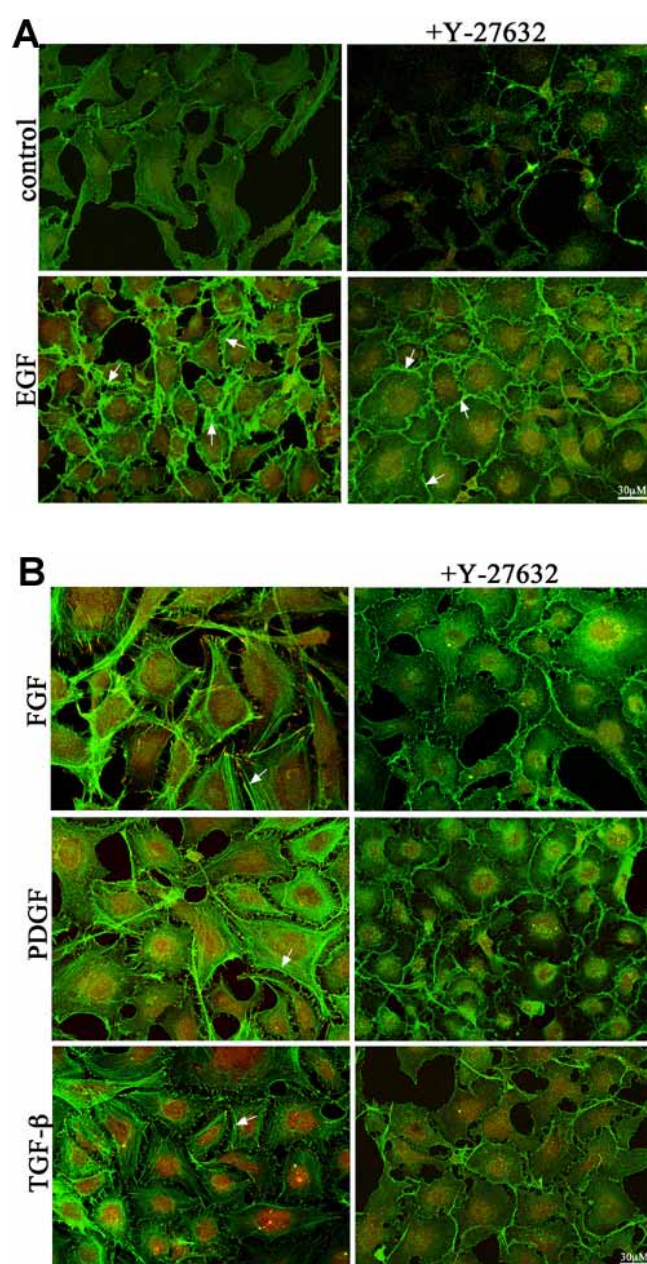


Figure 4. LPA and Thrombin induced cytoskeletal changes. Human lens epithelial cells maintained in 1% serum for 24 h were treated for 1 h with either LPA (5 μ g/ml) or thrombin (1 Unit/ml) in the absence of serum, then fixed and stained for detection of actin stress fibers and focal adhesions, as described in methods. LPA and thrombin both induced formation of actin stress fibers (indicated with arrows in the left panel) and focal adhesions (indicated with arrows in the right panel), however, as compared to thrombin, LPA induced very intense cortical stress fibers whereas thrombin caused formation of transverse actin stress fibers. LPA treated cells with intense cortical actin stress fibers retracted from each other compared to controls and to thrombin treated cells. As in the case of TGF- β , FGF, and PDGF, increased actin stress fibers are associated with increased focal adhesions at leading edges of the cell membrane in LPA treated cells.

lipid growth factor present in aqueous humor [27] which is known to regulate various cellular processes including proliferation, cell survival, and differentiation [28]. Treatment of lens epithelial cells (maintained under serum deprivation) with LPA (5 μ g/ml) or with thrombin (1 Unit/ml) for 1 h led to a strong induction of actin stress fiber formation (Figure 4). In LPA treated cells, however, the actin stress fibers exhibited very distinct localization to cortical regions accompanied by retraction of cells from each other, in contrast to the actin stress fibers formed in response to thrombin (Figure 4). LPA and thrombin exerted similar effects on focal adhesion formation (Figure 4, labeled for vinculin). Again, as in the case of growth factors, thrombin- and LPA-induced focal adhesions were found to be co-localized to stress fibers at the leading edges of cells.



To explore the involvement of the Rho/Rho kinase signaling pathway in growth factor-induced effects on lens epithelial cells, selective inhibitors of Rho GTPase (C3-exoenzyme) [29], Rho kinase (Y-27632) [30], and isoprenylation (lovastatin) were utilized [19]. Lens epithelial cells maintained in 1% serum were pretreated with 5 μ M Y-27632 for 20 min prior to stimulation with various growth factors. Formation of actin stress fibers and focal adhesions were evaluated as described under the methods section. Figure 5 and Figure 6 illustrate the effects of Y-27632 pretreatment on growth factors-, LPA-, and thrombin-induced cytoskeletal changes, respectively. Consistent with data presented in Figure 3 and Figure 4, EGF, b-FGF, PDGF, TGF- β , LPA, and thrombin stimulated increases in the formation of actin stress fibers and focal adhesions were markedly reduced in cells pretreated with the Rho kinase inhibitor-Y-27632 (Figure 5A, Figure 5B, and Figure 6). Cells pretreated with Y-27632 fail to prevent membrane ruffling induced by EGF (Figure 5A). Pretreatment of lens epithelial cells with either lovastatin (20 μ M for 18 h) or C3-exoenzyme (10 μ g/ml for 48 h) also blocked growth factor effects on actin cytoskeletal organization (Figure 7). Since Y-27632 abolished formation of both actin stress fibers and the focal adhesions induced by different growth factors, in the case of C3 exoenzyme and lovastatin, we chose to evaluate only actin stress fiber formation in response to a subset of the growth factors of interest (b-FGF, EGF, and PDGF). However, as shown in Figure 7, C3-treated cells appear quiescent with flat cell morphology like in the case of Rho kinase inhibitor treated cells (Figure 5), while lovastatin treated cells were retracted from each other and cell morphology appears slightly rounded. Both LPA and thrombin also failed to stimulate actin stress fiber formation in lens cells pretreated with lovastatin and C3-exoenzyme (data not shown).

Figure 5. Effect of Rho kinase inhibitor on growth factor-induced actin cytoskeletal organization and focal adhesion formation. Serum starved human lens epithelial cells were initially treated for 20 min with 5 μ M Y-27632, a specific inhibitor of Rho kinase (the downstream effector of Rho GTPase), followed by treatment for 1 h with different growth factors. Pretreatment of cells with Y-27632 (right panel) abolished growth factor-mediated induction of stress fiber and focal adhesion formation, as compared to cells stimulated with growth factors alone (left panel). While growth factor treated cells appeared to undergo morphological changes and exhibited clear increases in actin stress fibers (indicated with arrows), cells pretreated with Y-27632 and stimulated with growth factors appear flat with a non-rigid morphology (**B**). Unlike other growth factors, cells pretreated with inhibitor, followed by EGF stimulation, exhibit a relatively high number of membrane ruffles, a hallmark of Rac GTPase activation and not affected by Y-27632 (**A** indicated with arrows). Images shown here indicate the double labeling of actin stress fibers (FITC staining) and focal adhesions (vinculin staining with TRITC conjugated-secondary antibody). While actin fibers were prominent, focal adhesions staining was weak in these images. However, direct viewing with confocal microscopy showed a definite positive staining for vinculin.

DISCUSSION

Data presented in this study reveal that the actin cytoskeleton constitutes one of the critical down stream targets of growth factor activity in lens epithelial cells, and that growth factor stimulated reorganization of the actomyosin cytoskeleton and focal adhesion formation is mediated through the Rho/Rho kinase pathway in these cells. Further, to our knowledge, this is the first study to provide a detail profile of activation of Rho and Rac GTPases in lens epithelial cells by different growth factors, suggesting their potential role in variety of cell function.

Growth factors and hormones derived from the aqueous and vitreous humors regulate several aspects of lens growth and development including lens epithelial cell proliferation, migration, and differentiation [1-3]. Although cytoskeletal reorganization is thought to be very critical for lens fiber cell elongation and migration, the direct effect of growth factors on lens epithelial cell cytoskeletal organization has not been understood. Therefore, in this study we have examined the effects of different growth factors on actin cytoskeletal organization and focal adhesion formation in lens epithelial cells, and explored the possible involvement of the Rho/Rho-kinase signaling pathway in mediating such responses. Organization of the actin-based cellular cytoskeleton, including actomyosin mediated cellular contraction, actin polymerization, and

the interaction of actin stress fibers with extracellular matrix components at sites of focal adhesion plays a critical role in modulation of cell shape, migration, gene expression, and survival [12-16]. Amongst the various growth factors tested in the current study, b-FGF, TGF- β , and PDGF were found to strongly induce cortical actin stress fiber formation in the human lens epithelial cell line (SRA01/04), while EGF stimulated both, membrane ruffling and actin stress fiber formation. Intriguingly, induction of stress fibers was not evident in lens cells upon IGF-I stimulation (Figure 3). Similarly, except for IGF-1, all other growth factors tested including b-FGF, TGF- β , PDGF, and EGF stimulated the formation of focal adhesions, cell-ECM interactions (Figure 3) which are critical for cell migration and cell survival [12-16].

Since Rho GTPases are thought to play a key role in orchestrating the dynamics of the actin cytoskeleton and its interaction with integrins and ECM constituents, we were interested in identifying whether this signaling pathway mediated the effects of growth factors on cytoskeletal organization in lens epithelial cells [12-14]. EGF, b-FGF, PDGF, and TGF- β treatment of lens cells led to a clearly detectable activation of Rho GTPase as determined by GTP-loading in pull down assays [31]. Interestingly, many of these growth factors including EGF, PDGF, FGF, and TGF- β also activated Rac GTPase in lens cells. However, only EGF treated cells exhibited membrane ruffles at the cell borders, a characteristic feature of Rac activation [12-14]. On the other hand, TGF- β , b-FGF, and

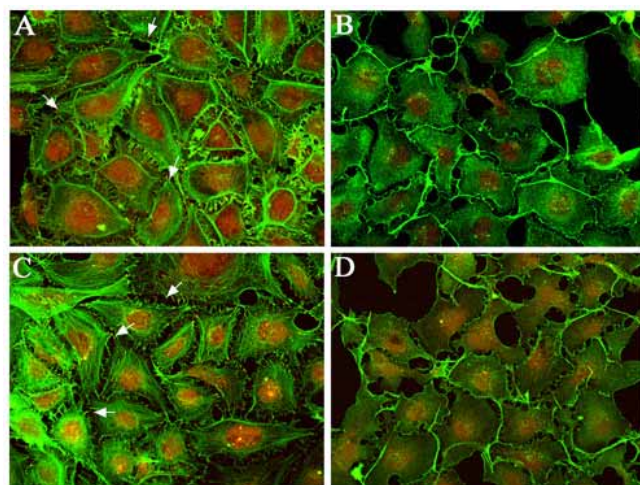


Figure 6. Effects of Rho kinase inhibitor on LPA- and thrombin-induced cytoskeletal reorganization. Pretreatment with Rho kinase inhibitor (Y-27632) prevents LPA and thrombin induced formation of actin stress fibers and focal adhesions in human lens epithelial cells. As compared to cells stimulated with LPA (A) and thrombin (C) alone, cells pretreated with Y-27632 (5 μ M for 20 min) fail to respond to these growth factors (B and D), as evidenced by the lack of actin stress fiber and focal adhesion formation. These observations indicate involvement of the Rho/Rho kinase pathway in mediating the effects of LPA and thrombin on cytoskeletal reorganization in lens cells. Cells were double-labeled for actin stress fibers (FITC-phalloidin) and focal adhesions (vinculin primary antibody with TRITC-conjugated secondary antibody). Punctate yellow-orange staining at the leading edges of cell membrane represents staining of focal adhesions (shown with arrows).

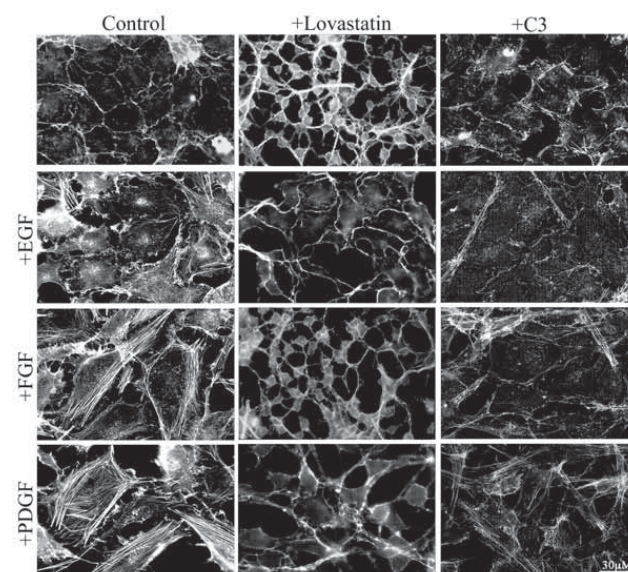


Figure 7. Lovastatin and C3-exoenzyme prevent growth factor-induced cytoskeletal reorganization. Pretreatment of lens epithelial cells with either C3-exoenzyme, a specific inhibitor of Rho GTPase, or lovastatin, a general isoprenylation inhibitor, abolished EGF, FGF and PDGF induced actin cytoskeletal reorganization. As in the case of Rho kinase inhibitor treated cells, C3-treated cells appear flat with no cortical rigidity but still maintained cell-cell contacts, while lovastatin treated cells underwent significant change in cell shape and appear rounded. Cells in this figure were stained only for F-actin, with rhodamin-phalloidin.

PDGF caused very strong cortical actin stress fibers, which are a characteristic response of Rho GTPase activation [12-14]. IGF-1 has also been reported to activate Rho and Rac and influence actin cytoskeletal organization in certain non-lenticular cells [32,33]. However, under our experimental conditions, IGF-1 had no obvious influence on either actin stress fibers or focal adhesions despite its activation of Rho and Rac GTPases. Two different batches of human IGF-1 showed a similar response on the actin cytoskeleton. The reasons for this discrepancy are not clear at present. Increased formation of focal adhesions, a response shared between both Rho and Rac-dependant signaling events was elicited by b-FGF, EGF, TGF- β , and PDGF [12-16]. In addition, unlike EGF, which induces activation of both Rho and Rac in lens cells and stimulates membrane ruffle and stress fiber formation, TGF- β , PDGF, and FGF only stimulated actin stress fiber formation, despite its apparent ability to activate both Rho and Rac. These divergent responses to different growth factors most likely would arise from cross talk between Rho- and Rac-dependant signaling pathways or between intracellular signaling mechanisms distinct from Rho/Rho kinase. Further, the activity of Rho can be negatively regulated by Rac [16] and the balance between the guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) activation exerted by different growth factors is also a critical determinant of specifying the final cytoskeletal organization [16]. In this study we have not tested the early response of growth factors on lens epithelial cell actomyosin organization and focal adhesion formation and it is quite possible that the early response effects may vary with the results reported here with one hour treatment.

Lysophospholipid, a bioactive lipid growth factor, regulates various cellular events including actin cytoskeletal organization, proliferation, migration, and survival [28]. Although there is not much known about the effects of LPA in the lens, our recent unpublished work demonstrated expression of various edg receptors (LPA and sphingosine 1-phosphate receptors) in lens epithelial cells. LPA induced very strong cortical actin stress fibers and focal adhesions in serum starved lens epithelial cells, in association with activation of Rho GTPase in these cells. This effect of LPA on cytoskeletal organization appeared to be similar to the effects of b-FGF, TGF- β , and PDGF, suggesting a potential role for this lipid agonist in epithelial cell migration, proliferation, and cell survival.

Interestingly, the effects of most of the growth factors (including b-FGF, TGF- β , PDGF, EGF, and LPA) on actomyosin organization and formation of focal adhesion in lens cells was found to be sensitive to Rho GTPase inhibitor, C3-exoenzyme, and Rho kinase inhibitor-Y-27632, confirming a requirement for Rho/Rho kinase activity for these events. In addition, lovastatin pretreatment prevented growth factor- as well as LPA- and thrombin-induced effects on actomyosin organization and focal adhesion formation. This observation further reinforces our previous observations on the significance of isoprenylated proteins (such as Rho GTPases) [19] and Rho kinase [34] in maintaining lens epithelial cell morphology and cytoskeletal organization, and suggests that Rho and Rac,

which are isoprenylated proteins, act downstream to growth factor receptors to regulate actomyosin organization and cell adhesion in response to external cues.

Although the focus of this study was primarily on cytoskeletal reorganization, there exists ample evidence for the significance of actin cytoskeletal organization and cell-ECM interaction in cell proliferation, migration, cell survival, and trafficking [12-16]. Additionally, activation of Rho and Rac GTPases has been reported to influence transcriptional activation and cell cycle progression, independent of actin cytoskeletal remodeling, through the activation of serum response factor (SRF) [12] and by the regulation of CDK kinases activity [35] and cell cycle inhibitor expression [36]. Furthermore, our previous study has demonstrated that inactivation of Rho GTPase in the mouse lens impairs lens growth and integrity [20].

Collectively, the data presented in this study demonstrate the influence of growth factors on lens epithelial actomyosin cytoskeletal reorganization and properties of cell adhesion, and confirm that the Rho/Rho kinase signaling pathway is an important downstream effector in growth factor-mediated cytoskeletal reorganization.

ACKNOWLEDGEMENTS

This study was supported by the National Institutes of Health grants EY12201 (PVR), EY013573 (PVR) and EY 00484 (VNR).

REFERENCES

1. Piatigorsky J. Lens differentiation in vertebrates. A review of cellular and molecular features. *Differentiation* 1981; 19:134-53.
2. McAvoy JW, Chamberlain CG, de Iongh RU, Hales AM, Lovicu FJ. Lens development. *Eye* 1999; 13 (Pt 3b):425-37.
3. Sue Menko A. Lens epithelial cell differentiation. *Exp Eye Res* 2002; 75:485-90.
4. Quinlan RA, Sandilands A, Procter JE, Prescott AR, Hutcheson AM, Dahm R, Gribbon C, Wallace P, Carter JM. The eye lens cytoskeleton. *Eye* 1999; 13 (Pt 3b):409-16.
5. Bassnett S, Missey H, Vucemilo I. Molecular architecture of the lens fiber cell basal membrane complex. *J Cell Sci* 1999; 112 (Pt 13):2155-65.
6. Ferreira-Cornwell MC, Venezia RW, Grunwald GB, Menko AS. N-cadherin function is required for differentiation-dependent cytoskeletal reorganization in lens cells in vitro. *Exp Cell Res* 2000; 256:237-47.
7. Beebe DC, Vasiliev O, Guo J, Shui YB, Bassnett S. Changes in adhesion complexes define stages in the differentiation of lens fiber cells. *Invest Ophthalmol Vis Sci* 2001; 42:727-34.
8. Mousa GY, Trevithick JR. Differentiation of rat lens epithelial cells in tissue culture. II. Effects of cytochalasins B and D on actin organization and differentiation. *Dev Biol* 1977; 60:14-25.
9. Blankenship TN, Hess JF, FitzGerald PG. Development- and differentiation-dependent reorganization of intermediate filaments in fiber cells. *Invest Ophthalmol Vis Sci* 2001; 42:735-42.
10. Woo MK, Lee A, Fischer RS, Moyer J, Fowler VM. The lens membrane skeleton contains structures preferentially enriched in spectrin-actin or tropomodulin-actin complexes. *Cell Motil Cytoskeleton* 2000; 46:257-68.
11. Ramaekers FC, Boomkens TR, Bloemendal H. Cytoskeletal and contractile structures in bovine lens cell differentiation. *Exp Cell*

- Res 1981; 135:454-61.
12. Hall A. Rho GTPases and the actin cytoskeleton. *Science* 1998; 279:509-14.
 13. Van Aelst L, D'Souza-Schorey C. Rho GTPases and signaling networks. *Genes Dev* 1997; 11:2295-322.
 14. Kaibuchi K, Kuroda S, Amano M. Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. *Annu Rev Biochem* 1999; 68:459-86.
 15. Ridley AJ. Rho GTPases and cell migration. *J Cell Sci* 2001; 114:2713-22.
 16. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. *Nature* 2002; 420:629-35.
 17. Rao PV, Robison WG Jr, Bettelheim F, Lin LR, Reddy VN, Zigler JS Jr. Role of small GTP-binding proteins in lovastatin-induced cataracts. *Invest Ophthalmol Vis Sci* 1997; 38:2313-21.
 18. Maddala R, Peng YW, Rao PV. Selective expression of the small GTPase RhoB in the early developing mouse lens. *Dev Dyn* 2001; 222:534-7.
 19. Maddala RL, Reddy VN, Rao PV. Lovastatin-induced cytoskeletal reorganization in lens epithelial cells: role of Rho GTPases. *Invest Ophthalmol Vis Sci* 2001; 42:2610-5.
 20. Rao V, Wawrousek E, Tamm ER, Zigler S Jr. Rho GTPase inactivation impairs lens growth and integrity. *Lab Invest* 2002; 82:231-9.
 21. Ibaraki N, Lin LR, Reddy VN. A study of growth factor receptors in human lens epithelial cells and their relationship to fiber differentiation. *Exp Eye Res* 1996; 63:683-92.
 22. Wride MA. Cellular and molecular features of lens differentiation: a review of recent advances. *Differentiation* 1996; 61:77-93.
 23. Ibaraki N, Chen SC, Lin LR, Okamoto H, Pipas JM, Reddy VN. Human lens epithelial cell line. *Exp Eye Res* 1998; 67:577-85.
 24. Rao PV, Deng PF, Kumar J, Epstein DL. Modulation of aqueous humor outflow facility by the Rho kinase-specific inhibitor Y-27632. *Invest Ophthalmol Vis Sci* 2001; 42:1029-37.
 25. Essler M, Amano M, Kruse HJ, Kaibuchi K, Weber PC, Aeppelbacher M. Thrombin inactivates myosin light chain phosphatase via Rho and its target Rho kinase in human endothelial cells. *J Biol Chem* 1998; 273:21867-74.
 26. Jalink K, van Corven EJ, Hengeveld T, Morii N, Narumiya S, Moolenaar WH. Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. *J Cell Biol* 1994; 126:801-10.
 27. Pages C, Simon MF, Valet P, Saulnier-Blache JS. Lysophosphatidic acid synthesis and release. *Prostaglandins Other Lipid Mediat* 2001; 64:1-10.
 28. Hla T, Lee MJ, Ancellin N, Paik JH, Kluk MJ. Lysophospholipids—receptor revelations. *Science* 2001; 294:1875-8.
 29. Rubin EJ, Gill DM, Boquet P, Popoff MR. Functional modification of a 21-kilodalton G protein when ADP-ribosylated by exoenzyme C3 of *Clostridium botulinum*. *Mol Cell Biol* 1988; 8:418-26.
 30. Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 1997; 389:990-4.
 31. Ren XD, Schwartz MA. Determination of GTP loading on Rho. *Methods Enzymol* 2000; 325:264-72.
 32. Cheng HL, Steinway ML, Russell JW, Feldman EL. GTPases and phosphatidylinositol 3-kinase are critical for insulin-like growth factor-I-mediated Schwann cell motility. *J Biol Chem* 2000; 275:27197-204.
 33. Taya S, Inagaki N, Sengiku H, Makino H, Iwamatsu A, Urakawa I, Nagao K, Kataoka S, Kaibuchi K. Direct interaction of insulin-like growth factor-1 receptor with leukemia-associated RhoGEF. *J Cell Biol* 2001; 155:809-20.
 34. Khurana RN, Maddala RL, Shimokawa H, Samuel Zigler J, Epstein DL, Vasantha Rao P. Inhibition of Rho-kinase induces alphaB-crystallin expression in lens epithelial cells. *Biochem Biophys Res Commun* 2002; 294:981-7.
 35. Tatsuno I, Hirai A, Saito Y. Cell-anchorage, cell cytoskeleton, and Rho-GTPase family in regulation of cell cycle progression. *Prog Cell Cycle Res* 2000; 4:19-25.
 36. Hill CS, Wynne J, Treisman R. The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* 1995; 81:1159-70.