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Protein kinase C alpha and epsilon differentially modulate hepatocyte growth factor-induced epithelial proliferation and migration

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Abstract

Protein kinase C (PKC) isoenzymes require membrane translocation for physiological activation. We have recently shown that the growth factors epidermal growth factor and hepatocyte growth factor (HGF), but not keratinocyte growth factor (KGF), regulate PKC α activation to promote epithelial wound healing (Sharma, G. D., Ottino, P., Bazan, H. E. P., 2005. Epidermal and hepatocyte growth factors, but not keratinocyte growth factor, modulate protein kinase C alpha translocation to the plasma membrane through 15(S)-hydroxyicosatetraenoic acid synthesis. *J. Biol. Chem.* 280, 7917-7924).

Protein kinase C alpha (PKC α) and protein kinase C epsilon (PKC ϵ) are two differentially regulated isoenzymes. While PKC α requires Ca²⁺ for its activation, PKC ϵ is Ca²⁺ independent. However, growth factor-induced activation of these enzymes and their specific regulation of epithelial migration and proliferation have not been explored.

In the present study, we overexpressed PKC ϵ fused to green fluorescent protein to examine its translocation in real time to the plasma membrane in living human corneal epithelial cells. Stimulation with HGF and KGF demonstrated translocation of PKC ϵ to the plasma membrane.

Because HGF activates both PKCs, this growth factor was used to stimulate wound healing. PKC α or PKC ϵ genes were knocked down individually without affecting the basal expression of the other PKC isoform. Gene-knockdown of PKC α significantly inhibited HGF-stimulated proliferation of human corneal epithelial cells. In contrast, PKC ϵ -gene silencing severely impaired the HGF-stimulated migratory ability of human corneal epithelial cells. When migrating epithelial cells in the cornea wound bed after injury were transfected with specific PKC α - or PKC ϵ -siRNA, there was a significant delay in wound healing. Corneal wound healing stimulated with HGF in similar conditions was also inhibited. On the other hand, over expression of PKC α or PKC ϵ genes fused with green fluorescent protein in migrating corneal epithelium accelerated repair of the epithelial defect.

Our findings demonstrate that PKC α and PKC ϵ modulate different stages of wound healing stimulated by HGF and contribute to epithelial repair by playing selective regulatory roles in epithelial proliferation and migration, both crucial to corneal wound healing.

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Keywords

Corneal epithelial proliferation; migration; protein kinase C alpha; protein kinase C epsilon; growth factor; wound healing; siRNA

1. Introduction

Corneal epithelial wound healing is a dynamic process driven by growth factors which, through tyrosine kinase receptors, regulate cell signaling that in turn modulate corneal repair. One of the first events is the initial coverage of the defective epithelium by migration of the residual cells. Thus, rapid cell migration avoids tissue opacity and impaired vision. The second important process is cell proliferation in order to obtain the 5-7 layers of stratified epithelium that make the surface of the cornea smooth and give its high refractive properties. It had been shown that migration and proliferation occurs at different sites of the epithelium. While migratory cells do not proliferate, cells distal from the area of the wound showed an increased proliferative rate (Zieske, 2000). To respond differently to these processes, the epithelial cells confronted with injury will activate specific signaling cascades. In fact, we have shown previously that the paracrine growth factors, hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF), activate the mitogen-activated kinases p38 and ERK1/2 in corneal epithelial cells and that a cross-talk exists between these kinases. While p38 activation promotes migration, ERK1/2 activation induces proliferation (Chandrasekher et al., 2001; Sharma et al., 2003). An important family of enzymes that contributes to the regulation of cell responses is the family of protein kinase C (PKC). Individual PKC isoenzymes have been implicated in many cellular responses such as migration, proliferation, differentiation and gene expression (Nishizuka, 1988). Several PKCs are expressed in corneal epithelium, and changes in their expression and cellular localization occur during wound healing (Chandrasekher et al., 1998). PKC α increases its activity after epithelial damage, and inhibition of its expression delays wound closure (Chandrasekher et al., 1998; Lin and Bazan, 1992). More recently we have shown that epidermal growth factor (EGF) and HGF can induce translocation (activation) of PKC α to the cell membrane while KGF does not (Sharma et al., 2005). This selective activation points to a specific function of PKC α upon stimulation with growth factors.

Here we investigate the role of PKC α and PKC ϵ in migration and proliferation on human corneal epithelial (HCE) cells stimulated by HGF. Activities of PKC α and PKC ϵ are regulated differently. PKC α requires Ca²⁺, diacylglycerol and phosphatidylserine for activation. However, PKC ϵ activation requires diacylglycerol and phosphatidylserine, but is independent of Ca²⁺. PKC ϵ is a PKC isoform that also changes its expression and distribution during *in vivo* corneal epithelial wound healing (Chandrasekher et al., 1998). We used both PKC α and PKC ϵ specific siRNA mediated gene-silencing and their overexpression with a construct containing full-length PKC α - and PKC ϵ -tagged to green fluorescent protein (PKCs-GFP) to monitor subcellular localization of PKCs as well as their specific involvement in epithelial migration and proliferation and corneal wound healing. We also show a distinction of PKC ϵ : both HGF and KGF stimulation of HCE cells lead to activation of PKC ϵ , and this kinase modulates HGF-stimulated cell migration. On the other hand, PKC α is mainly involved in the proliferative phase of corneal epithelial wound healing.

2. Materials and methods

2.1 Materials

Rabbit eyes were obtained from Pel-Freez Biologicals (Rogers, AR). Human recombinant double-chain HGF was a gift from Genentech (San Francisco, CA). Human recombinant KGF was purchased from Upstate Biotechnology (Charlottesville, VA). Specific PKC α -, PKC ϵ -

siRNA and scrambled control siRNA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The PKCs-GFP plasmids (a gift from Dr. Rosario Rizzuto, University of Ferrara, Italy) were constructs of pcDNA3 containing the PKC α or PKC ϵ gene fused with green fluorescent protein (GFP). X-tremeGENE and FuGENE 6 transfection reagents were from Roche Molecular Biochemicals (Indianapolis, IN). Anti-PKC α and anti-PKC ϵ antibodies were from Calbiochem (San Diego, CA). All SDS-PAGE reagents were from Bio-Rad (Hercules, CA). The biotinylated protein ladder detection pack was from Cell Signaling Technology (Beverly, MA). ECL Western Blotting Detection System was obtained from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). CyQuant cell proliferation assay kit was from Molecular Probes (Eugene, OR). β -Actin was obtained from Sigma (St. Louis, MA) and used as a loading control. Thermo Plate, a thermal stage to maintain the cells at 37 °C, was purchased from Nikon, Inc.

2.2 Cell culture

The human corneal epithelial (HCE) cell line was obtained from Dr. Roger Beuerman (LSU Eye Center, LSUHSC). The cell line was established using a HPV16-E6E7 vector (Nguyen et al, 2003). The cells had been characterized for epithelial keratins, receptors for several growth factors, and they responded in similar fashion to those primary corneal epithelial cultures (Sharma et al., 2003, Kakazu et al., 2004). The HCE cells were maintained in serum-free keratinocyte growth medium (KGM, Cambrex Bio Science, Walkersville, MD), essentially as described earlier (Sharma et al., 2003), and were used between 25-45 passages.

2.3 PKC ϵ -GFP overexpression in HCE cells

Transient transfections were performed in HCE cells with PKC ϵ -GFP (1 μ g DNA/dish) using FuGENE 6 transfection reagent (ratio 3:1, FuGENE: DNA) as described (Sharma et al., 2005). The transfected cells were incubated in KGM for an additional 24 h to allow the expression of GFP-tagged PKCs. Cells were examined under a fluorescence microscope to confirm the desired transfection efficiency (70-80%). Prior to the experiment, cells were starved in keratinocyte basal medium (KBM, Cambrex) for 16-18 h. An equal volume of FuGENE 6 was added to control cells (it had no adverse effect on the cells). Similarly, cells transfected with empty vector containing only GFP (EGFP) but no PKC genes were used as negative controls.

2.4 Real-time translocation of PKC ϵ -GFP to the plasma membrane

PKC ϵ -GFP transfected cells were starved as mentioned above and dishes were secured on the Thermo Plate kept at 37 °C and maintained at normal room air for the duration of the experiment. Cells were stimulated with growth factors (HGF or KGF [20 ng/ml]) and images were recorded prior to treatment (control, t = 0 min) at 60-second intervals up to 5 min, and at 5-min intervals up to 30 min as previously described (Sharma et al., 2005). The medium contained HEPES and the pH was maintained constant during the time of the recording. The initial microscope stage position remained unchanged so the same group of cells could be continuously monitored. Images were recorded by fluorescence microscope (Nikon Eclipse TE200), with a software-controlled shutter to minimize GFP photobleaching, and an attached Nikon digital camera (DXM1200) at 200 $\times$ magnification in a dark room using Meta Vue 5.0 (Nikon, Inc.) imaging software.

2.5 PKC α - or PKC ϵ -gene silencing with specific PKCs-siRNA transfection in HCE cells

Cells were cultured in 60-mm dishes (5 $\times$ 10⁵ cells/dish) in KGM and allowed to grow to 50-60% confluence. Transient transfection with PKC α -siRNA (1-3 μ g siRNA/dish) or PKC ϵ -siRNA (1-4 μ g siRNA/dish) was performed using X-tremeGENE siRNA transfection reagent according to manufacturer's protocol with some modifications. For 96-well plate experiments

(1.5×10^3 cells/well) the reagents were scaled-down and titrated for optimum transfection efficiency. Cells transfected with scrambled (scm) siRNA were used as negative control and control cells were added with the transfection reagent alone, which had no adverse effect on these cells. The transfected cells were fed with fresh KGM medium after 16-18 h and further incubated for 48 h to allow gene-knockdown, which was evaluated by analyzing the protein expression by Western blotting.

2.6 Knockdown/overexpression of PKC α - or PKC ϵ -gene in corneal epithelium in organ culture

An 8 mm de-epithelization wound was created in corneas of rabbit eyes using an Algerbrush II (Alger Co, Lago Vista, TX). Corneas were harvested and maintained in organ cultures in KBM as described earlier (Sharma et al., 2003). Transient transfection with PKC α -siRNA or PKC ϵ -siRNA (3 or 4 μ g siRNA/well/2corneas) or with PKCs-GFP (3 μ g/well/2corneas) for overexpression was performed at 12 h intervals after de-epithelization using X-tremeGENE or FuGENE 6 transfection reagent as described above. In each experiment, two additional corneas were transfected with scrambled siRNA (fluorescein conjugated) or EGFP alone, which were used as a negative control and also to monitor transfection in newly migrating epithelium. The transfected corneas were then incubated for 48 h with or without HGF (20 ng/ml). The corneas were stained with Alizarin red and the wounded area analyzed as described (Sharma et al., 2003).

2.7 Western blotting

To evaluate PKCs expression by immunoblotting, rabbit corneas in organ culture (8 to 10 per condition) were completely denuded without damaging the limbal area, and migrating epithelium was transfected with PKC α -, PKC ϵ -siRNA or scm siRNA as described above. Seventy two hours after creating the wound, newly formed epithelium was collected and assayed by Western blot (Sharma et al., 2003). Briefly, 30 μ g protein/well was separated on SDS-PAGE (10% gel) and then transferred to nitrocellulose membrane using a Bio-Rad Mini Trans Blot transfer unit. The membranes were blocked with Tris-buffered saline (TBS, 20 mM Tris-HCL, 150 mM NaCl, pH 7.4, 0.05% Tween 20) containing 5% nonfat dry milk for 1 hr at room temperature followed by incubation with specific primary anti-PKCs or anti- β -Actin antibody as a loading control. The membranes were washed five times (5 minutes per wash) with TBS (0.05% Tween 20) and further incubated with appropriate secondary antibodies. Membranes were stripped using a standard ECL-kit protocol and re-probed with other anti-PKCs or anti- β -Actin antibodies. The separated proteins were visualized by ECL kit according to the manufacturer's protocol. Densitometric analysis was performed using Quantity One (Bio-Rad).

2.8 Cell migration assay

HCE cells were seeded in 60-mm dishes and grown to 60-70% confluence. To study the effect of PKC α or PKC ϵ -gene knockdown on cellular migration, the cells were transfected with PKCs-siRNA (2-3 μ g/dish) or scm siRNA (negative control) as already described. Cells were incubated overnight in KBM. Then a scrape wound was performed with a sterile cell scraper, and cells were allowed to migrate with or without HGF stimulation (20 ng/ml) for 24 h. Images were taken at $t = 0$ h and after 24 h. Migrated cells were counted under the microscope as previously described (Sharma et al., 2003).

2.9 Cell proliferation assay

HCE cells were seeded in 96-well plates (1.5×10^3 cells/well) and allowed to grow to 50-60% confluence. Cells were then transfected with specific PKC-siRNA (1 μ g/ml and starved with KBM as described. Cells were then stimulated with 20 ng/ml HGF for 48 h to determine the

effect of PKC α or PKC ϵ gene-knockdown on cell proliferation. Each condition was performed in octuplicate and cellular proliferation was determined with CyQuant DNA binding dye as described earlier (Chandrasekher et al., 2002; Ottino et al., 2003). Fluorescence was measured on a Fluoroskan Ascent FL plate reader (Labsystems, Finland) with 485-nm excitation and 538-nm emission maximum filters. Cells were also transfected with either scm siRNA as a negative control, or with fluorescein-conjugated control siRNA and with transfection reagent alone as transfection controls.

2.10 Statistical analysis

The significance of data was analyzed by Student's *t*-test. Values with $p < 0.05$ were considered significantly different.

3. Results

3.1 Real-time translocation of PKC ϵ to the plasma membrane upon KGF or HGF stimulation

In previous studies we had shown that in HCE cells HGF induced translocation (and activation) of PKC α to the plasma membrane while KGF does not, suggesting that different signaling pathways are activated by these two paracrine growth factors (Sharma et al., 2005). To examine the kinetics of translocation of PKC ϵ to the plasma membrane upon KGF and HGF stimulation, we performed experiments using live-cell imaging to track real-time movement of PKC ϵ -GFP. The HCE cells were transfected with PKC ϵ -GFP (1 μ g/dish), starved overnight as described under "Materials and Methods" and then secured on the Thermo Plate at 37 °C. Transfected cells without stimulation ($t=0$) showed marked expression and distribution of GFP-PKC ϵ in the cytosolic region (Fig. 1). Following KGF treatment, increase in PKC ϵ intensity in the plasma membrane was first detected at 15 min (arrows) without accumulation in the perinuclear area (Fig. 1A). Increased accumulation of PKC ϵ in the plasma membrane occurred by 30 min. In cells stimulated with HGF, translocation of PKC ϵ to the plasma membrane also was noticed at 15 min, and the plasma membrane of the cell was more defined by 30 min (Fig. 1B). Observation of different fields at 30 min showed similar patterns of PKC ϵ movement. Table 1 shows the percentage of cells in which PKC ϵ was translocated upon stimulation with KGF and HGF for 30 min. Cells transfected with EGFP, but no PKC ϵ gene, were used as a negative control and showed no changes in GFP distribution upon growth factors stimulation (data not shown).

3.2 PKC α and PKC ϵ gene-silencing in cells in culture

In order to study the selective roles of the PKCs in wound repair, we knocked down PKC α - and PKC ϵ - genes using specific siRNAs as described under "Materials and Methods." Initially, conditions were optimized for the maximum transfection efficiency with minimal effect on cell viability. Control sets were transfected with a fluorescein-conjugated siRNA and observed under a fluorescence microscope. Transfection efficiency was very high with most of the cells shown fluorescence (Fig. 2A). Forty eight hours post transfection HCE cells transfected with PKC α -siRNA (3 μ g/60mm dish) showed 80% inhibition (average of two experiments) compared to control scrambled siRNA transfection (Fig. 2B, *upper* panel). When the same membrane was stripped and re-probed with anti-PKC ϵ antibody, there was no change in PKC ϵ expression, compared to control (Fig. 2B, *lower* panel). Similarly, when cells were transfected with PKC ϵ -siRNA (2 or 4 μ g/60mm dish) and analyzed for PKC ϵ protein expression, an inhibition in PKC ϵ expression was noticed with 2 μ g (30%) and 4 μ g (70%) PKC ϵ -siRNA compared to control scm siRNA transfection (Fig. 2C, *upper* panel). Upon re-probing the membrane with anti-PKC α antibody, no changes in PKC α was detected (Fig. 2C, *lower* panel). The transfection reagent X-tremeGENE at similar concentration did not affect the expression of either of the two PKCs. These results demonstrate the specificity of the PKCs-siRNAs transfected in HCE cells.

3.3 PKC ϵ -gene knockdown delays corneal epithelial wound closure

To examine the effect of PKC α and PKC ϵ gene-silencing or overexpression in a model of cornea wound healing, we transfected the migrating corneal epithelium of rabbit wounded corneas in organ culture experiments as described in "Materials and Methods." Corneas transfected with PKC α -siRNA (3 μ g/2 corneas), showed 73% inhibition (average of two samples) in PKC α expression compared to scrambled control after normalization with β -Actin (Fig. 3A, *left* panel). PKC ϵ -siRNA (4 μ g/2 cornea) transfection produced an inhibition of 56% of PKC ϵ protein expression compared to scrambled control after normalization with β -Actin (Fig. 3A, *right* panel). The results demonstrate that PKC α and PKC ϵ could be successfully knockdown in wounded cornea epithelium in organ culture.

Rabbit corneas transfected with PKCs-siRNA or GFP-PKCs were stimulated with HGF. Forty eight hours after the wound, a significant increase in wound healing (> 50%) was noticed in control corneas treated with transfection reagents (X-tremeGENE or FuGENE 6) (Fig. 3B) compared to control (t = 0 h, original wound). As previously reported (Chandrasekher et al., 2001), addition of HGF significantly (p<0.01) increased the wound healing compared to control corneas. Corneas transfected with PKC α -siRNA (3 μ g) or PKC ϵ -siRNA (4 μ g) showed a marked inhibition in wound healing compared to control corneas. In the presence of HGF, epithelial healing was significantly delayed in PKC α -siRNA or PKC ϵ -siRNA transfected corneas compared with scm siRNA. With or without HGF, the wounded area was significantly higher in corneas where PKC ϵ expression was inhibited, compared to PKC α knockdown. Overexpression of PKC α or PKC ϵ by itself also had a significant effect on wound healing, and stimulation with HGF showed a small but significant (p<0.05) increase in wound healing with respect to overexpression of PKC α alone. The effect was more pronounced (p<0.01) with PKC ϵ . EGFP transfection of cornea did not significantly change the wound healing under such conditions (data not shown).

3.4 PKC ϵ -gene knockdown decreases epithelial migration but does not affect proliferation

To obtain more clear evidence of the role of PKC α and PKC ϵ on corneal epithelial wound healing, *in vitro* assays for migration and proliferation were performed in HCE cells transfected with PKC α - or PKC ϵ -siRNA and stimulated with HGF as described in "Materials and Methods." Scrambled siRNA transfected cells were used as negative control in these experiments. Figure 4 shows the stimulation of cell migration in the presence of HGF; PKC α -siRNA transfection (2 μ g/dish) had no effect on HGF-stimulated migration of these cells. PKC ϵ -siRNA transfected cells, on the other hand, showed very dramatic inhibition in cell migration compared with HGF and scm siRNA transfected cells. The bars show the effect on migration of HCE cells with different concentrations of siRNA with or without stimulation with HGF. In these assays, HGF stimulates more than 3 times the migration of HCE cells. PKC α has no effect at 2 μ g of siRNA, but at 3 μ g it inhibits about 20% of the migration induced by HGF. On the other hand, inhibition of PKC ϵ almost completely blocked migration.

We also investigate the role of PKC α and PKC ϵ in cell proliferation. Cells were allowed to grow with or without HGF stimulation and cell proliferation was assayed after 48 h as previously reported (Sharma et al., 2003). As previously shown (Chandrasekher et al., 2002, Ottino et al. 2003), cells stimulated with HGF showed significant increase in cell proliferation compared to control cells (Fig. 5). Transfection with PKC α -siRNA inhibits proliferation in nonstimulated cells. In the presence of HGF, the PKC α -siRNA transfected cells also showed significant inhibition in proliferation compared to scm siRNA transfected cells and HGF. On the other hand, cells transfected with PKC ϵ -siRNA do not show significant effect on proliferation with or without HGF stimulation over control cells.

4. Discussion

One puzzle to elucidate is how growth factors, in our case HGF and KGF, produce signal specificity. That is, how are the signals transmitted to induce specific biological responses? Both HGF and KGF are released after corneal injury by stroma cells and the lacrimal gland and act on receptors in the epithelial cells (Wilson et al., 1999a; Wilson et al., 1999b). They activate ERK1/2 as well as the phosphatidylinositol-3 kinase (PI-3K) /Akt-1/ p70S6 kinase pathways, and stimulate epithelial wound healing (Chandrasekher et al., 2001; Liang et al., 1998). With the use of GFP-PKCs, it was possible to monitor PKC translocation through real-time visualization in living HCE cells stimulated by HGF and KGF. We previously demonstrated that PKC α -GFP (Sharma et al., 2005), and now show that PKC ϵ -GFP, translocates to the plasma membrane after growth factors stimulation. While PKC α is stimulated only by HGF, PKC ϵ is activated by both of the paracrine factors with KGF seen to be the stronger stimulator, suggesting differences in cell signaling involving these two PKCs. Therefore, the ability of different growth factors to selectively translocate isoforms of PKC to the membrane could be a mechanism by which cells can regulate where and which of the PKCs acts. Previous studies had shown that the 15 lipoxygenase product (15-(S) hydroxyeicosatetraenoic acid) is an intracellular mediator that facilitates PKC α translocation induced by HGF (Sharma et al., 2005). It is known that translocation, mediated by the C1 domain of the PKCs, is also affected by diacylglycerol (DAG), required by both isoenzymes for their activation (Tanimura et al., 2002; Stahelin et al., 2005). Two types of DAG appear to be important for the physiological activation of PKC. One is the rapid DAG produced from phosphatidylinositol 4,5-bisphosphate (PIP₂) by phospholipase C (PLC) upon stimulation of G protein-coupled receptors. The other results from hydrolysis of phosphatidylcholine. This latter DAG production occurs slowly and is more sustained (Shirai et al., 2002) and is induced by growth that activates PLC γ . It had been reported that HGF stimulates PLC γ in neocortical cells and generates DAG (Machide et al. 1998). Activation of KGF receptors produces subsequent activation of PLC γ that may increase intracellular levels of DAG culminating with PKC activation (Shaoul et al., 1995). Therefore, it is possible that signaling by KGF requires intermediate steps such as release of DAG. Further experiments will be needed to determine the DAG production in HCE cells.

The ability of HGF to translocate both PKC α and PKC ϵ to the membrane directs our studies to determine how these two enzymes are involved in epithelial wound healing stimulated by the growth factor. The major finding of this study is that each one of the isoenzymes had a defined role in two important processes that regulate epithelial wound healing activated by HGF. While PKC α is involved in proliferation, PKC ϵ affects migration.

Due to the lack of selectivity of pharmacological inhibitors of PKC isozymes, unwanted suppression of other non-specific PKCs, and possible cross talk between these pathways, we choose a selective and more specific gene-silencing approach as well as gene overexpression to dissect the exact role of PKC α and PKC ϵ in HGF stimulated cellular proliferation and migration.

Our studies of wounded corneas in organ culture indicate that although inhibition of both PKC α and PKC ϵ decrease wound closure, PKC ϵ is more potent. On the other hand, overexpression of PKC ϵ increases wound closure significantly faster than HGF alone. It is well documented that wound closure is a dynamic process in which migration is activated during the early phase of the healing while active proliferation occurs later (Zieske, 2000). The results suggest that PKC ϵ has an important role in migrating epithelium near the wound.

To determine the role of PKC ϵ in cell migration, we evaluate the motility of HCE cells in which the gene had been knocked down. We found that the action of HGF, a potent activator of cell

migration, was abolished over a 24 h period of HGF stimulation. Migration can be activated by the lipid product of the PI-3K, PI-3,4,5P3 (Derman et al., 1997) and both KGF and HGF stimulate PI-3K in HCE cells (Chandrasekher et al., 2001). On the other hand, PKC ϵ does not stimulate proliferation of the cells. This is in agreement with studies in hepatocyte cells in which PKC ϵ does not contribute to the PI-3K mitogenic signal (Balciunaite and Kazlauskas, 2001). Using inhibitors of PKC, it had been demonstrated that PKC plays a role in FGF-2 induced migration in corneal endothelial cells, although the study does not specify the isoform involved (Rieck et al., 2001). PKC ϵ positively regulates integrin-dependent adhesion, spreading, and migration of human glioma cells (Besson et al., 2002).

Previous work from our lab had shown that PKC α activity increased during a phase of wound healing characterized by active proliferation and that the use of antisense for 48 h delayed the process of epithelial wound healing in the organ culture model (Chandrasekher et al., 1998). In the current experiments, the decrease in the proliferative capacity observed in corneal epithelial cells in which PKC α expression had been inhibited demonstrates a role of this isoenzyme in the replication of HCE cells. Activation of PKC α at the plasma membrane could phosphorylate substrates that convey signals involved in proliferation. The downstream targets of PKC α could be diverse. PKC α has been shown in other systems to phosphorylate Raf-1 kinase (Kolch et al. 1993), which leads to the activation of ERK1/2 cascade. In fact, previous work (Liang et al., 1998) had shown that the PKC inhibitor calphostin had a significant inhibitory effect on ERK1/2 activation induced by HGF, but not by KGF, in HCE cells. This is consistent with our present results showing that KGF does not activate PKC α and with previous studies demonstrating that ERK1/2 is involved in proliferation of these cells (Sharma et al. 2003). PKC α had also been associated with cell cycle progression (Besson and Yong, 2000) and inhibition of PKC in a human lung cell line decrease HGF-induced thymidine uptake (Awasthi and King, 2000), which is in agreement with a role of the enzyme in proliferation.

PKC α and PKC ϵ had been reported to have different and sometimes opposite functions in another system. In human intestinal epithelial cells, PKC ϵ stimulates endocytosis while PKC α blocked the ability of remodel F-actin and opposed the action of PKC ϵ (Song et al., 2002). In glioma cells, PKC α regulates cell cycle and proliferation and negatively regulates adhesion and motility, while PKC ϵ activation increases motility (Besson et al. 2001). Other studies using inhibitors of PKC α and PKC ϵ found that both reduce endothelial cell proliferation (Graham et al., 2000).

In summary, this study links activation of PKC α and PKC ϵ with a tissue-repair response. We provide evidence that in corneal epithelium, activation of PKC ϵ is involved in the migration. Moreover, PKC ϵ mediates HGF-induced migration but not proliferation. In contrast, PKC α mediates HGF-stimulated proliferation but is not involved in migration. If one of the signals is interrupted, then the healing process is hampered. These findings provide new insights into this intricate process and raise the possibility of novel therapeutic strategies when wounds are difficult to repair.

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References

Awasthi V, King RJ. PKC, p42/p44 MAPK, and p38 MAPK are required for HGF-induced proliferation of H441 cells. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L942–L949. [PubMed: 11053031]

- Balciunaite E, Kazlauskas A. Early phosphoinositide 3-kinase activity is required for late activation of protein kinase Cepsilon in platelet-derived-growth-factor-stimulated cells: evidence for signalling across a large temporal gap. *Biochem J* 2001;358:281–285. [PubMed: 11513725]
- Besson A, Yong VW. Involvement of p21(Waf1/Cip1) in protein kinase C alpha-induced cell cycle progression. *Mol Cell Biol* 2000;20:4580–4590. [PubMed: 10848585]
- Besson A, Davy A, Robbins SM, Yong VW. Differential activation of ERKs to focal adhesions by PKC epsilon is required for PMA-induced adhesion and migration of human glioma cells. *Oncogene* 2001;20:7398–7407. [PubMed: 11704869]
- Besson A, Wilson TL, Yong VW. The anchoring protein RACK1 links protein kinase Cepsilon to integrin beta chains. Requirements for adhesion and motility. *J Biol Chem* 2002;277:22073–22084. [PubMed: 11934885]
- Chandrasekher G, Bazan NG, Bazan HEP. Selective changes in protein kinase C (PKC) isoform expression in rabbit corneal epithelium during wound healing. Inhibition of corneal epithelial repair by PKC alpha antisense. *Exp Eye Res* 1998;67:603–610. [PubMed: 9878223]
- Chandrasekher G, Kakazu A, Bazan HEP. HGF- and KGF-induced activation of PI-3K/p70 s6 kinase pathway in corneal epithelial cells: its relevance in wound healing. *Exp Eye Res* 2001;73:191–202. [PubMed: 11446769]
- Chandrasekher G, Ma X, Lallier TE, Bazan HEP. Delay of corneal epithelial wound healing and induction of keratocyte apoptosis by platelet-activating factor. *Invest Ophthalmol Vis Sci* 2002;43:1422–1428. [PubMed: 11980856]
- Derman MP, Toker A, Hartwig JH, Spokes K, Falck JR, Chen CS, Cantley LC, Cantley LG. The lipid products of phosphoinositide 3-kinase increase cell motility through protein kinase C. *J Biol Chem* 1997;272:6465–6470. [PubMed: 9045671]
- Graham MA, Rawe I, Dartt DA, Joyce NC. Protein kinase C regulation of corneal endothelial cell proliferation and cell cycle. *Invest Ophthalmol Vis Sci* 2000;41:4124–4132. [PubMed: 11095605]
- Kakazu A, Chandrasekher G, Bazan HEP. HGF protects corneal epithelial cells from apoptosis by PI-3K/Akt/Bad- but not the ERK1/2-mediated signaling pathway. *Invest Ophthalmol Vis Sci* 2004;45:3485–3492. [PubMed: 15452053]
- Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D, Rapp UR. Protein kinase C alpha activates RAF-1 by direct phosphorylation. *Nature* 1993;364:249–252. [PubMed: 8321321]
- Liang Q, Mohan RR, Chen L, Wilson SE. Signaling by HGF and KGF in corneal epithelial cells: Ras/MAP kinase and Jak-STAT pathways. *Invest Ophthalmol Vis Sci* 1998;39:1329–1338. [PubMed: 9660480]
- Lin N, Bazan HEP. Protein kinase C subspecies in rabbit corneal epithelium: increased activity of alpha subspecies during wound healing. *Curr Eye Res* 1992;11:899–907. [PubMed: 1330442]
- Machide M, Kamitori K, Nakamura Y, Kohsaka S. Selective activation of phospholipase C gamma1 and distinct protein kinase C subspecies in intracellular signaling by hepatocyte growth factor/scatter factor in primary cultured rat neocortical cells. *J Neurochem* 1998;71:592–602. [PubMed: 9681449]
- Nguyen, DH.; Buehrman, RW.; De Wever, B.; Rosdy, M. Three-dimensional construct of the human corneal epithelium for in vivo toxicology. In: Salem, H.; Katz, SA., editors. *Altern Toxicol Methods*. 2003. p. 147-159.
- Nishizuka Y. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* 1988;334:661–665. [PubMed: 3045562]
- Ottino P, Taheri F, Bazan HEP. Growth factor-induced proliferation in corneal epithelial cells is mediated by 12(S)-HETE. *Exp Eye Res* 2003;76:613–622. [PubMed: 12697425]
- Rieck PW, Cholidis S, Hartmann C. Intracellular signaling pathway of FGF-2-modulated corneal endothelial cell migration during wound healing in vitro. *Exp Eye Res* 2001;73:639–650. [PubMed: 11747364]
- Shaoul E, Reich-Slotky R, Berman B, Ron D. Fibroblast growth factor receptors display both common and distinct signaling pathways. *Oncogene* 1995;10:1553–1561. [PubMed: 7731710]
- Sharma GD, He JC, Bazan HEP. p38 and ERK1/2 coordinate cellular migration and proliferation in epithelial wound healing: evidence of cross-talk activation between MAP kinase cascades. *J Biol Chem* 2003;278:21989–21997. [PubMed: 12663671]

- Sharma GD, Ottino P, Bazan HEP. Epidermal and hepatocyte growth factors, but not keratinocyte growth factor, modulate protein kinase C alpha translocation to the plasma membrane through 15(S)-hydroxyeicosatetraenoic acid synthesis. *J Biol Chem* 2005;280:7917–7924. [PubMed: 15613483]
- Shirai Y, Saito N. Activation mechanisms of protein kinase C: maturation, catalytic activation, and targeting. *J Biochem (Tokyo)* 2002;132:663–668. [PubMed: 12417013]
- Song JC, Rangachari PK, Matthews JB. Opposing effects of PKC alpha and PKC epsilon on basolateral membrane dynamics in intestinal epithelia. *Am J Physiol Cell Physiol* 2002;283:C1548–C1556. [PubMed: 12372816]
- Stahelin RV, Digman MA, Medkova M, Ananthanarayanan B, Melowic HR, Rafter JD, Cho W. Diacylglycerol-induced membrane targeting and activation of protein kinase Cepsilon: mechanistic differences between protein kinases Cdelta and Cepsilon. *J Biol Chem* 2005;280:19784–19793. [PubMed: 15769752]
- Tanimura A, Nezu A, Morita T, Hashimoto N, Tojyo Y. Interplay between calcium, diacylglycerol, and phosphorylation in the spatial and temporal regulation of PKC alpha-GFP. *J Biol Chem* 2002;277:29054–29062. [PubMed: 11997388]
- Wilson SE, Chen L, Mohan RR, Liang Q, Liu J. Expression of HGF, KGF, EGF and receptor messenger RNAs following corneal epithelial wounding. *Exp Eye Res* 1999a;68:377–397. [PubMed: 10192796]
- Wilson SE, Liang Q, Kim WJ. Lacrima gland HGF, KGF, and EGF mRNA levels increase after corneal epithelial wounding. *Invest Ophthalmol Vis Sci* 1999b;40:2185–2190. [PubMed: 10476782]
- Zieske JD. Expression of cyclin-dependent kinase inhibitors during corneal wound repair. *Prog Retin Eye Res* 2000;19:257–270. [PubMed: 10749377]

Fig. 1. Real-time translocation kinetics of PKC ϵ in epithelial cells stimulated by KGF or HGF
HCE cells were seeded in 60 mm dishes, allowed to grow to 50-60% confluence and transfected with PKC ϵ -GFP (1 μ g/dish) as described under "Materials and Methods." Prior to the experiment, cells were serum-starved overnight then secured in the thermo plate, stimulated with KGF or HGF (20 ng/ml) and images were recorded (scale bar: 50 μ m).

A. KGF-induced movement of PKC ϵ to the plasma membrane (arrows) was observed as compared to control (t = 0 min). Images were recorded in a time-lapse series of 60 sec up to 5-min intervals, and thereafter, but only 15 and 30 min images are shown.

B. Similarly, cells stimulated with HGF were observed for 30 min for change in PKC ϵ localization compared to control (t=0 min). Once the images were recorded, five to six additional fields were examined that show similar pattern of PKC ϵ movement. The data is representative of three independent sets of experiments.

Fig. 2. PKC α - or PKC ϵ -gene knockdown in HCE cells

Cells were transiently transfected with scrambled (scm) siRNA, PKC α - or PKC ϵ -siRNA and analyzed for PKCs expression.

A. Cells transfected with fluoroscein-conjugated control siRNA. Arrows show examples of siRNA positive cells. Image is representative of more than 10 randomly chosen fields.

B. PKC α -siRNA transfected cells were immunoblotted with anti-PKC α and anti-PKC ϵ antibodies to analyze knockdown in PKC α or PKC ϵ expression.

C. PKC ϵ -siRNA transfected cells were immunoblotted and probed with anti-PKC α and anti-PKC ϵ antibodies to show the effect on PKC ϵ or PKC α expression. The experiment was repeated twice with similar results.

Fig. 3. PKC ϵ -gene knockdown produces more delayed wound closure than PKC α -gene knockdown

A. Migrating corneal epithelium after injury was transfected with scrambled (scm) siRNA, PKC α -siRNA, or PKC ϵ -siRNA in organ culture and collected and analyzed for PKCs expression after 72 h of epithelial debridement by Western blot. The membranes were stripped and immunoblotted with anti- β -Actin antibody and the percent of inhibition was calculated after normalization with β -Actin. Data is representative of two independent experiments.

B. An epithelial debridement wound (8 mm) was made with an Algerbrush II in rabbit corneas in organ culture and migrating corneal epithelium was transfected with PKC α -siRNA, PKC ϵ -siRNA or GFP-PKC α or GFP-PKC ϵ . Corneas were incubated for 48 h with or without HGF. Photomicrograph shows remaining wound area stained with Alizarin Red under the different

conditions; □ = controls, t = 0 h shows initial wound size; the others are 48 h controls with transfection reagents; ▨ = in the presence of scrambled (scm) siRNA and HGF. ■ = relative wound area in PKCs-siRNA or GFP-PKCs (OE) transfected corneas. *p < 0.01 compared to control (with X-tremeGENE or FuGENE 6), **p < 0.01 compared to HGF (with scm siRNA), #p < 0.05 compared to PKCα-siRNA or PKCα-siRNA+HGF. Values are average ± SEM of four corneas in three separate experiments.

Fig. 4. PKC ϵ -gene knockdown affects HCE cell migration

HCE cells were grown to 60-70% confluence in 60 mm dishes and transfected with PKC α -siRNA, PKC ϵ -siRNA or scrambled (scm) siRNA (2-3 μ g siRNA/dish). Scrape wound migration assays were performed after 24 h of stimulation with 20 ng/ml of HGF as described under "Materials and Methods."

A. The microphotographs show migration of PKCs-siRNA transfected corneal epithelial cells across the reference line with or without HGF-stimulation. Images were taken at 100x magnification.

B. The bars represent the number of migrated cells across the reference line after 24 h HGF-stimulation. * p <0.01, compared to scm control ($\square$), ** p <0.01, compared to HGF+scm-siRNA

(■). Data is average $\pm$ SEM of 10 randomly chosen areas in three independent sets of experiments.

Fig. 5. PKC α -gene knockdown affects cell proliferation

HCE cells were grown to 50-60% confluence in 96-well plate and transfected with PKC α -, PKC ϵ - or scrambled (scm) siRNA (1 μ g/ml). Cells were analyzed for proliferation after 48 h of HGF stimulation. * $p < 0.01$, compared to scm siRNA, ** $p < 0.01$, compared to HGF+ scm siRNA, ns = non-significant differences. The data corresponds to the average $\pm$ SEM of three independent experiments of octuplicate samples.

Table 1PKC ϵ translocation 30 min after KGF and HGF stimulation.

KGF	HGF
59 $\pm$ 3.8% (5)	39 $\pm$ 4.0% (6)

Values are average $\pm$ SD of percent of cells in which PKC ϵ translocation was observed with respect to total GFP-PKC ϵ transfected cells. In parentheses is the number of experiments. In each experiment, 8–10 fields were counted.