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Calcium-induced Human Keratinocytes (HaCaT) Differentiation Requires Protein Kinase B Activation in Phosphatidylinositol 3-Kinase-dependent Manner

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ABSTRACT. The survival and growth of epithelial cells depends on adhesion to the extracellular matrix. An adhesion signal may regulate the initiation of differentiation, since epidermal keratinocytes differentiate as they leave the basement membrane. A metabolically dead cornified cell envelope is the end point of epidermal differentiation so that this process may be viewed as a specialized form of programmed cell death. In order to investigate the precise cellular signaling events leading to terminal differentiation of keratinocytes, we have utilized HaCaT cells to monitor the biological consequences of Ca^{2+} stimulation and numerous downstream signaling pathways, including activation of the extracellular signal-regulated protein kinase (ERK) pathway and activation of phosphatidylinositol 3-kinase (PI3K). The results presented in this study show that Ca^{2+} function as potent agents for the differentiation of HaCaT keratinocytes, and this differentiation depends on the activation of ERK, Protein kinase B (PKB) and p70 ribosomal protein S6 kinase (p70S6K). Finally, the results show that the expression of Activator protein 1 (AP-1; c-Jun and c-Fos) increased following Ca^{2+}-mediated differentiation of HaCaT cells, suggesting that ERK-mediated AP-1 expression is critical for initiating the terminal differentiation of keratinocytes.

Keywords: Terminal differentiation, Keratinocytes, HaCaT cell, PI3K, PKB, S6.

INTRODUCTION

Epidermis is a layered tissue, which forms the uppermost, multilayered compartment of the skin and has evolved to provide a physical and permeability barrier, which is essential for survival as an adaptation to terrestrial life in mammals (Kalinin *et al.*, 2001). This barrier against the environment - which excludes foreign substances and organisms and prevents the loss of vital fluids - is provided, and continuously regenerated, by terminally differentiating keratinocytes. This process is known as cornification and is highly organized, both in space and in time (Candi *et al.*, 2005; Kalinin *et al.*, 2002). Keratinocytes move from a proliferative cell type in the basal cell layer of the epidermis through the granular layer where the cornified envelope is formed. At the cellular level, cornification begins with the synthesis of an immature type of envelope underneath the plasma membrane, and this envelope then undergoes maturation by the covalent attachment of preformed dedicated molecules to produce a rigid structure that fulfils its main physiological functions - that is, providing physical resistance and acting as a water barrier (Eckert *et al.*, 1997; Kalinin *et al.*, 2002).

In the biology of mammalian skin, there are two distinct types of cell death, apoptosis and terminal differentiation (or cornification), which occur in the lower and upper layers of the skin, respectively (Candi *et al.*,

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2005; Gandarillas *et al.*, 1999). However, some authors believe that terminal differentiation of keratinocytes is a specialized form of programmed cell death since a metabolically dead cornified cell envelope is the end point of epidermal differentiation (Maruoka *et al.*, 1997; Polakowska *et al.*, 1994). Moreover, the apoptotic program and keratinocyte differentiation share overlapping signaling effector mechanisms (Gandarillas, 2000). *In vivo*, lack of caspase 3 cysteine protease, an key component of the cell death signaling, resulted in increased proliferation and decreased embryonic keratinocyte differentiation via downstream of Notch1 (Okuyama *et al.*, 2004). Additionally, the group of Tschachler shows that caspase-14 is dispensable for keratinocyte apoptosis, suggesting that caspase-14 plays a part in terminal keratinocyte differentiation and skin barrier formation (Rendl *et al.*, 2002). Nevertheless, "classical" apoptosis and epidermal differentiation are distinct processes, with diverse execution times and biological outcomes; the former leads to the elimination of individual dead cells from tissues within hours, whereas the latter relies on the survival and synchronized maturation of whole sheets of cells over the course of weeks.

HaCaT cells are spontaneously immortalized keratinocytes (Rossler and Thiel, 2004) that have been extensively used as an established in vitro model system of keratinocyte cell biology. Many of these studies have analyzed keratinocyte growth and cell death, differentiation, or signal transduction. HaCaT cells are derived from an adult donor and are nontumorigenic in experimental animals (Rossler and Thiel, 2004).

As a first step toward understanding the precise cellular signaling events leading to terminal differentiation of keratinocytes, we have utilized HaCaT cells to monitor the biological consequences of Ca^{2+} stimulation and numerous downstream signaling pathways, including activation of the extracellular signal-regulated protein kinase (ERK) pathway and activation of phosphatidylinositol 3-kinase (PI3K). It has been suggested that activation of ERK and/or activation of PI3K is essential for controlling cell survival and proliferation of epithelial cells (Dufourny *et al.*, 1997; Kaufmann and Thiel, 2002). The results presented in this study show that Ca^{2+} function as potent agents for the differentiation of HaCaT keratinocytes, and this differentiation depends on the activation of ERK, Protein kinase B (PKB) and p70 ribosomal protein S6 kinase (p70S6K). The crucial role of ERK, PKB and p70S6K was further demonstrated in HaCaT cells using specific chemical inhibitors and Proteome Profiler™ Array. Finally, the results show that the expression of Activator protein 1 (AP-1; c-Jun and c-Fos) increased following Ca^{2+}-mediated differentiation of HaCaT cells, suggesting that ERK-mediated AP-1 expression is critical for initiating the terminal differentiation of keratinocytes.

MATERIALS AND METHODS

Chemical and reagents

Keratinocyte serum-free medium (KSFM), gentamicin, trypsin, and Lipofectamine were obtained from Life Technologies, Inc. LY294002, PD90859 and rapamycin were from Calbiochem. $CaCl_2$, $MnCl_2$, $MgCl_2$ and $CaCO_3$ were from Sigma. Goat polyclonal antibodies against fillagrin were from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against total PKB protein, Ser473-phosphorylated (active) PKB, total ERK 1/2 protein, Thr202-, Tyr204-phosphorylated (active) ERK, total S6 protein, Ser223-, Ser225-phosphorylated (active) S6, c-fos, c-jun were from Cell Signaling Technology. Mouse polyclonal antibodies against involucrin and loricrin were from Babco/Covance.

Cell culture

Immortalized human HaCaT keratinocytes were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco BRL). For experiments, cells were plated at $2 \times 10^4/cm^2$ in KSFM, supplemented keratinocyte basal medium containing 0.07 mM Ca^{2+}, 0.5 ug/ml hydrocortisone, 2 ug/ml transferrin, and 1 nM all-trans-retinoic acid plus 10 ng/ml EGF and 5 ug/ml insulin, and cultured for at least 24 h before use.

Induction of HaCaT cell differentiation

To prevent self differentiation by cell-cell contact, the cell number for plating was restricted. HaCaT cells were plated at a density of 1×10^5 cells/cm^2 and with KSFM containing 0.07 mM calcium and used in the subsequent experiments. To initiate differentiation, the concentration of $CaCl_2$ was raised to 1.2 mM. At indicated time, cells were washed with phosphate-buffered saline (PBS) and lysates prepared as described below. Keratinocytes in cell culture plates were washed twice with PBS and then incubated in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 25 mM NaF, 40 mM β-glycerol phosphate (pH 7.5), 1% NP-40, 120 mM NaCl and complete protease inhibitors for 20 min. Cells were scraped into microfuge tube, incubated at 4°C for 30 min, and pelleted by centrifuge. The supernatant was collected.

Gel electrophoretic methods and immunological detection

The protein concentration of the lysates was measured by a bovine serum albumin protein assay. Equal

amounts of protein were prepared in sample buffer (Laemmli, 1970) in the presence of 2% β-mercaptoethanol and electrophoresed through reducing SDS-PAGE and electroblotted onto polyvinylidene fluoride microporous membrane (Immobilon-P, 0.45 μm; Millipore). Involucrin, loricrin, c-Fos, c-Jun, PKB, pS473-PKB, p70S6K, pS299, ERK, pERK, S6, pS6 and Actin were detected by immunoblotting. For immunoblot, the membrane was blocked with 5% skim milk in tri-buffered saline (TBS) containing 0.02% Tween 20 and incubated for 12~15 h at 25C with each primary antibody with proper dilution. After incubation with a horseradish peroxidase-conjugated goat secondary anti-rabbit IgG antibody (Amersham Pharmacia Biotech) for 1 h at a dilution of 1 : 10,000, the blots were washed, and visualized using chemiluminesence detection reagents (Amersham Pharmacia Biotech). Quantification was performed using Tina 2.0 Program (Raytest GmbH).

Proteome Profiler™ Array

Proteome Profiler™ Array was carried out using the Human Phospho-MAPK array kit according to the manufacturer's instructions (R&D System). Briefly, cell lysates was placed into each well of the 4-well Multi-dish. The lysate-conjugated membranes were incubated with Anti-Phospho-MAPK Detection Antibody Cocktail and streptavidin-HRP for 1 hour. Then, cover with plastic wrap and expose to X-ray film for 1~10 min.

Immunostaining and indirect immunofluorescence microscopy

HaCaT cells were plated on sterile 24 × 24 mm coverslips at the density of 1×10^5 per six-well dish and cultured to desired points in growth media prior to staining. Cells were fixed with 3% formaldehyde/PBS for 1 h at room temperature (RT) and extensively washed with cold PBS. The cell membrane was permeabilized at RT with PBS containing 0.1% Triton and 0.1% Tween (5 min each) and cells were washed twice in PBS. The cover slips were incubated with primary antibodies overnight at 4 C, followed by FITC-conjugated goat anti-mouse or goat anti-rabbit secondary antibody incubation (Molecular Probes). Cells were mounted fluoromount-G solution (Southern Biotechnology Associates, Inc.) prior to fluorescence microscopy.

RESULTS

Ca^{2+} promote the differentiation of HaCaT cells

A calcium gradient is known to be present in the epidermis with relatively low calcium concentrations in the basal layer and higher concentrations in the suprabasal layer (Menon *et al.*, 1985; Pillai *et al.*, 1990). The presence of this in vivo gradient suggests a role for calcium in regulating keratinocyte differentiation. To examine the effect of divalent cations on keratinocyte differentiation, HaCaT cell were grown in KSFM containing one of three divalent salts ($MgCl_2$, $MnCl_2$, $CaCl_2$) for 12 hr following serum-starvation for 18 hr. Western blot analysis of keratinocyte differentiation marker reveal that calcium was the most potent agent to induce the differentiation of keratinocyte (Fig. 1A). Then we further evaluate the possibility if this ability for inducing keratinocyte differentiation was due to chloride instead of divalent cat-

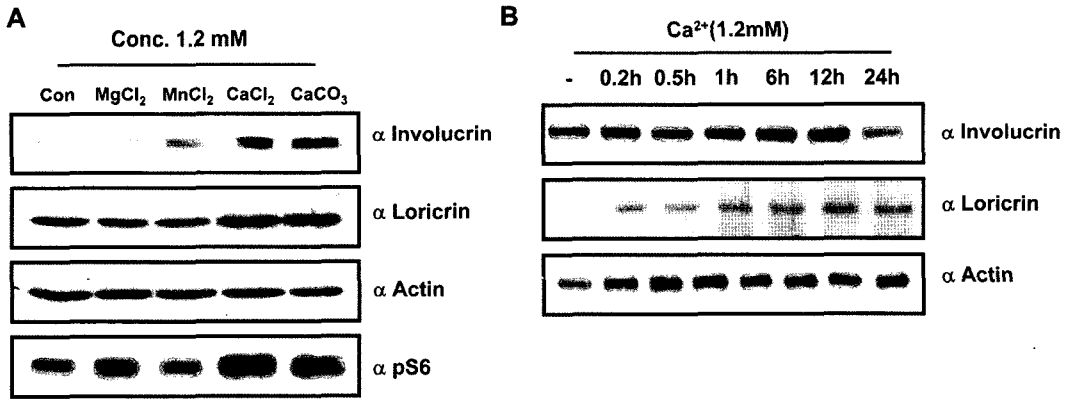

Fig. 1. Changes of involucrin and loricrin expression in Calcium-treated HaCaT cells. (A) HaCaT cells were grown until 50% confluent in DMEM supplemented with 10% serum, followed by the starvation of cells in KSFM for 18 hr. Cells were then left for 12 hr in same medium (control) or treated with 1.2 mM $CaCl_2$, 1.2 mM $ZnCl_2$, or 1.2 mM $MgCl_2$. Total cell lysates were harvested and subjected to a 12% SDS-polyacrylamide gel. The expression of involucrin, loricrin, phosphor-S6 or actin was analyzed by Western-blot analysis. (B) Time course of Ca^{2+}-induced keratinocytes differentiation. After the starvation of HaCaT cells in KSFM for 18 hr, cells were treated with 1.2 mM $CaCl_2$ for the indicated time. Blots were incubated with an antibodies directed against involucrin, loricrin or actin.

ions. Treatment of HaCaT cells with 1.2 mM of $CaCO_3$ also gave a similar result with the previous result (Fig. 1A). Additionally, phosphorylation of S6 was monitored in the above condition, since p70S6K is involved in the keratinocyte differentiation (Segrelles *et al.*, 2004). As expected, S6 phosphorylation was also remarkably increased (Fig. 1A). Next, we monitored the differentiation of HaCaT cells in presence of 1.2 mM of $CaCl_2$ in indicated time point by using antibodies directly against Involucrin or Loricrin which are induced following the differentiation process of keratinocyte. As shown in figure 1B, Involucrin was expressed at low level in untreated cell, was induced after 0.2 hr and was increased until 12 hr. These results suggest that calcium is indeed a potent inducer for keratinocyte differentiation.

Up-regulation of PKB, p70S6K and ERK activity in the process of keratinocyte differentiation

It has been suggested that Protein kinase C/Ras/ERK pathway is involved to induce the expression of differentiation marker gene, such as involucrin (Dashti *et al.*, 2001a, b; Efimova and Eckert, 2000). Therefore, we checked the status of ERK activation during keratinocyte differentiation induced by $CaCl_2$. ERK activity was stayed low until 1hr and was increased after 6hr incubation of HaCaT cells with $CaCl_2$, which is similar to the induction profile of involucrin expression (Fig. 1B and 2A). Adhesion to the basement membrane is a negative regulator of keratinocyte differentiation, and the disruption of adhesion signals triggers terminal differentiation (Watt *et al.*, 1988). PI3K is a fundamental signaling molecule that regulates the adhesion signal. To check the possible involvement of PI3K signaling in keratinocyte differentiation, we have monitored the phosphorylation status of PKB and S6, which is the downstream of PI3K signaling pathway. Phosphorylation of PKB was induced starting from 6 hr and increased until 24 hr. However, S6 phosphorylation was induced immediately and stayed high until 24 hr (Fig. 2A). These results indicated that Ras/ERK pathway and PI3K/PKB pathway somehow contribute to make keratinocyte to be differentiated.

To further evaluate the involvement of Ras/ERK pathway and PI3K/PKB pathway in keratinocyte differentiation, we have employed the specific inhibitor for each signaling molecule, such as ERK for PD98059, PKB for LY294002 and p70S6K for Rapamycin. Since S6 phosphorylation represents the status of keratinocyte differentiation (Fig. 1), we have monitored the status of S6 phosphorylation as a differentiation marker in this experiment condition. Calcium-induced differentiation of HaCaT cells were blocked by the pretreatment of cells with the specific inhibitors in time-dependent manner (Fig. 2B).

Global changes of enzymatic activity for various protein kinase sets in undifferentiated and differentiated HaCaT cells

It has been well established that phosphorylation and dephosphorylation is a key mechanism by which eukaryotic cells respond to extracellular stimuli (Johnson and Hunter, 2005). Therefore we have utilized the Proteome Profiler™ Array (developed from R&D System) which allows us to monitor the several sets of kinase activity in one membrane. With help of this method, we

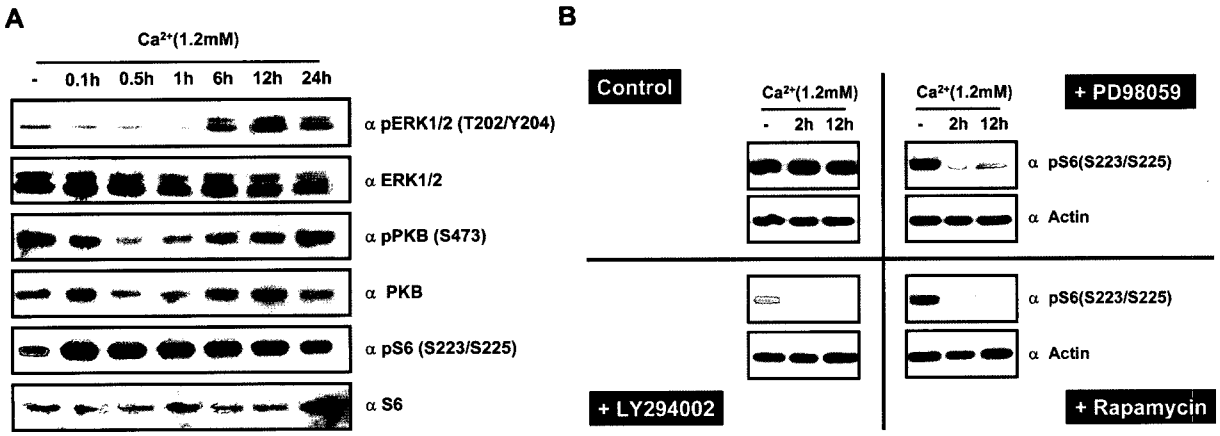

Fig. 2. Activation of PKB and ERK in the differentiated HaCaT cell. (A) HaCaT cells were serum starved for 18 hr, treated with 1.2 mM $CaCl_2$ for the indicated time. Total cell lysates were prepared and subjected to Western blot analysis. Blots were incubated with a first antibodies directed against S6, phosphorylated S6, ERK1/2, phosphorylated ERK1/2, PKB and phosphorylated PKB. (B) HaCaT cells were pretreated with LY294002 (50 nM) for 15 min, PD98059 (1 mM) for 30 min, Rapamycin (1 mM) for 1 hr or left untreated (Control), following the treatment of HaCaT cell with 1.2 mM $CaCl_2$ for the indicated time.

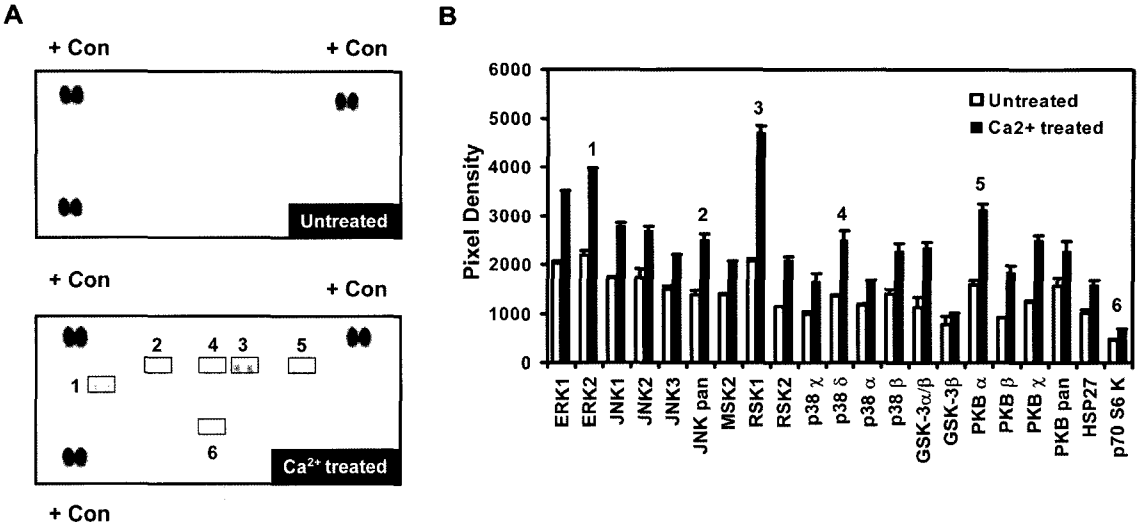

Fig. 3. Profiling of several protein kinase activity in the process of HaCaT cells differentiation. (A) HaCaT cells were left untreated (Untreated) or treated with 1.2 mM $CaCl_2$ for 24 hr (Ca^{2+} treated), following the serum starvation of cells for 18 hr. Total cell lysates was placed into each well of the 4-well multi-dish. The lysate-conjugated membranes were incubated with Anti-phospho-MAPK detection antibody cocktail, washed with PBS and visualized. (B) Semi-quantitative analysis obtained by densitometry of the corresponding western dot blot data.

can easily summarize the difference of phosphorylation status of several kinases. As shown in Fig. 3A and 3B, ERK2 (1), c-Jun N-terminal kinase (JNK) pan (2), p38d (4), p90 ribosomal protein S6 kinase 1 (3), PKBa (5), and p70S6K (6) were increased the phosphorylation after Ca^{2+}-mediated keratinocyte differentiation, which is consistent with the previous results (Fig. 2A and 2B). Additionally, we found the stress-activated protein kinase, such as JNK and p38 also involved in the process of keratinocyte differentiation, suggesting that additional signaling molecules will play a potential role in the keratinocyte differentiation.

Expression levels of AP-1 proteins are increased in time-dependent manner during keratinocyte differentiation

The AP-1 transcription factor consists of homo- or heterodimers of members of the Fos (c-Fos, FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD) proteins (Shaulian and Karin, 2001). It is well known that AP-1 regulates many genes encoding critical players of skin homeostasis (Angel *et al.*, 2001), including struc-

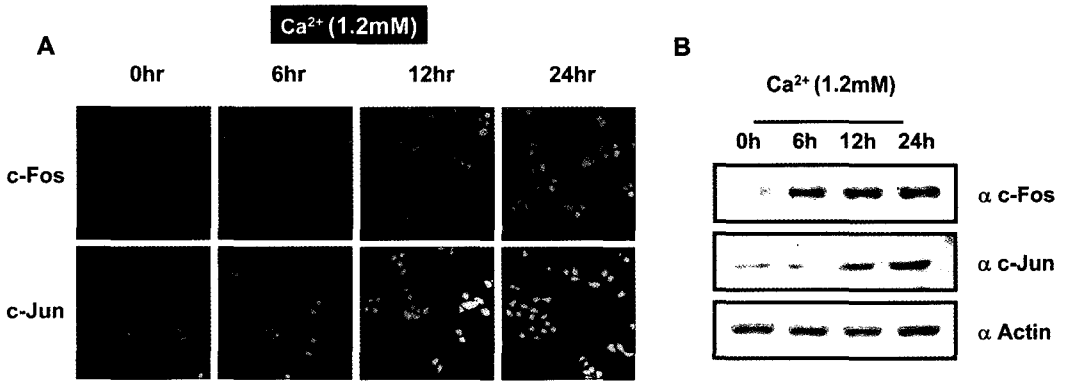

Fig. 4. Changes of AP-1 expression during Ca^{2+}-mediated differentiation of HaCaT cells. HaCaT cells were serum starved for 18 hr, treated with 1.2 mM $CaCl_2$ for the indicated time. Cells were either fixed on cover slips or lysed at the indicated time points. (A) Immunostaining of c-Fos and c-Jun proteins in monolayer culture was performed at the indicated time points. (B) Western blot analysis of c-Fos and c-Jun expression at the indicated time points of HaCaT cell differentiation. Fifty micrograms of nuclear extracts were analyzed on 12% SDS-polyacrylamide gels and transferred to PVDF membrane. Blots were incubated with an antibodies directed against c-Fos, c-Jun or actin.

al., 2004). Consistent with previous reports (Florin *et al.*, 2006; Mehic *et al.*, 2005; Zenz and Wagner, 2006), the expression of AP-1 (c-Jun and c-Fos) were induced in calcium-mediated HaCaT cell differentiation, monitored by immunofluorescence and biochemical analysis (Fig. 4A and 4B). Although in mice inactivation of some AP-1 proteins did not reveal an overt skin phenotype (Angel *et al.*, 2001), it is difficult to speculate about AP-1 functions in mouse skin as loss of JunB and Fra-1 results in embryonic lethality (Eferl and Wagner, 2003). Furthermore, it has been suggested that AP-1 proteins are expressed in a highly specific manner during terminal differentiation of keratinocytes and that the enhanced expression of c-Jun in basal and suprabasal keratinocytes might contribute to the pathogenesis of psoriasis (Mehic *et al.*, 2005).

Notably, ribosomal S6 protein, which is one of major downstream targets for p70S6K, gets phosphorylation during calcium mediated HaCaT cells differentiation (Fig. 1A and 2A). There is an evidence that p70S6K is also activated by PKB-dependent pathway (Goncharova *et al.*, 2002) and that a marked increase in 4E-binding protein 1 (4E-BP1; on Ser 65) and p70S6K (on Thr389) phosphorylation was evident in tumors derived from PKB-transfected keratinocytes compared with those derived from control keratinocytes (Segrelles *et al.*, 2004). However, others role out a regulatory role for p70S6K in keratinocyte differentiation, since it has been reported that constitutively active p70S6K or specific inhibitor produced relatively minor changes in human Involucrin promoter activity (Efimova and Eckert, 2000).

In summary, during keratinocytes travel across the epidermis towards the stratum corneum, calcium initiated the keratinocyte differentiation through the activation of Ras/ERK pathway and likely induced the expression of differentiation-related genes, mediated by AP-1 transcription factor. Additionally the activation of PI3K/PKB pathway is needed for the survival of differentiating keratinocytes during this apoptosis-like process. Although murine knock-outs of the p110a and p110b genes result in embryonic lethality, PKBa and PKBb double knockout animals die perinatally and display severe atrophy and impaired differentiation of the epidermis, suggesting essential function of these molecules in the tissue homeostasis (Peng *et al.*, 2003). However whether PKB proteins have specific roles in differentiation or simply affect cell proliferation still needs to be defined.

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