CK2 Is Acting Upstream of MEK3/6 as a Part of the Signal Control of ERK1/2 and p38 MAPK during Keratinocytes Autocrine Differentiation

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Protein kinase CK2 (formerly termed "casein kinase II") is a ubiquitously in mammalian cells distributed Ser/Thr kinase, with global role in cell regulation. Although, the involvement of CK2 in cell signalling is vast-investigated, virtually nothing is known about its contribution to signal control of keratinocytes differentiation. Here we show that, in autocrine differentiating keratinocytes, inhibition of the CK2 activity induced by 4,5,6,7-tetrabromobenzotriazole (TBB) causes reciprocal changes in the activities of major signal transduction regulators of keratinocytes differentiation, *i.e.* ERK1/2 and p38 MAPK, without affecting their protein levels. The ERK1/2 activity is strongly suppressed, while the activity of p38 is increased. We have also found that the activity of upstream and specific for p38 MAPK kinase MEK3/6 is also stimulated by TBB. These original results clearly demonstrate the participation of CK2 in the signal transduction pathway controlling MEK3/6, p38 MAPK, and ERK1/2 in the used model system.

Key words: CK2, MAPK, Keratinocytes Differentiation

Introduction

Protein kinase CK2 (formerly termed "casein kinase II") is an extremely conserved and second messenger-independent Ser/Thr kinase. CK2 occurs ubiquitously in mammalian cells, where it most often appears to exist in tetrameric complexes, consisting of two catalytic subunits ($\alpha\alpha$, $\alpha\alpha$' or α'α') and two non-catalytic subunits (β). The holoenzyme and the isolated catalytic subunits are constitutively active, even in the absence of any stimulus, which is provided by special structural features (Niefind *et al.*, 2009). Accumulated biochemical, genetic, and cell biological evidence indicates that CK2 plays a global role in cell regulation, however, the precise molecular mechanisms are still poorly understood (Filhol and Cochet, 2009). We have previously reported the implication of CK2 in the signal transduction that regulates keratinocyte autocrine proliferation (Isaeva *et al.*, 2007; Isaeva and Mitev, 2009). Here, taking advantage of the CK2 cell-permeable inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) (Sarno *et al.,* 2001), we demonstrate that CK2 participates in keratinocyte autocrine differentiational signalling and, most importantly, that its activity is a part of the signal transduction controlling ERK1/2, p38 MAPK, and its upstream and regulatory kinase MEK3/6.

Keratinocyte physiology is subject to sophisticated control by a signal transduction network, in which the mitogen-activated protein kinase (MAPK) cascades take a central place. Each MAPK pathway contains a three-tiered kinase cascade comprising a MAP kinase kinase kinase (MAPKKK or MEKK) that phosphorylates and activates a MAP kinase kinase (MAPKK or MEK), which in turn activates MAP kinase (MAPK). Five families of MAPKs have been defined in mammalian cells: extracellular signal-regulated kinases (ERK1 and ERK2), Jun N-terminal kinases (JNK1, JNK2, and JNK3), p38 kinase isozymes [p38α, p38β, and p38δ are expressed in epidermal keratinocytes (Dashti *et al.*, 2001a)], ERK3/ERK4, and ERK5. Phosphorylation of both threonine and tyrosine residues at the dual phosphorylation motif is required for MAPK activation (Chang and Karin, 2001).

Material and Methods

Primary keratinocyte culture and treatment

Normal human epidermal keratinocytes were derived from adult skin obtained from surgery

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(Dr. D. Hristov, Clinic for Plastic and Aesthetic Surgery, University Hospital "Aleksandrovska", Sofia, Bulgaria) and cultured in a defined keratinocyte growth medium (KGM) (Cambrex Bio Science, Walkersville, MD, USA). The cell cultures were incubated at 37 °C and 5% CO_2 in a humidified incubator. Third passage keratinocytes at 60% confluence were switched to KBM (Cambrex Bio Science), the same medium as KGM but without supplements, and cultured to monolayer to obtain confluent cells. The confluent keratinocytes were treated 24 h later with increasing concentrations of TBB (Calbiochem, Darmstadt, Germany) as described in the text and the figure legend. Control cells were treated with equal amounts of the solvent DMSO. TBB was dissolved in DMSO (the content of DMSO added to cells never exceeded 0.1%, v/v) according to manufacturer's instructions and stored frozen as stock solutions at –80 °C.

Western blot analysis

Western blot analysis was performed as described previously (Isaeva *et al.*, 2007). Briefly, whole cell lysates (containing 50–100 μg protein) of TBB-treated or untreated primary keratinocytes were obtained, proteins resolved by SDS-PAGE and transferred to PVDF membranes (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The membranes were then probed with anti-phospho-ERK1/2 ab and anti-ERK1/2 ab (Upstate Biotechnology, Charlottesville, VA, USA), anti-phospho-p38 MAPK ab (Cell Signaling Technology, Danvers, MA, USA) and anti-p38 MAPK ab (Biosource Technologies, Vacaville, CA, USA), anti-phospho-MEK3/6 ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA). For control of protein loading, after membrane-stripping, β-actin was detected with a monoclonal anti-β-actin antibody (Sigma, St. Louis, MO, USA). Primary antibodies were revealed with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) and SuperSignal reagent (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA).

Determination of cell viability by the MTT assay

The MTT assay was performed as described previously (Isaeva and Mitev, 2009). Briefly, TBB-treated or untreated primary keratinocytes were cultured in the presence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT) (Sigma) solution for 2 h in the dark at 37 °C. Formed formazan crystals were dissolved in isopropanol for 30 min, and the absorbance at 570 nm was spectrophotometrically measured and converted into percentage of cell viability, using control cultures to determine 100% of cell viability. The results are expressed as mean ± SD, and were calculated from three experiments conducted on quadruplicate samples. Significance of differences to the control was determined using ANOVA ($p < 0.05$).

Results and Discussion

The autocrine differentiating keratinocytes were treated with increasing concentrations of TBB for 8 h, and activation states and protein levels of ERK1/2 and p38 MAPK were analysed by Western blot. The ERK1/2 activity was strongly suppressed by TBB, without changes in its protein level. Simultaneously, the p38 MAPK activity was stimulated, again without any effect on its level. Next, since functioning of p38 MAPK is regulated by its upstream and specific kinase MEK3/6, the effect of TBB on the activity of MEK3/6 was also investigated. MEK3/6 activation state was increased in TBB-treated keratinocytes (Fig. 1A). The concentrations of TBB used did not affect the keratinocytes viability (Fig. 1B).

In epidermal keratinocytes a unique ERK1/2-p38δ complex operates (Efimova *et al.*, 2003, 2004). In the complex, the ERK1/2 activity and p38δ activity are coordinately and inversely regulated by novel protein kinase C (PKC) isoforms (δ, ε, and η), Ras, MEKK1, MEK3, MEK6, and MEK7, and also by subcellular localization of the complex (Efimova and Eckert, 2000; Dashti *et al.*, 2001a, b; Balasubramanian *et al.*, 2002; Efimova *et al.*, 1998, 2002, 2004). High ERK1/2 activity – low p38δ activity maintains keratinocyte proliferation, while low ERK1/2 activity – high p38δ activity induces differentiation and apoptosis.

In contrast to the widely accepted concept of predominant implication of CK2 in proliferation signalling (St-Denis and Litchfield, 2009), our study reveals its important participation also in the signal transduction in keratinocyte maturation. Since TBB-mediated inhibition of the CK2 activity causes stimulation of the MEK3/6 activity, resulting in upregulation of the p38 MAPK

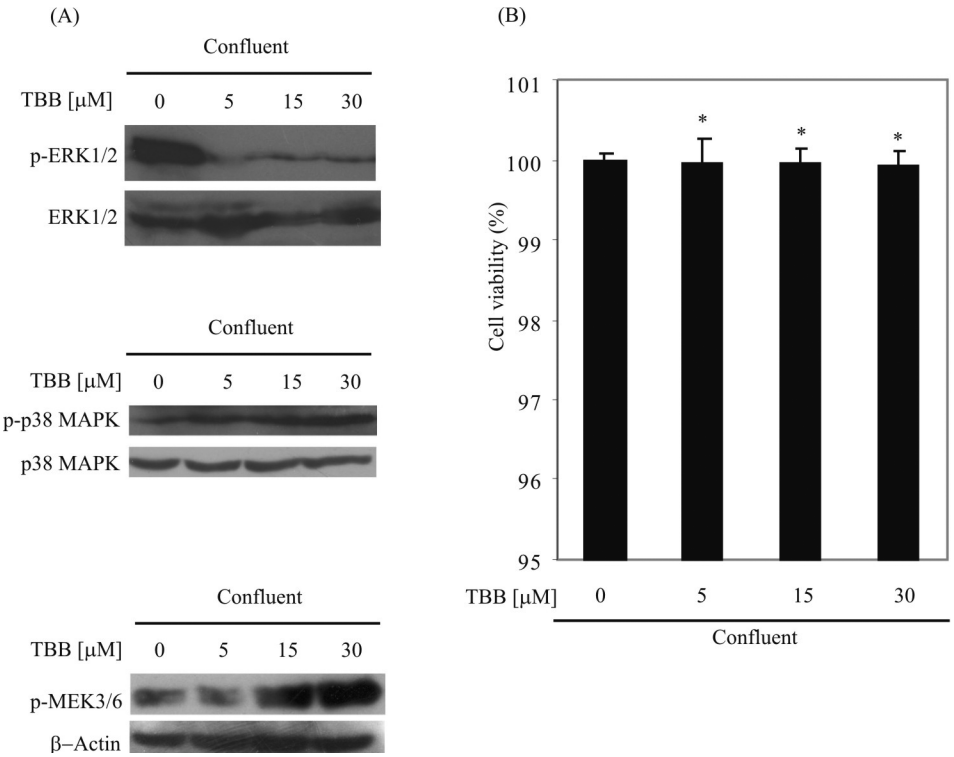

Fig. 1. Inhibition of CK2 activity by TBB suppresses ERK1/2 activity, stimulates p38 MAPK and MEK3/6 activities, and has no effect on keratinocyte viability. Autocrine differentiating keratinocytes were treated with the indicated concentrations of TBB for 8 h. (A) Whole cell lysates were obtained and analysed by Western blot for basal and phosphorylated ERK1/2, p38 MAPK, and MEK3/6, respectively. The above results are representatives of three independent experiments. (B) Keratinocyte viability was determined as described in Material and Methods.

activity and simultaneous downregulation of the ERK1/2 activity, we conclude that CK2 acts upstream of MEK3/6 in the regulation of ERK1/2 and p38 MAPK functioning. Even though, the specific affects of CK2 on p38 kinase isozymes activities remain to be identified, in view of our results revealing opposite effects on ERK1/2 and p38 activity, respectively, we are attempted to speculate that CK2 actually participates in the control of the ERK1/2-p38δ complex.

This is, to our knowledge, the first report for implication of CK2 in the signal transduction pathway controlling the functioning of MEK3/6, p38 MAPK, and ERK1/2 in autocrine differentiating normal human epidermal keratinocytes.

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