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Dermokine-β impairs ERK signaling through direct binding to GRP78

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ABSTRACT

Dermokine-β is abundant in stratified epithelia and in differentiating cultured keratinocytes. In this study, we investigated the role of dermokine-β in differentiation of keratinocytes. Treatment of keratinocytes or skin tumor cells with dermokine-β attenuated phosphorylation of extracellular-signal-regulated kinase (ERK). Exposure of cells to dermokine-β, as well as its carboxyl-terminus domain peptide, interrupted phosphorylation of ERK and stimulated dermokine gene expression. Inhibition of ERK signaling by its specific inhibitor also increased dermokine expression level. A combination of chemical cross-linking and immunoprecipitation, followed by proteomics analyses, identified glucose-regulated protein 78 (GRP78) as a dermokine-β-associated protein. Blockage of GRP78 expression by a specific siRNA abrogated actions of dermokine-β. These findings provide novel insights into the physiological significance of dermokine-β in the epidermis.

Structured summary of protein interactions:
dermokine-Beta physically interacts with GRP78 by cross-linking study (View interaction)
dermokine-Beta(Glo2) and GRP78 physically interact by competition binding (View interaction)
dermokine-Beta binds to GRP78 by anti bait coimmunoprecipitation (View interaction)

1. Introduction

The epidermis is a keratinized, stratified squamous epithelium composed of basal, spinous, granular, and cornified cell layers [1]. Keratinocytes differentiate in the basal layer, where epidermal stem cells are abundant, and migrate gradually to the cornified layer [2]. Numerous studies of spatial and temporal changes in gene expression patterns during keratinocyte differentiation have revealed layer-specificity. Involucrin, transglutaminases, loricrin, cystatin, filaggrin and desmoplakin have been identified as upper spinous and granular layer-specific proteins [3].

Dermokine was first identified as a spinous layer-specific gene by a signal trap expression screening strategy in mice [4,5]. Northern blotting and quantitative RT-PCR revealed that dermokine is abundant in stratified epithelia and in differentiating primary human keratinocytes [4]. Human dermokine has been reported to be encoded by 25 exons, spanning 17 kb of genomic DNA [4–6]. Its expression leads to four groups of transcripts according to three different transcriptional start sites, two termination sites and several alternative coding exons [7]. Unlike the α, β, and γ isoforms, dermokine-δ mRNAs do not encode a putative signal peptide and are predicted to produce cytosolic proteins [8]. Although precise physiological contributions of dermokine have yet to be revealed, Leclerc et al. demonstrated that dermokine-δ activates Rab5 and thus is involved in early endosomal trafficking [8]. Dermokine-β, a major isoform in epidermis, does not have significant homology to other proteins. It is generally known that many cytokines, like bone morphogenetic proteins (BMPs), eotaxin, fibroblast growth factors (FGFs), interferon-β, interleukins, platelet-derived growth factor (PDGF), and Wnt proteins (Wnts), have high p*I* values [4]. The carboxyl terminal domain of dermokine-β also has a high p*I* value, suggesting some biological functions of dermokine-β.

We have recently shown that the expression levels of dermokine-β were increased in inflammatory skin disorders, while decreased in skin cancers [9]. Proinflammatory cytokines such as interleukin-1β,

Abbreviations: ERK, extracellular-signal-regulated kinase; MEK, mitogen-activated protein kinase; SCC, squamous cell carcinoma; GRP78, glucose-regulated protein 78

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interleukin-12, and tumor necrosis factor-α augmented dermokine expression in cultured human keratinocytes. In contrast, growth factors including epidermal growth factor, insulin-like growth factor-I, keratinocyte growth factor, and transforming growth factor-α significantly reduced dermokine expression [9]. The present study was conducted to reveal the physiological significance of dermokine-β in the epidermis.

2. Materials and methods

2.1. Cell cultures and reagents

Normal human epidermal keratinocytes (Kurabo, Osaka, Japan) were grown in serum-free medium, Humedia KG2 (Kurabo), in 5% CO_2 at 37 °C. Human squamous cell carcinoma (SCC) and G-361 melanoma cells were obtained from Health Science Research Resources Bank (Osaka, Japan), and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Bacterial expression plasmids were prepared by ligating the respective coding sequence into pGEX-6P-1 (GE Healthcare, Buckinghamshire, England). A rat anti-human dermokine-β/γ monoclonal antibody raised against amino-terminus region of dermokine-β was prepared as previously described [9]. Anti-extracellular-signal-regulated kinase (ERK)1/2, phospho-ERK1/2 and GRP78 antibodies, and mitogen-activated protein kinase kinase (MEK)1/2 inhibitor U0126 were purchased from Cell Signaling Technology (Danvers, MA). Glutathione sepharose beads and PreScission protease were purchased from GE healthcare. A cross-linker, dithio-bis-maleimidoethane (DTME), was obtained from Thermo Scientific (Rockford, IL).

2.2. Transfection of siRNA

The sense strand sequence of the siRNA used for targeting GRP78 was UGAAGAACUCUUUAACCAGUUGCUG. Stealth RNAi Negative Control Medium GC Duplex was used as a control (Invitrogen, Carlsbad, CA). Normal human keratinocytes were transfected with 2 μg of siRNA using Lipofectamine 2000 (Invitrogen) as previously described [10]. Seventy-two hours later, cells were incubated with recombinant dermokine-β.

2.3. Expression analysis

The amounts of mRNA were quantified using MESA Blue qPCR Mastermix (Eurogentec, Seraing, Belgium) as previously described [9]. Relative mRNA expression levels of each gene were normalized against those of the glyceraldehyde-3-phosphate dehydrogenase gene in the same RNA preparation. The primers used were as follows: dermokine-β/γ; forward primer, 5′-GGCAATGGAGGGCCACCAAAC-3′, reverse primer, 5′-GGGATTCGTGCACCCTTCATTC-3′, involucrin; forward primer, 5′-GGAGCTCCTCAAGACTGTTCCT-3′, reverse primer, 5′-GCTCGACAGGCACCTTCTG-3′, *GAPDH*; forward primer, 5′-AGCGACACCCACTCCTCCAC-3′, reverse primer, 5′-GAGGTC CACCACCCTGTTGC-3′.

2.4. Recombinant protein

The secreted form of dermokine-β, its partial peptides, and GRP78 fused to glutathione S-transferase (GST) were expressed in *Escherichia coli* and purified as previously described [11]. Through the purification on glutathione sepharose beads and PreScission protease cleavage, recombinant dermokine-β, partial peptides, and GRP78 with high purity were obtained.

2.5. Chemical cross-linking, immunoprecipitation, and microsequencing

Chemical cross-linking reactions were performed as previously described [12]. Briefly, human keratinocytes cultured in the media without bovine pituitary extract (BPE) for 3 days were incubated with 10 μg/ml of dermokine-β for 30 min, and then DTME (0.2 mM) was added for 30 min at 37 °C. After the cross-linking reaction, cell lysates were prepared and immunoprecipitation using rat anti-dermokine-β/γ monoclonal antibody was performed as previously described [11]. Proteins bound to beads were resolved by 5–10% SDS–PAGE, and then visualized by silver staining. Desired bands were excised, in-gel digested with trypsin and solvent extracted, and the resulting MS/MS spectra were searched using the MASCOT search engine as previously described [13].

2.6. Western blot

Whole cell extracts were resolved by SDS/PAGE, transferred to a PVDF membrane, and blocked with 5% bovine serum albumin for 30 min at room temperature as previously described [13]. After incubation with primary antibodies, the proteins of interest on immunoblots were detected using an enhanced chemiluminescence detection system.

2.7. Caspase activity

The apoptotic response was measured by Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega GmbH, Mannheim, Germany) in accordance with the instructions of the manufacturer.

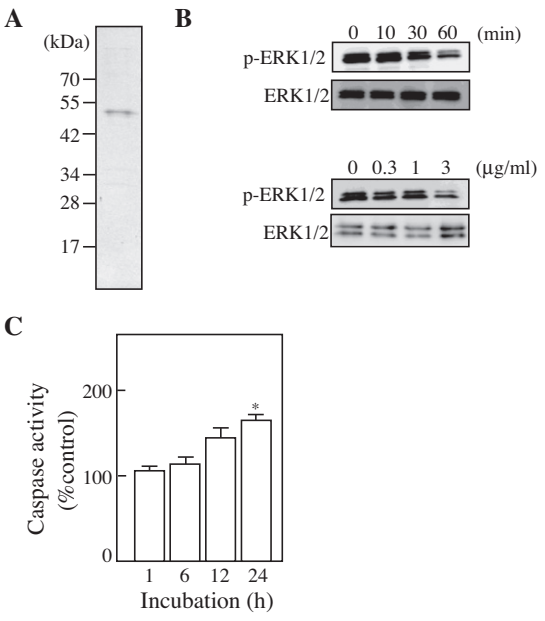

Fig. 1. Dermokine-β inhibits phosphorylation of ERK1/2 in cultured keratinocytes. (A) Recombinant human dermokine-β was prepared, and stained with Coomassie Brilliant Blue. Note that only one band was observed, with molecular weight of 50 kDa. (B) Normal human keratinocytes were treated with dermokine-β for various periods of incubation at 3 μg/ml (upper panel) or at various concentrations for 60 min (lower panel). The levels of phosphorylated ERK1/2 and ERK1/2 were detected in the same Western blot membrane. Similar results were obtained in three independent experiments. (C) Human keratinocytes were treated with dermokine-β for various periods of incubation, and the caspase-3/7 activity was determined. Data shown are mean ± S.D. values ($n = 3$). *, $P < 0.05$.