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14-3-3σ controls corneal epithelial cell proliferation and differentiation through the Notch signaling pathway

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ABSTRACT

14-3-3σ (also called stratifin) is specifically expressed in the stratified squamous epithelium and its function was recently shown to be linked to epidermal stratification and differentiation in the skin. In this study, we investigated its role in corneal epithelium cell proliferation and differentiation. We showed that the 14-3-3σ mutation in *repeated epilation* (*Er*) mutant mice results in a dominant negative truncated protein. Primary corneal epithelial cells expressing the dominant negative protein failed to undergo high calcium-induced cell cycle arrest and differentiation. We further demonstrated that blocking endogenous 14-3-3σ activity in corneal epithelial cells by overexpressing dominative negative 14-3-3σ led to reduced Notch activity and Notch1/2 transcription. Significantly, expression of the active Notch intracellular domain overcame the block in epithelial cell differentiation in 14-3-3σ mutant-expressing corneal epithelial cells. We conclude that 14-3-3σ is critical for regulating corneal epithelial proliferation and differentiation by regulating Notch signaling activity.

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Introduction

The corneal epithelium is a stratified squamous epithelium that undergoes constant renewal with an estimated turnover time of about 7–10 days in mammals [1]. The corneal epithelium is composed of a mitotically active basal monolayer and differentiating suprabasal layers. The edge of the cornea at the junction with the sclera is comprised of a limbal region which is a niche for corneal stem cells that generate new basal cells [2–4]. Recent studies have shown that some stem-like cells scattered throughout the corneal epithelium can regenerate the corneal and conjunctival epithelia [5]. The corneal epithelial progenitor cells in the basal layer of the normal corneal epithelium generate transient amplifying (TA) cells that undergo multiple divisions and gradually migrate to the upper layer. During this process, the differentiating epithelial cells switch expression of differentiation-specific cytokeratins from the universal stratified epithelial cytokeratin pair of keratin-5 (K5) and K14 to cytokeratin-3 (K3) and K12 [6]. The terminally differentiated epithelial cells in the most superficial layer form desmosomes and tight junctions, which act as a barrier to protect the cornea from environmental insults [7,8]. The same corneal epithelial differentiation process can be largely achieved in cell culture using culture conditions that allow the culture, expansion, and differentiation of corneal epithelial progenitor cells. The molecular machinery that controls and maintains corneal epithelial cell homeostasis is still poorly understood.

14-3-3σ belongs to a seven member family of highly conserved phosphoserine/phosphothreonine-binding proteins regulating cell signaling of numerous processes that control the cell cycle, cell growth, differentiation, apoptosis, and cell migration [9]. In contrast to other 14-3-3 proteins, 14-3-3σ is specifically expressed in the stratified squamous epithelium and forms homodimers with a target specificity different from those of dimers of other family members [10]. Because of its unique target substrates, 14-3-3σ has specific cellular functions; for example, it is the only tumor suppressor protein in the family. It is also an important G2M cell cycle checkpoint regulator and its expression is induced by DNA damage stress through a p53-dependent pathway [11]. Epigenetic silencing of the 14-3-3σ gene through promoter methylation has been reported in various tumors of epithelial origin [12].

14-3-3σ is a key regulator of stratified squamous epithelial homeostasis. In skin, after exiting from the stem cell compartment, differentiating keratinocytes show increased expression of

Abbreviations: TA, transient amplifying; K5, keratin-5; K14, keratin-14; K3, cytokeratin-3; K12, keratin-12; NICD, Notch intracellular domain; RBP1, retinol binding protein 1; WT, wild type; Er, repeated epilation; PFA, paraformaldehyde; BrdU, 5-bromo-2′-deoxyuridine; DAPI, diamidinophenylindole; Q-PCR, quantitative polymerase chain reaction; GFP, green fluorescent protein

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14-3-3σ [13], suggesting that 14-3-3σ is involved in initiation of differentiation of epithelial cells. Studies in *Er* mice, in which *14-3-3σ* is mutated, have clearly demonstrated that 14-3-3σ is essential for skin epithelial differentiation [14,15]. We previously showed that, in *Er* mice, a single nucleotide insertion in the 14-3-3σ coding region leads to a truncated protein lacking the C-terminal 40 amino acids, which form part of the ligand-binding domain [15]. Theoretically, the mutant 14-3-3σ could still dimerize with wild type 14-3-3σ, but the resulting complex would be inactive due to lacking any ligand binding ability, suggesting that the mutant 14-3-3σ would act as a dominant negative protein.

In this study, we showed that 14-3-3σ is a key regulator of corneal epithelial cell homeostasis. Blocking 14-3-3σ activity with the dominant negative mutant 14-3-3σ resulted in increased cell proliferation and diminished differentiation of corneal epithelial cells. These defects were associated with reduced Notch activity and Notch receptor expression. Furthermore, reintroduction of an active form of Notch restored the differentiation of the mutant cells. Our data suggest that 14-3-3σ regulates corneal epithelial cell proliferation and differentiation by maintaining Notch activity.

Materials and methods

Animals. C57BL/6J mice were purchased from the Jackson Laboratory and housed under pathogen-free conditions in accordance with institutional guidelines. Animal care and use were approved by the Institutional Animal Care and Use Committee of the University of Louisville.

Corneal epithelial cell culture. Eyes were collected from euthanized mice and treated overnight at 4 °C with dispase (10 mg/ml in 1640 medium) to disrupt the basement membrane, then the epithelial sheets were peeled off and digested in 0.25% trypsin–EDTA at 37 °C for 5–10 min. The cells were washed in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (HyClone) and resuspended in keratinocyte serum-free medium (KSFM; Invitrogen) containing 0.05 mM calcium and plated on collagen-coated tissue culture plates. To induce differentiation, the cultures were exposed to 1.6 mM calcium for 48 h, then were fixed in 4% paraformaldehyde (PFA) for immunostaining or collected for Western or Q-PCR analysises.

Immunohistological analysis. The PFA-fixed cells were treated for 25 min at 95 °C with Tris–EDTA buffer (10 mM Tris base, 1 mM EDTA, pH 9). All subsequent steps were done at room temperature. The treated cells in 24-well plates were incubated for 1 h with 5% donkey serum, then for 90 min with primary antibodies and for 30 min with a fluorescein-conjugated secondary antibody, both in PBS containing 1% BSA and 0.1% Triton X-100. The primary antibodies used were mouse anti-p63 (1:200, sc-8431, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rat anti-BrdU (1:800, MAS 250c, Harlan-Sera Lab, Loughborough, UK), and rabbit anti-ZO-1 (1:600, Cat #61-7300, Zymed Laboratories, Inc., San Francisco, CA). Secondary Cy3- or FITC-conjugated antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Nuclei were stained with diamidinophenylindole (DAPI).

293T culture, transfection, and co-immunoprecipitation. 293T human embryonic kidney cells (Invitrogen, Carlsbad, California) in 10-cm plates were maintained in DMEM medium containing 10% FBS and were transfected by the calcium phosphate method. Two days after transfection, the cells were lysed in cold RIPA buffer [20 mM Tris–HCl, 100 mM NaCl, 0.2% deoxycholic acid, 0.2% Triton X-100, 0.2% NP-40, and protease inhibitor cocktail (Roche)]. After clearance by incubation for 30 min with 50 μl of normal goat IgG (from Santa Cruz, both 1 μg/ml) and 100 μl of protein G-coupled agarose beads slurry (Amersham Biosciences), the lysates were incubated for 2 h at 4 °C with 30 μl of protein G-coupled agarose beads slurry goat antibodies against the C-terminus of 14-3-3σ (1:200, sc-7683) or normal goat IgG (sc-2028) (both from Santa Cruz, both 1 μg/ml). The beads were then washed five times before the antibody–antigen complexes were released from the beads with SDS lysis buffer and loaded on 10% SDS–polyacrylamide gels and subjected to Western analysis with goat antibody against the N-terminus of 14-3-3σ (1:200, sc-7681, Santa Cruz).

Western blotting for NCID. Please see details in the Supplementary material.

Quantitative polymerase chain reaction. Total RNA was isolated from mouse primary corneal epithelial cell cultures using RNeasy Protect Mini Kit (Qiagen). Reverse transcription was performed with 2 μg of total RNA using random hexamers and a cDNA synthesis kit (SuperScript First-Strand Synthesis System, Invitrogen, Carlsbad, CA). The quantitative polymerase chain reaction (Q-PCR) was performed according to the manufacturer's instructions (Stratagene Mx3000P QPCR System, Stratagene, San Diego, California) using a primer set for actin as the internal standard. Primers for mouse *Notch1* (ID 13177625a1), *Notch2* (ID 33859592a3), *Jagged1* (ID 7305197a1), *Jagged2* (ID 2765404a1), and *RBP1* (ID 6755300a1) were designed based on the PrimerBank database (Harvard Medical School). The *Hes1* primer sequences have been published previously [16].

Lentiviral production. The lentivector EF.hiCN1.CMV.GFP was obtained from Addgene. Details of the production of the lentiviral expression vectors CMV.GFP and CMV.14-3-3σMut have been published previously [15]. Lentiviruses were produced by co-transfection with lentiviral vector and the packaging vectors pMDL, pRev, and pVSVG (K4975-00, Invitrogen, Carlsbad, California) as described previously [17].

Results

The mutant 14-3-3σ has a dominant negative effect

It is thought that the 14-3-3σ mutant might act as a dominant negative, as it retains the homodimerization domain that can dimerize with wild type (WT) 14-3-3σ, but lacks the region that participates in phospho-binding motif formation [15] and therefore the complex is inactive. To determine whether mutant 14-3-3σ functions as a dominant negative, we first asked whether there was an interaction between WT 14-3-3σ and mutant 14-3-3σ. 293T cells were transfected with WT 14-3-3σ cDNA and/or mutant 14-3-3σ cDNA, then the cells were solubilized and the lysates subjected to immunoprecipitation with antibodies recognizing the C-terminus of WT 14-3-3σ, but not the 14-3-3σ mutant, which lacks this region. Western blots of the immunoprecipitated protein using antibodies against the N-terminus of 14-3-3σ (recognizing both WT and mutant 14-3-3σ) showed that the mutant 14-3-3σ co-immunoprecipitated with WT 14-3-3σ (Fig. 1, top panel, lane 7), thus demonstrating that the mutant 14-3-3σ forms heterodimers with WT 14-3-3σ. In the lysate of the cell co-transfected with equal amounts of the WT and mutant cDNAs, more WT protein was immunoprecipitated than mutant protein, indicating that both WT homodimers and WT/Mut heterodimers were formed, but heterodimers were dominant. Next, we asked whether the WT/mutant 14-3-3σ dimer could bind ligands. A well-known ligand for 14-3-3σ is Cdc2, a cyclin-dependent kinase that is critical for G2M cell cycle progress [11,18]. By Western blotting using anti-Cdc2 antibody, we showed that Cdc2 was bound to the WT 14-3-3σ homodimer (Fig. 1, lane 3 vs lane 4), but was absent in the sample containing dominantly WT/Mut heterodimers (Fig. 1, Lane 7 vs lane 3). These data demonstrate that mutant 14-3-3σ indeed functions as a dominant negative binding partner blocking the function of WT 14-3-3σ.