Journal of Dermatological Science 78 (2015) 189–196

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

NF-κB-regulated transcriptional control of CLCA in a differentiated mouse keratinocyte line

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ARTICLE INFO

Article history:
Received 25 November 2014
Received in revised form 13 February 2015
Accepted 5 March 2015

Keywords:
CLCA
Keratinocyte
NF-κB
Differentiation

ABSTRACT

Background: CLCA was postulated to be a calcium-activated chloride channel accessory protein. Recent reports indicate that CLCA isoforms are likely to be expressed in different layers of the stratified epithelium of the skin.
Objective: The present study investigated the transcriptional mechanism by which murine CLCA2 (mCLCA2) is expressed in the transformed keratinocyte line Pam212 that can differentiate.
Methods: A luciferase reporter assay, chromatin immunoprecipitation (ChIP) assay, reverse transcription-PCR, and immunocytochemistry were performed using Pam212 cells.
Results: Promoter activity of mCLCA2 was inhibited profoundly by site-directed mutagenesis of a putative nuclear factor-κB (NF-κB) binding site and by treatment with siRNA against p65. ChIP and transcription factor assays showed the specific association of endogenously activated p65 protein with the NF-κB binding domain. As confirmed by the nuclear translocation of p65, tumor necrosis factor α and caffeic acid phenethyl ester (CAPE) increased and decreased mCLCA2 promoter activity, respectively, but exhibited modest effects on endogenous mCLCA2 expression in cells in culture medium containing 0.05 mM Ca^{2+}. When the Ca^{2+} concentration was raised to 1.0 mM, the mRNA and protein levels of mCLCA2 increased as well as those of the differentiation markers keratin 1 (K1) and K10. CAPE profoundly suppressed only the Ca^{2+}-triggered expression of mCLCA2, not K1 or K10. Immunohistochemistry of native skin and organotypic 3D cultures confirmed the distribution of the CLCA2 homolog in differentiated cells.
Conclusion: The present study revealed for the first time that basal NF-κB activity is involved in the Ca^{2+}-dependent regulation of mCLCA2 expression in a mouse keratinocyte line.

1. Introduction

CLCA was postulated to be a calcium-activated chloride channel accessory protein that modulates the channel pore [1,2]. Previously, we identified a rat CLCA homolog (rCLCA, rCLCA2) that is responsible for modulating Ca^{2+}-dependent Cl^- transport in salivary ductal cells of the rat submandibular gland [3]. More recently, we have shown that isoforms of rCLCA exhibit specific localization and function on epithelial cells in rat skin [4].

Among the 8 mouse *Clca* genes located on chromosome 3, mCLCA2, an isoform sharing a 83% amino acid identity with rCLCA, was shown to be expressed in lactating and involuting mammary glands, suggesting its involvement in stage-specific organogenesis [5,6]. Another mouse isoform, mCLCA1, shares a 95% amino acid sequence identity with mCLCA2, but its tissue distribution is quite distinct [7]. These observations suggest that specific transcriptional regulation occurs for the distinct expression and function of CLCA isoforms [6].

Several CLCA isoforms are reportedly expressed in the stratified epithelium. Human CLCA2 (hCLCA2) is localized along the basal membrane of basal epithelial cells of the cornea and skin and its expression is elevated during epithelial stratification [8,9]. Porcine CLCA2 was detected in the granular layer [10]. Among the mouse

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http://dx.doi.org/10.1016/j.jdermsci.2015.03.007
0923-1811/

isoforms, mCLCA5 was shown to be expressed in the granular layer of the skin and oral cavity, especially in keratohyalin granules [11]. We demonstrated the expression of the full-length isoform of rCLCA in granular and spinous layers of the skin [4]. Recently, Bart et al. [12] have shown that rCLCA and mCLCA2 are novel target genes of ultraviolet (UV) radiation and may play a role in epidermal differentiation. Although these CLCA isoforms are likely to be expressed in keratinocytes, specific regulation of their gene expression has not yet been elucidated.

To clarify the transcriptional mechanism underlying the expression of these CLCA isoforms in differentiated epidermis, we searched for a suitable cell line to achieve this purpose. In a preliminary experiment, we found the expression of mCLCA2, which is highly homologous to rCLCA, in the transformed mouse keratinocyte line Pam212 [13]. In addition, this cell line is reportedly an appropriate model for keratinocyte differentiation. Like normal keratinocytes and other cell lines [14,15], Pam cell lines have the capacity to grow in culture medium containing a low Ca^{2+} concentration (0.02–0.09 mM) and demonstrate the differentiation characteristics of normal keratinocytes when the Ca^{2+} concentration is raised to 1.4 mM. The present study revealed for the first time that basal nuclear factor-κB (NF-κB) activity is involved in the Ca^{2+}-dependent regulation of mCLCA2 expression in a mouse keratinocyte line.

2. Materials and methods

2.1. Reagents and cell culture

The primary antibodies used were rabbit monoclonal anti-p65 (D14E12; Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal anti-keratin 10 (K10; Dako, Glostrup, Denmark), mouse monoclonal anti-RNA pol II (Active Motif, Carlsbad, CA, USA), rabbit polyclonal anti-CLCA [4,12], and mouse monoclonal anti-β-actin (sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Caffeic acid phenethyl ester (CAPE) and tumor necrosis factor α (TNF-α) were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Calbiochem (San Diego, CA, USA), respectively. The other compounds were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA).

The Pam212 cell line derived from mouse keratinocytes was a kind gift from Dr. Yuspa (Center for Cancer Research, National Cancer Institute, MD, USA) [13]. They were grown in Joklik modification of minimum essential medium Eagle supplemented with 10% Ca^{2+}-stripped fetal bovine serum (FBS) in low Ca^{2+} concentration (0.05 mM) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The cells were seeded at a density of 5.0×10^4 cells/cm^2 and cultured for 48 h before the assay. Cells were switched from a medium with low Ca^{2+} to one with 1.0 mM Ca^{2+} and cultured for 24 h before the assay to obtain a differentiated phenotype.

2.2. Reverse transcription (RT)-PCR

Pam212 cells were homogenized in an RNA extraction reagent (Isogen; Nippon Gene, Tokyo, Japan). Total RNA was isolated and the mRNA was reverse transcribed into cDNA using a PrimeScript II 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. PCR was performed with a pair of primers (Table 1) specific to mCLCA2, mCLCA1, mCLCA5, keratin 1 (K1), p65 (RelA), and hypoxanthine phosphoribosyltransferase (Hprt) and Taq polymerase (Ex-Taq; Takara) under the following thermal cycling conditions for 40 cycles: 94 °C for 30 s; 54 °C for 30 s; and 72 °C for 60 s. PCR products were analyzed on an ethidium bromide-stained agarose (1.5%) gel.

2.3. Luciferase assay

Fragments of the mCLCA2 5′-flanking region were cloned into a luciferase reporter (pGL3-Basic vector; Promega, Madison, WI, USA). Pam212 cells were transiently transfected with pGL3-Basic or the mCLCA2 reporter construct and with pRL-SV40 (transfection efficiency control) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). The cells were harvested at 24 h after transfection, and luciferase activity was determined with a Dual-Luciferase Reporter Assay System (Promega). Activity was shown as x-fold activity relative to the value for the pGL3-Basic control vector.

2.4. RNA interference

A small interfering RNA (siRNA) to target the mouse p65 gene was purchased (#6337; Cell Signaling). Scrambled RNA control was used as a negative control (#6568; Cell Signaling). The cells were lysed at 48 h after transfection with Lipofectamine 2000 (Life Technologies). The western blot procedure used to confirm the ability of the siRNA to suppress expression is described in the Supplemental Materials.

2.5. Chromatin immunoprecipitation (ChIP)

Nuclear protein and DNA complexes were cross-linked in the culture medium containing 1% formaldehyde for 10 min at 21–25 °C, and were then homogenized in NP-40 buffer for 5 min at 21–25 °C. After centrifugation, the pellet was resuspended in SDS lysis buffer, followed by a 5-fold dilution in ChIP dilution buffer. Ten micrograms of soluble sheared chromatin were incubated overnight at 4 °C with the anti-p65 or anti-Pol II antibody, or control rabbit IgG bound to protein G magnetic beads (#9006; Cell Signaling). After washing, immune complexes were eluted by incubation for 20 min at 65 °C with ChIP direct elution buffer, and then the cross-links were reversed by an overnight incubation at 65 °C. DNA was purified using a GenElute PCR Clean-up Kit (Sigma–Aldrich), and PCR was performed for DNA amplification specific to the mCLCA2 promoter. The buffers used for ChIP are listed in the Supplemental Materials.

2.6. Transcription factor assays

A nuclear extract was obtained from Pam212 cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. For the enzyme-linked immunosorbent assay (ELISA)-based

Table 1
Sequences of the PCR primers used in the present study.

Name (GenBank accession number)	Forward (5′–3′)	Reverse (5′–3′)
mCLCA1 (NM_009899)	CCATCTCCGGTGGGGAGTAT	ACACAGTTGCCTCTCTCACAG
mCLCA2 (NM_030601)	GTGTGATGCAGGTTCACTTTAC	TCAGTTGTAAAAGCAGGTAGCA
mCLCA5 (NM_178697)	CAACTTTTGGATGGCGGAGC	CTAGTGTGCTTGTGCTGGGA
Hprt (NM_013556)	GGCCAGACTTTGTTGGATTTG	TGCGCTCATCTTAGGCTTTGT
p65 (NM_009045)	ATCCACATGGAATCGAGAGC	CAGGAAGGGATATGGAAGCA
K1 (NM_008473)	GACCAGTCACGGATGGATTC	CGAACTCATTCTCTGCGTTG