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Skn-1a/Oct-11 and ΔNp63α exert antagonizing effects on human keratin expression

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ABSTRACT

The formation of a stratified epidermis requires a carefully controlled balance between keratinocyte proliferation and differentiation. Here, we report the reciprocal effect on keratin expression of ΔNp63, pivotal in normal epidermal morphogenesis and maintenance, and Skn-1a/Oct-11, a POU transcription factor that triggers and regulates the differentiation of keratinocytes. The expression of Skn-1a markedly downregulated ΔNp63-driven K14 expression in luciferase reporter assays. The extent of downregulation was comparable to the inhibition of Skn-1a-mediated K10 expression upon expression of ΔNp63. ΔNp63, mutated in the protein–protein interaction domain (SAM domain; mutated in human ectodermal dysplasia syndrome), was significantly less effecting in downregulating K10, raising the possibility of a direct interaction among Skn-1a and ΔNp63. Immunolocalization in human skin biopsies revealed that the expression of the two transcription factors is partially overlapping. Co-immunoprecipitation experiments did not, however, demonstrate a direct interaction between ΔNp63 and Skn-1a, suggesting that the antagonistic effects of Skn-1a and p63 on keratin promoter transactivation is probably through competition for overlapping binding sites on target gene promoter or through an indirect interaction.

1. Introduction

The epidermis is a renewable tissue composed of keratinocytes that provide protection against external insults. Its protective barrier function is dependent on a tightly regulated differentiation program that results in distinct epidermal layers. The basal layer, which is in contact with the basement membrane, is composed of undifferentiated proliferating cells. The suprabasal layers consist of cells progressively differentiating as they move outward, achieving terminal differentiation in the outermost cornified layer [1,2]. Keratinocytes undergo a specialized form of programmed cell death (PCD) while they undergo these coordinated progressive changes [3–5].

Epidermal homeostasis is maintained by the orchestrated expression of different genes. The proliferating basal cells are characterized by the expression of cytokeratins 5 (K5) and 14 (K14), which are then repressed in the suprabasal cell layers where keratins 1 (K1) and 10 (K10), and involucrin are expressed [6]. Although several transcription factors (for example: AP-1, AP-2, Mad/Max, RARs and HNF-4) have been identified as being important at different stages of skin maturation [7–10] it remains to be established precisely how they drive specific gene expression and keratinocyte differentiation. Among several candidates, the POU domain proteins and p63 have been found to be transcription factors involved in regulation of keratinocyte specific genes.

POU domain factors are characterized by a conserved DNA-binding domain (called the POU domain) [11], which binds to a specific octamer DNA motif [12]. Several octamer binding proteins (called Oct-1 to Oct-10) are present in several tissues at different stages of development [13–15]. To date, three major POU domain factors have been demonstrated to be present in normal mammalian epidermis [16–19]: Oct-1, Oct-6 (also named Tst-1) and Oct-11 (also named Epoc-1/Skn-1a and hereafter referred as Skn-1a) [18]. The ubiquitous Oct-1 is expressed in both proliferating and differentiating epidermal keratinocytes, whereas Oct-6 and Skn-1a are primarily expressed in suprabasal layers [16,18,20,21]. In particular, Skn-1a is epidermis-restricted, as it is expressed almost exclusively in interfollicular epidermis and cortical cells of the hair, while Oct-6 has also been found in other epithelia (oesophagus and vagina) and in the nervous system (glial cells) [19,22,23]. The hypothesis that Skn-1a is involved in regulating keratinocyte differentiation is supported by two findings. Firstly, Skn-1a is involved in cell differentiation and tissue specification [15,24–27].

Abbreviations: K, keratin; SAM, sterile alpha motive; PCD, programmed cell death; TGs, transglutaminases; CBP, CREB-binding protein; NHEKs, normal human epidermal keratinocytes; AEC, ankyloblepharon, ectodermal defects and cleft lip and palate.

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Secondly, *in vitro* studies have shown that Skn-1a can transactivate the K10 and SPRR-2A promoters [16,28,29] while inhibiting the expression of involucrin [30], profilaggrin [31] and K14 [32]. The repression of the K14 promoter is independent of DNA binding, but is achieved by interfering with the activity of the CREB-binding protein (CBP) co-activator [33]. Mice lacking the *Skn-1* gene did not, however, reveal a specific function for this gene as they develop normally; this is probably due to redundancy with Oct-6 [18].

p63 is a member of the p53 family, a family of transcription factors that shows a high degree of molecular similarity (reviewed in Refs. [33,34]). Unlike p53, both p63 and p73 give rise to multiple protein isoforms due to alternative promoter utilization and/or alternative mRNA splicing. Differential splicing of the p63 messenger results in different C-termini (α, β and γ-isoforms), all of which extend the protein sequence well beyond the region that is homologous to p53. Differential use of the two promoters results in transcription of two classes of proteins: the full length (TAp63) isoforms, containing an N-terminal, transactivation domain (able to activate downstream target genes and induce apoptosis) and the N-terminally truncated variants (ΔNp63 isoforms), acting as pro-survival proteins [33–36], controlling stemness [37,38]. In addition, the carboxy-terminal spliced region in the α-variant contains a sterile α-motif (SAM domain), a protein–protein interaction domain, and a trans-inhibitory (TI) domain that are absent in the p53 gene. In mice and human epithelia, the ΔNp63 isoforms are the main isoforms expressed in the basal proliferating cell layer and their expression level is repressed during keratinocyte differentiation [39,40]. Although the N-terminal domain is missing, the ΔN isotypes are, nonetheless, transcriptionally active and can upregulate markers of the basal compartment such as K14 [41]. Indeed, p63 KO mice give a clear demonstration of its vital role in epithelial, craniofacial and limb development. Epithelial loss in p63-deficient mice reflects an inability to sustain multilayered physiological asymmetrical division. The mechanism through which p63 carries out this remarkable tasks is unknown: p63 could be essential in maintaining the stem cell population or the transient amplifying cells, which are proliferative cells already committed to differentiate [42,43].

Here, we report the reciprocal effect on keratin expression of ΔNp63 and Skn-1a/Oct-11.

2. Materials and methods

2.1. Cell lines

HEK293 (ATCC, Manassas, VA) cells were grown in DMEM medium, supplemented with 10% foetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 1.2 g/L Na-bicarbonate, at 37 °C with 5% CO_2 in a humidified atmosphere.

NHEKs were obtained from Clonetics (San Diego, CA) and grown in serum-free keratinocyte growth medium (SFM; Invitrogen) at 0.05 mM calcium, supplemented with 60 μg/ml of bovine pituitary extract. Primary keratinocytes were induced to differentiate by growth factor withdrawal (lowering FBS concentration from 10% to 2%) and shifting of calcium concentration in the culture medium from 0.05 to 1.5 mM.

2.2. Plasmids and transfections

The K14 and K10 luciferase plasmids were a gift of Dr. B. Andersen (University of California, Irvine) (Andersen et al., 1997; Sugihara et al., 2001). pCMV/Oct-11 encoding Skn-1a was a gift of Dr. T. Kanda (National Institute of Infectious Diseases, Tokyo, Japan). All p63 constructs TA- and ΔN-p63α, β, and γ were generated by PCR and cloned into pCDNA3.1-HA (Clontech). SAM domain mutations in ΔNp63α cDNAs were obtained by inverse PCR using wild type HA-tagged cDNA (cloned in pcDNA3.1) as template and overlapping primers containing the required point mutations. Mutations in the SAM domain are: G530V, T533P, Q536L and I537T. All constructs were checked by sequencing.

HEK293 cells were cultured in 12-well dishes and transfections were performed with Effectene (Qiagen, Italy), according to the manufacturer's recommendations. We used a 1:3 ratio between the reporter plasmid (containing the firefly luciferase gene under the control of either the K14 or K10 promoters) and the given expression vectors encoding for all p63 isoforms and mutants, and Skn-1a. In co-transfection experiments, the relative ratio between Skn-1a and p63 isoforms or mutants was 1:1; in competitive luciferase assays, ratios varied from 1:3 to 2:1. When needed, empty vector was added to keep the total amount of DNA used in each transfection constant. In all cases, 10 ng of *Renilla* Luciferase Vector (pRL-CMV; Promega, Madison, WI) was co-transfected as a control of transfection efficiency. Twenty-four hours after transfection, luciferase activities in cellular extracts were measured by using a Dual Luciferase Reporter Assay System (Promega); light emission was measured over 10 s using an OPTOCOMP I luminometer.

2.3. RT-PCR

RNA was extracted using the RNAeasy kit (Qiagen) and converted to cDNA using InPromII kit (Promega). Normalization was performed amplifying human β-actin housekeeping mRNA using the following primers: forward 5′-CTGGCACCACACCTTCTACA ATG-3′ and reverse 5′-AATGTCACGCACGATTTCCCGC-3′. For detection of TAp63 the primers used were: forward 5′-CCCAGAGCACACA GACAAAT-3′ and reverse 5′-GTTCAGGAGCCCCAGGTTCG-3′; for ΔNp63 were: forward 5′-AATGCCCAGACTCAATTTAGTGA-3′ and reverse 5′-CATCACCTTGATCTGGATGGGGC-3′, for K14 were: forward 5′-TTTGGTGGTGGCTTTGCTGG-3′ and reverse 5′-AACTCTGTC TCATACTTGGTGCGG-3′.

2.4. Western blot

Cells were resuspended in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS) plus protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Specific primary antibodies used: anti-Skn-1a (Santa Cruz Laboratories; Santa Cruz, CA), anti-HA-7 monoclonal mouse antibody (Sigma, St. Louis, MO), anti-p63 (4A4 monoclonal mouse antibody; NeoMarkers, Fremont, CA) and anti-K10 (BD Pharmingen). Actin (C-11 goat polyclonal antibody; Santa Cruz Biotechnology) was used as an internal control. Signal detection was performed with the Amersham ECL™ Western Blotting Detection System (GE Healthcare).

2.5. Immunostaining and confocal microscopy

Skin biopsies were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were deparaffinized (Histolemon; Carlo Erba, Italy) and rehydrated stepwise in alcohol/distilled water. Microwave-assisted antigen retrieval was performed in 0.01 M sodium citrate for three cycles of 5 min. Primary antibodies used: anti-p63 (Ab4; NeoMarkers, 1:500 dilution), anti-Skn-1a (Santa Cruz Biotechnology; 1:100 dilution) and anti-K10 (BD Pharmingen; 1:100 dilution). Secondary antibodies used were conjugated with Alexa-488 or Alexa-568 (Molecular Probes). The tissue sections were then mounted by using Prolong Antifade kit (Molecular Probes), and fluorescence was evaluated by a Nikon Instruments "C1" confocal microscope mounted on Eclipse TE200.