A Functional Enhancer of Keratin14 Is a Direct Transcriptional Target of ΔNp63

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Keratin14 (K14) is a prototypic marker of dividing basal keratinocytes where its gene is transcribed at high levels. Transcriptional regulation of K14 is governed by an evolutionarily conserved functional enhancer marked by DNase 1 hypersensitive sites present upstream of the gene. This enhancer is sufficient to confer epidermal-specific gene expression, which is mediated in part by binding of members of activator protein-2 (AP)-2, AP-1, Ets, and Sp1 families of transcription factors. Here we provide evidence that a keratinocyte-specific nuclear protein identified as ΔNp63 binds to a conserved motif within this enhancer. Interestingly, the selective expression profile of ΔNp63 in various cell lines correlates with both the nuclear complex and the expression of K14. Biochemical studies reveal that ΔNp63 can bind to a specific DNA sequence present in the K14 enhancer and this binding leads to transactivation. In addition, chromatin immunoprecipitation experiments with ΔNp63-specific antibodies demonstrate that the enhancer is occupied by ΔNp63 in cultured keratinocytes and in mouse skin epidermal cells *in vivo*. Finally, we show that ectopic expression of either p63 isoform (ΔN or TA) can induce *de novo* expression of K14. These studies provide a potential mechanism by which ΔNp63 directly governs the expression of K14 in a keratinocyte-specific manner.

Journal of Investigative Dermatology (2007) **127,** 1175–1186. doi:10.1038/sj.jid.5700652; published online 7 December 2006

INTRODUCTION

Keratins constitute the largest subgroup of the family of intermediate filaments and represent the most abundant proteins in epithelial cells (Fuchs and Weber, 1994). These proteins are divided into type I (acidic) and type II (basic and neutral) classes based on their sequence. Keratins from these two classes form obligate heterodimers and are expressed in tissue- and differentiation-specific manners in epithelial tissues of various types including simple epithelia, internal stratified epithelia, outer stratified epithelia, and hair follicles (Coulombe and Omary, 2002). This distinctive expression of specific keratin pairs depends largely on the tissue-type, differentiation status, and the physiological state. For example, dividing basal keratinocytes of the skin epidermis express keratin5 (K5) and keratin14 (K14). As these cells exit the cell cycle and embark on a program of differentiation, expression of K5 and K14 is downregulated and a new set of keratins, keratin1 and keratin10 are expressed in the suprabasal spinous layer (Byrne *et al*., 1994). The differential expression of keratins has been useful in marking the boundaries of the different layers of the skin epidermis and has often served as surrogate markers to distinguish proliferation and differentiation states of the skin epidermis. Therefore, deciphering the molecular mechanisms governing keratin gene expression is important for better understanding the regulatory network that underlies epidermal development and homeostasis.

To characterize transcriptional regulatory mechanisms that govern the expression of keratin genes, many studies have focused on the identification of *cis* and *trans* regulatory elements (Byrne, 1997; Eckert *et al*., 1997). The restricted expression of K14 in the basal layer of the skin epidermis is primarily controlled at the level of transcription and regulation of this process has been extensively studied using various complementary approaches. DNAse I hypersensitive site (Hs) mapping of the human *K14* gene has identified several Hs in the 5′ region that are present selectively in keratinocytes (Sinha *et al*., 2000; Sinha and Fuchs, 2001). Among these Hs elements are those which correspond to the minimal promoter (Hs I) and two closely spaced Hs (Hs II and III) at the –1700 and –1400 region. Interestingly, the Hs elements are organized similarly in the mouse *K14* gene, and there is high level of sequence conservation in the Hs regions, further supporting the functional relevance and importance of these elements in controlling K14 expression. This has been experimentally proven by extensive analysis of these Hs segments by reporter gene assays in keratinocytes grown in culture as well as in transgenic mice. Indeed these studies have clearly demonstrated that the K14 Hs II and III regions act as an enhancer element that confers high levels of gene expression selectively

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Abbreviations: AP-1, activator protein-21; MK, mouse keratinocyte; ChIP, chromatin immunoprecipitation; GST, glutathione S-transferase; Hs, hypersensitive site; KSC, keratinocyte-specific complex; PBS, phosphate-buffered saline; TK, thymidine kinase

Received 23 May 2006; revised 6 October 2006; accepted 10 October 2006; published online 7 December 2006

in keratinocytes when assayed by reporter gene assays in transient transfection experiments. More importantly, this enhancer can target basal keratinocyte-specific expression of a reporter gene in transgenic mice. Similar investigative studies of the K14 partner gene, K5 have also lead to the identification of several Hs elements that function as critical regulatory modules, some of which have been characterized in detail (Kaufman *et al.*, 2002). Collectively these studies have led to the conclusion that the epidermal and differentiation-specific transcription of these two keratin genes is governed by complex regulatory elements.

To identify transcription factors that mediate the expression of the basal keratin genes, these Hs elements have been subjected to biochemical analysis. These studies show that basal-specific and differentiation-specific gene expression in keratinocytes relies on a complex array of regulatory elements that bind diverse transcription factors belonging to many families including activator protein-2 (AP)-2, AP-1, ETS, Sp1/Sp3 and as yet unidentified factors. Many of the transcriptional activators and repressors that interact with these Hs sites are quite ubiquitous in their expression pattern and only a few exhibit some level of keratinocyte-restricted expression. This has raised an important question, that is, how is epidermal-specific gene expression achieved? One hypothesis is that gene expression in basal epidermis is governed by broadly expressed factors working in a combinatorial manner. Alternatively, a mechanism may exist that confers keratinocyte-specific activity on an otherwise ubiquitously expressed transcription factor by virtue of specific post-translational modification or interaction with a co-activator. Finally, it is possible that transcription factors that are generally restricted to keratinocytes interact directly with these regulatory elements and mediate cell-type specific gene expression.

p63 is a p53 homolog that is critical for the development of the stratified epithelium of the skin as shown by several recent studies (Mills *et al.*, 1999; Yang *et al.*, 1999, 2002). Indeed, both loss-of-function and gain-of-function experiments have demonstrated that p63 is important for maintaining epithelial homeostasis (Koster and Roop, 2004b). One complicating issue in deciphering the biological role of p63 is the fact that the *p63* gene contains two distinct promoters, resulting in the expression of two major classes of proteins, one with an amino-terminal trans-activation domain (TAp63) and one without (ΔNp63) (Yang *et al.*, 1998). Knockout studies in mice so far have not examined the relative contribution of these two major isoforms in orchestrating the balance between keratinocyte proliferation and differentiation. However, several recent studies utilizing expression analysis and ectopic overexpression experiments have led to the postulation of contradictory roles for the TAp63 and ΔNp63 isoforms (Koster *et al.*, 2004; Candi *et al.*, 2006; Laurikkala *et al.*, 2006). What is unequivocally clear however, is the fact that epidermal cells that lack p63 exhibit dramatic downregulation in the levels of K14. Although, there has been a recent report suggesting that loss of expression of K14 in the absence of p63 is indirectly mediated by the transcription factor AP-2γ, the possibility that p63 can directly target K14 gene expression has not been examined thoroughly (Koster *et al.*, 2006).

We describe here a nuclear complex that is restricted to keratinocytes and binds to a specific conserved sequence present in the enhancer segment corresponding to Hs II of the *K14* gene. We provide *in vitro* and *in vivo* evidence that ΔNp63 is a component of this unique complex and that ΔNp63 binding is important for the K14 enhancer activity. Furthermore, our data suggests that contrary to published data, not only TAp63 but also all isoforms of ΔNp63 are capable of inducing K14 gene expression, when ectopically expressed in cells that normally do not express p63 or K14. The fact that ΔNp63 is the predominant isoform expressed both in cultured keratinocytes and in skin epidermis suggests it is the critical p63 isoform that governs K14 gene expression. Our data strongly argues that transcription factors working in a combinatorial manner govern gene expression in epidermis and that p63 is a keratinocyte-specific transcription factor that potentially serves an important role as a master regulator in this process.

RESULTS

A novel KSC binds to the K14 enhancer

Previously we characterized an enhancer element located ~1.4 kb upstream of the *K14* gene, delineated by a keratinocyte-specific DNAse I Hs site (Sinha *et al.*, 2000). We showed that binding of AP-1, AP-2, and the Ets family of proteins was important for enhancer activity. We identified an additional nuclear complex binding to an oligonucleotide containing K14 enhancer sequences from −1433 to −1407 that showed an intriguing behavior in electrophoretic mobility shift assay (EMSA) (Figure 1a). We named this slow migrating band keratinocyte-specific complex (KSC), as it was present only in nuclear extracts from mouse and human keratinocytes and not from any other cell types including HepG2 (liver), HeLa (epithelial), NIH3T3 (fibroblast), or B16 (melanoma). The nuclear extracts from the different cell types were prepared in a similar manner and showed comparable DNA–protein complexes when tested with other oligonucleotides, suggesting that the extracts were functionally similar (data not shown). Additional faster moving complexes that bound to the oligonucleotide containing the KSC-binding sequence were either also present in other cell types or were nonspecific as they disappeared in competition experiments with random oligonucleotides or with high amounts of competitor DNA. Additionally, KSC binding was stable in the presence of 0.5 M NaCl suggesting that there is a tight interaction between KSC and its DNA-binding site (data not shown).

Binding of KSC is dependent upon specific DNA sequences

To precisely identify the residues that were critical for DNA binding of KSC, we generated several mutant oligonucleotides (MTs) and performed direct EMSA and competition assays (see Figure S1 for oligonucleotide sequences). Two of the single base pair mutations (MT5: GGGCCTG > GGG*A*CTG) and (MT8: CCTGTCT > CCT*A*TCT) failed to bind KSC when using either HaCaT or MK nuclear extracts in