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p63 and p73 Transactivate Differentiation Gene Promoters in Human Keratinocytes

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p53 and its two homologues, p73 and p63, share considerable structural similarities, an ability to interact between themselves and to transactivate the same promoters, including for example p21. Furthermore, p73 can induce cell death via its interaction with c-Abl. In contrast, p63 has been demonstrated to be essential for limb and skin formation. We evaluated the expression of p63 and p73 in differentiating human keratinocytes *in vitro*. Skin biopsy and primary cultures of normal human epidermal keratinocytes (NHEK) express both p73 and p63. NHEK induced to differentiate *in vitro* by high calcium exposure show induction of p73 δ and downregulation of all isoforms of p63. This latter gene is predominantly expressed in its transcriptionally inactive form, ΔNp63. We further evaluated the effect of either p73s or p63 transfected in either NHEK or transformed human keratinocytes (HaCat cells). p73 γ, δ, and p63 were able to transactivate the promoters of loricrin and involucrin in both NHEK and HaCat cells. These results suggest the involvement of both p73 and p63 genes in keratinocyte terminal differentiation.

Key Words: **p73; p63; p53; apoptosis; differentiation; keratinocytes; skin.**

Until very recently, p53 was a unique tumour suppressor gene (1). Two new genes related to p53 have now been identified, p63 (2–5) and p73 (6). This new family shares both structural and functional similarity. Indeed, the sequence identity between the three genes reaches 63% in the DNA binding domains, 29% in the transactivation domain, and 38% in the oligomerization domain. The latter homology is probably responsible for the homotypic and heterotypic interactions between the three members of the family. This structural similarity results, therefore, in often comparable functional effects. Indeed, both p53 and p73 are able to transactivate the promoters of some common genes, including p21, bax, mdm2, cyclin-G, gadd45, and IGFBP3 (6–10). The possible formation of hybrid tetramers between different members of the same family results in further cross-talk between the proteins engendering additional functional complexity (reviewed in 11–14).

At the functional level both p53 and p73 have been shown to induce cell death (7), even though this seems to occur by different mechanisms. In fact, p73 can induce apoptosis in the absence of p53 via a physical interaction with c-Abl (11, 15–18).

Despite these similarities at a structural and functional level, two lines of evidence would suggest that p53 family members may also have discrete roles. First, viruses differentiate between p53 and p73 (19). Second, tumors show frequent mutation in p53 but not in p73 and p63 (13, 20–22). Preliminary data also indicate a potential involvement of p63 and p73 in differentiation. Neurons from p53 null, and wild type mice cultured with p53 antisense oligonucleotides *in vitro,* show accelerated spontaneous differentiation (23). This suggests that loss of the p53 heterodimeric partners may liberate more uncomplexed or homodimerized p63/p73 and allow expression of their differentiation-inducing function. Second, both p63 and p73 contain a structural motif, sterile alpha motif (SAM), found in proteins involved in differentiation (24) and which is absent in p53. Finally, mice with a disrupted p63 gene show abnormal limb and skin de-

Abbreviations used: NHEK, normal human epidermal keratinocytes; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified essential medium; KBM, keratinocyte basal medium.

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velopment (25, 26). Consistent with this, mutations in p63 have been very recently identified as causing the limb mammary syndrome, knows as ectrodactyly, ectodermal dysplasia, and cleft lip (EEC) syndrome (27). Eight patients showed a mutation in the p63 DNA binding region, resulting in loss of transactivational function; the last patient contained a frameshift affecting p63α, but not the β and γ isoforms. It is therefore possible that the role of p63 in limb formation may be distinct from its role in epidermal differentiation.

Accordingly, we evaluated the expression and the effect of p63 and p73 in differentiating keratinocytes *in vitro*.

MATERIALS AND METHODS

Plasmids, constructs, and RT–PCR. Human p73s and p53 cDNAs in pCDNA3 have been described (8, 28). The construction of the plasmids containing 1.2 kbp of loricrin promoter (Lor-pCAT) and 0.9 kbp of involucrin promoter (Inv-pCAT) upstream of the CAT reporter gene is also described elsewhere (29). RT–PCR was performed as described (8).

Cell cultures, transfections, and protein assays. Cryopreserved normal human epidermal keratinocytes (NHEK) were obtained from Clonetics (San Diego, CA) and grown in calf skin collagen (Sigma) coated dishes in serum free keratinocyte basal medium (KBM, Clonetics) at 0.05 mM Ca^{2+}. Third passage NHEK cells were used for transfection experiments. HaCaT cells were grown in Dulbecco's modified Eagle's medium supplemented with 4.5 g/liter glucose, 10% fetal bovine serum and non essential amino acids (Life Technologies Inc., Gaithersburg, MD). Transient transfections were performed in duplicate using Effectene transfection reagent (Qiagen, Hilden, Germany) following the manufacturer's instructions. Typically 2–3.5 $\times$ 10^5 cells were plated in six-well culture plates 24 h before transfection. Transfection was performed when cultures reached 60–70% confluence. Transfection efficiency was monitored by use of 0.5 mg of a thymidine kinase β-galactosidase construct (Clontech, Palo Alto, CA). After transfection the calcium in the medium was adjusted to 1.2 mM for NHEK. Cells were harvested 48 h after transfection, and the CAT activity was assayed as described (30). Cellular extracts were prepared and used for CAT assays, β-galactosidase (β-gal) activity determination and protein quantitation (31). CAT and β-gal activities were determined by fluorodiffusion (32), using chloramphenicol and [^{3}H]acetyl-CoA (DuPont NEN) as substrates, and β-galactosidase enzyme system (Promega, Madison, WI) respectively. The values for CAT were normalized by b-gal activity and protein content. The relative CAT values are the average of six independent experiments, each performed in duplicate.

RESULTS AND DISCUSSION

We have previously reported that human skin biopsies express different isoforms of p73 (8). We have now evaluated by RT–PCR the expression of p73 and p63 and their different isoforms in primary human keratinocytes (NHEK). To this end, NHEK were exposed to high exogenous concentrations of calcium. Figure 1A shows that at 1 and 4 days the expression of p73 particularly the δ isoform is significantly increased. In contrast, the expression of p63 is decreased during *in vitro* differentiation of NHEK. Indeed, both α and β isoforms show a significant down-regulation at both 1 and 4 days, as shown in Fig. 1B. p63 is predominantly expressed in the transcriptionally inactive form, ΔNp63 (see Fig. 1B) which was also downregulated following high Ca^{2+} treatment. Finally, p53 showed a comparable down-regulation under the same conditions (see Fig. 1C).

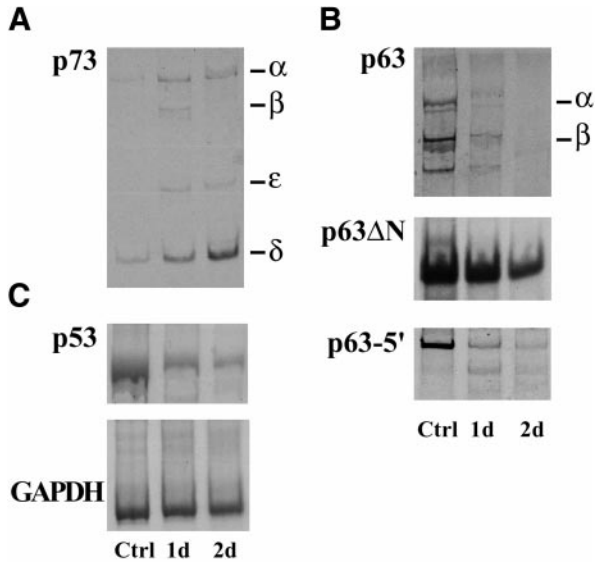

FIG. 1. Expression of p63 and p73 in differentiating keratinocytes. Primary cultures of normal human epidermal keratinocytes (NHEK) were induced to differentiate *in vitro* by calcium for 2 or 4 days; RT–PCR as performed to evaluate the differential expression of p73 and p63. (A) Expression of p73 isoforms α, β, ϵ, δ. (B) Expression of p63. To evaluate both the C-terminal variants (α, β) and the N-terminal isoforms (p63 and ΔNp63) different RT–PCR were performed. (C) Control RT–PCRs to evaluate the expression of p53 and GAPDH. Experimental details are described under Materials and Methods.

p63 has been demonstrated to be essential in skin formation and limb development. Indeed, KO mice with disrupted p63 fail to maintain the apical ectodermal ridge essential for limb development, and have truncated limbs. They also have defective stratified epithelial differentiation with no hair follicles, teeth, mammary, lachrymal and salivary glands (25, 26). In keeping with these data, we demonstrated differential expression and modulation of the various p63 isoforms during *in vitro* keratinocyte differentiation.

The differential expression in keratinocytes of all three members of the family increases the degree of functional complexity. While in control cells the predominant isoform is ΔNp63, in calcium-differentiated keratinocytes the most abundant is p73δ. To determine whether this relative increase in p73δ expression was of functional significance, we transfected different isoforms in human keratinocytes.

Both NHEK and transformed human keratinocytes, HaCat cells, were transiently transfected with differ-

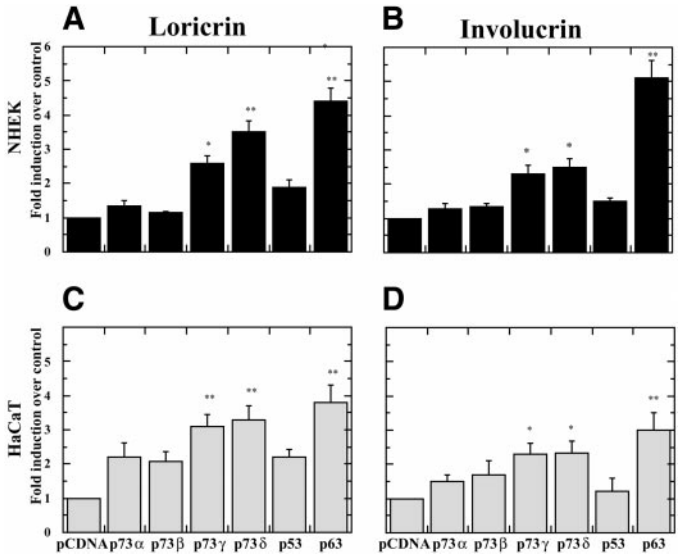

FIG. 2. Effect of p73 α, β, γ and δ, p53 and p63 on the activity of the Lor-pCAT and Inv-pCAT constructs. Lor-pCAT (A and C) and Inv-pCAT (B and D) constructs were co-transfected into NHEK (A and B) or HaCaT cells (C and D) together with plasmid DNA expressing p73 α, β, γ and δ, p53 or p63 cDNA or expression vector DNA only (pCDNA). The relative values of the transient CAT activity are presented as a fold change over the activity of Lor-pCAT or Inv-pCAT cotransfected with the pCDNA. * represents $P < 0.01$; ** represents $P < 0.001$.

ent isoforms of p63 or p73. To evaluate the effect at the differentiation level, we performed CAT assays using the promoters of loricrin and involucrin. These two genes are induced during keratinocyte differentiation and are recognized as molecular markers of epidermal differentiation (33). Figures 2A and 2B show that p73 δ is effective in inducing transactivation of both loricrin and involucrin in NHEK cells. Comparable results were obtained using HaCat cells, as reported in Figs 2-C and 2D. p63α and p73 γ (whose expression relatively declines in differentiating NHEK; Fig. 1A and B) also transactivate these promoters to roughly the same extent. This suggests that p63α and p73 γ and δ may act at a common promoter site although the physiological significance of p63α and p73 γ transactivation in cells differentiated in high Ca^{2+} remains obscure.

In vivo, since many isoforms are present, the effects of p73 isoforms on loricrin and involucrin expression are likely to be complex. For example, decline in dominant negative ΔNp63 may result in greater increases in involucrin and loricrin expression than seen by single transfection experiments *in vitro.*

Our results indicate that both p63 and p73 are (i) expressed in keratinocytes, (ii) differentially modulated during *in vitro* differentiation, (iii) able to transactivate two gene promoters, loricrin and involucrin, markers of epidermal differentiation. Recently, Parsa *et al.* (34) have shown by *in situ* hybridization that p63 is predominantly expressed in proliferating keratinocytes. However, the predominant form is the dominant negative ΔNp63. The cyclin kinase inhibitor p21 Cip1/WAF1 plays a relevant role in the differentiation of primary mouse keratinocytes, and this function is independent of cell cycle control (35). Since p21 can be transactivated by p53, p63 and p73 (6–8), our results suggest a clear molecular link that activates keratinocyte differentiation. The present working hypothesis is therefore that ΔNp63 down regulation is a crucial event to possibly allow p73 δ to trigger differentiation.

In contrast to p53, both p73 and p63 have an extended C-terminal region containing a protein-protein interaction module, the SAM domain (24, 36), which is usually found in proteins related to differentiation (37). This structural property led to the suggestion that these two proteins could be related to development and differentiation (37). Indeed, our data provide evidence that, while p53 and p73 can induce cell death, p63 and p73 can also be associated with differentiation. The abnormalities observed in the p63 knockout mouse (25, 26) further support the hypothesis that p63 and possibly p73, are involved in differentiation.

Ikawa *et al.* (13) have recently detected an increased expression of p63 during the myoblastic differentiation of the C2C12 cell line, exposed for 1–2 days at low serum concentration. They also have preliminary data indicating that transfected p63 can induce erythroid differentiation as indicated by the enhanced production of hemoglobin and non-genotoxic benzidine (13). We have recently demonstrated that p73 can trigger neuronal differentiation in a neuroblastoma cell line (38), inducing the expression of NCAM, p21, and neurofilaments. Furthermore, dominant negative p73 blocks NCAM promoter transactivation induced by retinoids, a well known trigger of neuronal differentiation. In addition, p73 KO mice show among others also neurological defects (39). These, together with the data presented here, support a consistent role for p63 and p73 in differentiation, in addition to the involvement of p73 in apoptosis. The differentiation-related role of p63 and p73 functionally discriminates these genes from their original homolog p53.

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