Retinoic Acid Inhibits Downregulation of ΔNp63α Expression During Terminal Differentiation of Human Primary Keratinocytes

Casimir Bamberger, Dieter Pollet,* and Hartwig Schmale

Institut für Zellbiochemie und Klinische Neurobiologie, Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany; *Paul Gerson Unna-Skin Research Center, Beiersdorf AG, Hamburg, Germany

Recently, the p53 homolog p63 has been implicated in sustaining the epidermal stem cell population. The p63 gene encodes six major products with transactivating or dominant-negative properties. The expression pattern of these isoforms in keratinocytes was investigated here. Northern blot, ribonuclease protection assay, reverse transcription–polymerase chain reaction, and western blot techniques sensitive for all six p63 isotypes verified the predominant expression of the truncated and potentially dominant-negative isotype ΔNp63α in human keratinocytes. The expression of this isoform is downregulated when proliferating human primary keratinocytes begin to differentiate after growth factor withdrawal. The onset of differentiation does not change the ratio of two other weakly expressed isotypes ΔNp63γ and TAp63α relative to ΔNp63α. Treatment of primary human keratinocytes with all-*trans* retinoic acid does not alter the expression pattern of p63 isotypes but prevents its downregulation as observed in control cell cultures. These data suggest that p63 expression in human keratinocytes is affected by all-*trans* retinoic acid and this influence might contribute to the fine tuned keratinocyte proliferation and differentiation equilibrium in the mammalian epidermis. ***Key words: involucrin/p53 protein family/p63 splice variants. J Invest Dermatol 118:133–138, 2002***

p63 shows remarkable structural homology to the related protein p73 and the tumor suppressor p53 (Yang and McKeon, 2000; Strano *et al*, 2001). Whereas only a single isoform of p53 is known, multiple p63 and p73 transcripts are observed. At least two promoters exist in the p63 gene, yielding transactivating (TA, transactivation domain) as well as transactivating (ΔN, n-terminally truncated) isotypes. A conserved DNA binding domain and a basic tetramerization domain are present in all p63 isotypes. In conjunction with the three different carboxy-termini, α, β, and γ, six major p63 isoforms are possible. For clarification, a full length cDNA or protein is described by its specific start point (TA or ΔN) followed by the gene name (p63) and the specific endpoint (α, β, or γ). The gene name followed by one specific isoform attribute refers exclusively to this isoform irrespective of other possible isoforms at the alternative end. TAp63γ resembles most p53, whereas the p63α and p63β splice variants extend the p63 protein sequence considerably. The full length p63α protein contains a sterile α motif-like sequence and a negatively charged C-terminus. As a result of exon 13 deletion, only the N-terminal part of the sterile α motif-protein domain is expressed in the p63β isoform. All p63 isoforms potentially bind to p53 responsive promoter elements but only TAp63γ and TAp63β transactivate reporter genes at levels comparable with wild-type p53 (Celli *et al*, 1999). TAp63α, possibly as consequence of an inhibitory effect of the C-terminus, and all p63ΔN isoforms completely lack this ability.

Basal keratinocytes in normal skin express detectable levels of ΔNp63α and its expression is associated with the proliferative potential in normal and neoplastic human keratinocytes (Parsa *et al*, 1999). ΔNp63α overexpression in mouse epidermis using the loricrin promoter results neither in an overt phenotype nor in an altered proliferation rate (Liefer *et al*, 2000).

Vitamin A and its major biologically active form all-*trans* retinoic acid have been appreciated as critical regulators of growth and differentiation in the developing and adult skin (Fisher and Voorhees, 1996). Proliferating keratinocytes in the basal layer of the skin leave the cell cycle and move on to an early phase of differentiation. Subsequently, keratinocytes form cross-linked envelopes in a late stage of differentiation (Green and Watt, 1982). Involucrin is a precursor protein of the cornified envelope and a marker for early keratinocyte differentiation.

In vitro, addition of retinoic acid to primary human keratinocyte cell cultures, which start to differentiate after growth factor withdrawal, efficiently retards cornified envelope formation and marginally stimulates proliferation. During the first 14 d of retinoic acid supplementation the amount of involucrin is comparably high in treated and untreated keratinocyte cultures (Cline and Rice, 1983), whereas the number of cornified envelopes is drastically reduced.

In vivo, retinoids do not suppress keratinocyte terminal differentiation but stimulate keratinocyte proliferation. Therefore,

Manuscript received July 19, 2001; revised September 20, 2001; accepted for publication October 16, 2001.

Reprint requests to: Hartwig Schmale, Institut für Zellbiochemie und Klinische Neurobiologie, Universitätsklinikum Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany. Email: schmale@uke.uni-hamburg.de

Abbreviations: b, bases; BPE, bovine pituitary extract; HGPRT, hypoxanthine guanine phosphoribosyl transferase; KGM, keratinocyte growth medium; ΔN, N-terminally truncated; RPA, ribonuclease protection assay; TA, transactivation.

retinoic acid is thought to modify the finely tuned balance between these two cellular processes.

Because of the ability of retinoids to modify keratinocyte differentiation, we investigated p63 splice variant expression in human primary keratinocytes. As an experimental approach, we used cell cultures cultivated under differentiation promoting conditions (Poumay *et al*, 1999) to study the development of p63 expression in detail over time during terminal differentiation.

MATERIALS AND METHODS

Cell culture Normal human primary keratinocytes (Clonetics, CellSystems, St. Katharinen, Germany) were cultivated on cell culture plates (Nalge Nunc Int., Rochester, NY) at 37°C and 5% CO_2. For all assays, keratinocytes in the third or fourth passage were used and media were renewed every second day. All experiments were repeated three times with different healthy donors for primary keratinocytes.

A simple change of culture media from proliferation- to differentiation-promoting cell culture conditions started the differentiation experiments. In detail, the proliferation-promoting keratinocyte growth medium (KGM-2, Clonetics) included bovine pituitary extract, insulin, transferrin, epinephrine, epidermal growth factor, and 0.15 mM $CaCl_2$ according to manufacturer's instructions. These supplements were omitted in the differentiation promoting keratinocyte basal medium-2 (Clonetics), whereas the calcium concentration was kept.

Keratinocytes at 50% cell density were washed twice with keratinocyte basal medium-2, cells were subsequently refed every second day with keratinocyte basal medium-2 and grown under differentiation promoting conditions for 11 d at 37°C and 5% CO_2.

For retinoic acid treatment, keratinocytes grown to 50% cell density were washed twice with KGM-2 medium without bovine pituitary extract (KGM-2-BPE). Cells were cultivated for 10 further days at 37°C and 5% CO_2 in KGM-2-BPE supplemented with 0.1 μM all-*trans* retinoic acid (Sigma, Deisenhofen, Germany). Retinoic acid stock solutions were prepared in dimethyl sulfoxide (Sigma) and control cells were grown in KGM-2-BPE, including 0.01% dimethyl sulfoxide for the same time span.

HaCaT cells kindly provided by Dr. N. Fusenig (Boukamp *et al*, 1988) and HEK293 cells (Graham *et al*, 1977) were cultivated in DMEM (Gibco, Eggenstein, Germany), including 10% fetal bovine serum (Gibco).

Preparation of human epidermis Skin from circumcisions (kindly provided by the children's hospital, Universitätsklinikum-Hamburg Eppendorf) was washed three times in wash buffer (1 × phosphate-buffered saline without calcium and magnesium supplemented with 0.5 mg per ml gentamicin (Gibco)); 0.25 cm^2 skin segments were dispase I digested (2.4 U per ml dispase I in wash buffer) overnight at 4°C. Using forceps, the epidermis was carefully separated from the dermis and stored at –70°C.

RNA preparation Human tissues used for RNA isolation were removed at autopsy and stored at –70°C. For RNA preparation from human cell culture cells, any liquid was removed from the Petri dishes, they were quickly cooled on dry ice and subsequently stored at –70°C. Total RNA was isolated using RNAclean (Hybaid-AGS, Heidelberg, Germany) according to the manufacturer's instructions.

Northern blot, ribonuclease protection assay (RPA), and reverse transcription–polymerase chain reaction (reverse transcription–PCR) The northern blot technique was employed for quantitative detection of human mRNA with the following α-^{32}P-deoxycytidine triphosphate labeled cDNA fragments (Amersham, Freiburg, Germany). Labeling was carried out with the Prime it II Random Primer Labeling Kit (Stratagene, La Jolla, CA), cDNA fragments were purified by gel filtration through Nuctrap Push columns (Stratagene) and quantified in a Wallac 1409 scintillation counter (Wallac, Freiburg, Germany). The p63core probe of 232 bp length (nt 964–1195; GB: AF075430) was used to detect all human p63 mRNA splice variants. Involucrin expression was analyzed by a 1647 bp long involucrin cDNA fragment (nt 183–1830; GB: M13902). The β-actin mRNA was identified with a 377 bp long β-actin probe (nt 1418–2234, excluding intron C; GB: M10277). Glyoxal denatured total RNA (15 μg) was separated in a phosphate-buffered 1.2% agarose gel. The RNA was transferred overnight on to a Hybond-N nylon membrane (Amersham) according to the manufacturer's instructions. The RNA molecules were heat cross-linked to the nylon membrane (80°C, 2 h) and prehybridization was performed at 42°C in ULTRAhyb buffer (Ambion, Austin, TX) for 2 h. The denatured radioactive labeled cDNA probe (95°C, 10 min, followed by 4°C, 5 min) was added to ULTRAhyb buffer (preheated to 68°C) to a final concentration of 2×10^7 cpm per ml. The northern blot was incubated overnight at 42°C in hybridization buffer. After washing twice for 15 min at 42°C in 2 × sodium citrate/chloride buffer/0.1% (wt/vol) sodium dodecyl sulfate (SDS) followed by 15 min at 42°C in 0.2 × sodium citrate/chloride buffer/0.1% (wt/vol) SDS blots were analyzed with the phosphoimager system Fuji BAS-1800II reader and the AIDA software package (Raytest, Straubenhardt, Germany). The northern blot was stripped in 0.1 × sodium citrate/chloride buffer/50% formamide (75°C, 1 h) and rehybridized with another radioactively labeled probe as described. Because of mRNA length and signal strength RNA were detected in the following order: p63, involucrin and β-actin.

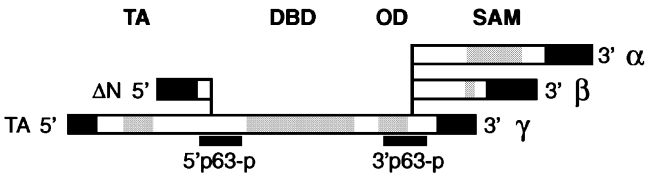

Figure 1. Schematic of the main p63 mRNA isotypes. Untranslated mRNA sequence is indicated in black. Gray areas highlight the conserved TA domain, the DNA binding domain (DBD), the oligomerization domain (OD) and the sterile α motif domain (SAM). Positions of the complementary RPA probes 5′p63-p and 3′p63-p are indicated.

For RPA, contaminating DNA in tissue and cell culture total RNA preparations was removed by DNaseI digestion (1 U per μg RNA, 15 min at 37°C in 1 × transcription buffer; Roche, Mannheim, Germany). A total of 15 μg tissue RNA or 10 μg cell culture RNA per assay was incubated with 5×10^5 cpm α-^{32}P-uridine triphosphate (Amersham) labeled probe at 42°C overnight in hybridization buffer (RPA-III-Kit, Ambion). A β-actin or hypoxanthine guanine phosphoribosyl transferase (HGPRT) specific probe (5×10^4 cpm per total RNA probe) was included as internal control. Riboprobes have the suffix "-p" and were generated by *in vitro* transcription of 1 μg linearized plasmid DNA with T7 RNA polymerase (Roche). 5′p63-p of 296 b length was used to hybridize to 236 b of the human p63TA mRNA (nt 166–401; GB: AF075430). Owing to the probe design (**Fig 1**), simultaneous detection of p63ΔN was possible as a 195 b long protected complementary RNA fragment. The p63α and p63γ splice variants were detected by the 3′p63-p of 262 b length, resulting in protected complementary RNA fragments of 164 b and 201 b (nt 904–1104; GB: AF075429), respectively. A 335 b long HGPRT RNA fragment (nt 2324–568; GB: NM_000194) was protected by the 421 b long transcript named HGPRT-p. Act-p of 160 b length hybridized to 125 b of the human β-actin mRNA (nt 821–945; GB X00351; pTRI-β-actin-125-Human Anti-sense Control Template plasmid; Ambion; T3 RNA polymerase (Roche) transcript). For p63 5′-end and 3′-end detection, RNase treatment was carried out for 40 min at 37°C with RNaseA/T_1 (1:100 dilution) and RNaseT_1 (1:50 dilution; RPA-III-Kit; Ambion), respectively. After separation of complementary RNA fragments in a 8% polyacrylamide gel, radioactive bands were analyzed on the dried gel with the phosphoimager system Fuji BAS-1800II reader and the AIDA software package (Raytest).

For reverse transcription–PCR, 2 μg of total RNA was reverse transcribed using Superscript II reverse transcriptase (Gibco) and random hexamer primers (Amersham). A PCR approach with a p63 5′-end forward and 3′-end reverse oligonucleotide identified the expressed p63 5′/3′-end combination. Specific p63 cDNA sequences were analyzed with the forward and reverse oligonucleotides listed in **Table I**. Primers were selected using the Lasergene program package (DNASTAR, Madison, WI). The HGPRT and one specific p63 splice variant cDNA were amplified from 1/20 of the reverse transcribed total RNA with the ELONGASE Enzyme Mix (BRL Life Technologies). PCR was performed in 35 cycles (94°C for 2 min for the initial denaturation, 94°C for 30 s and 55°C for 30 s and 68°C for 2 min 30 s for each cycle and 68°C for 10 min for the final elongation). PCR fragments were visualized with ethidium bromide after separation of equal volumes of each reaction on a 1.2% (wt/vol) agarose gel.

Western blot For SDS gel electrophoresis, cultured cells were lyzed in 1 × gel-loading buffer (2% SDS/60 mM Tris (pH 7.5)/100 mM dithiothreitol). The proteins were heat denatured (96°C, 5 min) and

Table I. Reverse transcription–PCR amplification primers used for detection of human HGPRT and p63 splice variants[a]

Identification	Forward primer	Reverse primer	Size (bp)	Position (nt)	GB
TAp63α	5′-gcc cat tga ctt gaa ctt tgt g-3′	5′-gtc tgg cgg agg gtg aat -3′	1727	92-1818	AF075430
TA1p63α	5′-aga gag ggg gaa gaa caa cag-3′	5′-gtc tgg cgg agg gtg aat -3′	1924	42-1965	(AF075430)
TA2p63α	5′-ttt gca aat atg tat gaa gga gag aag-3′	5′-gtc tgg cgg agg gtg aat -3′	2111	76-2186	(F075430)
ΔNp63α	5′-ggg gtt ggc aaa atc ctg-3′	5′-gtc tgg cgg agg gtg aat -3′	1727	55-1781	(F075431)
TAp63γ	5′-gcc cat tga ctt gaa ctt tgt g-3′	5′-gcc cag cag tac act tga ca-3′	1807	92-1898	AF075428
TA1p63γ	5′-aga gag ggg gaa gaa caa cag-3′	5′-gcc cag cag tac act tga ca-3′	2003	42-2044	(F075428)
TA2p63γ	5′-ttt gca aat atg tat gaa gga gag aag-3′	5′-gcc cag cag tac act tga ca-3′	2190	76-2265	(F075428)
ΔNp63γ	5′-ggg gtt ggc aaa atc ctg-3′	5′-gcc cag cag tac act tga ca-3′	1806	55-1860	(F075429)
HGPRT	5′-cgt cgt gat tag tga tga tga-3′	5′-ttc aaa tcc aac aaa gtc tgg c-3′	526	106-631	NM00194

[a]Numbers given in brackets indicate that the amplified PCR product is identical to a part of the Gene Bank cDNA entry.

after determination of protein concentration 10 μg per mm^2 protein was separated in a 8% SDS–acrylamide gel and blotted on to Immobilon-P (Millipore, Eschborn, Germany) according to standard procedures. Primary antibodies were diluted 1:200 for p63 monoclonal antibody 4A4 (Santa Cruz Biotechnology, Santa Cruz, CA), 1:10,000 for involucrin monoclonal antibody SY5 (Sigma) and 1 : 40,000 for β-actin monoclonal antibody 15 (Sigma) in blocking buffer (5% dried skimmed milk/1 × Tris-buffered saline/0.2% Nonidet P-40). After incubation with primary antibody (4°C, o/n), blots were washed five times in 1 × Tris-buffered saline (room temperature, 10 min) and bound antibodies were detected with horseradish coupled anti-mouse IgG (dilution 1 : 2500 in blocking buffer, room temperature, 2 h, ECL-System, Amersham). The final washing was performed as described and the horseradish peroxidase activity was detected with the SuperSignal Substrate (Pierce, Rockford, IL), measured with a Lumi-Imager (Roche) and quantified with the provided software.

RESULTS

Tissue distribution of p63 splice variants p63 expression was analyzed by RPA in different human tissues and cell lines with splice variant specific probes (**Fig 1**). The RPA probe 5′p63-p was used to detect p63TA (236 b). Protected complementary RNA fragments 41 b shorter than the expected p63TA fragment indicated p63ΔN (195 b) expression. In analogy, 3′p63-p was designed for the two alternative p63 3′-ends, p63α and p63γ. 3′p63-p protected 164 b common to all p63 splice variants and therefore indicated p63α and p63β expression. We did not differentiate between these isoforms because p63β is a minor transcript showing the same expression profile as p63α (Bamberger and Schmale, 2001). Additional 37 b are included in the case of p63γ (201 b) expression. HGPRT expression was monitored as positive control in all tissues and cell lines.

As indicated by RPA analysis, the existence of all four investigated p63 splice variants ΔN, TA, α, and γ was proven in human tongue consisting of multiple tissues such as epithelium, tongue muscle, and salivary glands (**Fig 2*A***, ***B***). RPA detected weakly but consistently the existence of TAp63γ mRNA in human skeletal muscle tissue. Very weak expression of p63ΔN was also found in skeletal muscle. The same RPA experiments suggest ΔNp63α expression in human placenta. In a spontaneously transformed human epithelial cell line from adult skin termed HaCaT (Boukamp *et al*, 1988) ΔNp63α was detectable in 25% higher levels than in proliferating primary human keratinocytes when normalized to HGPRT expression. As a negative control, the human embryonic kidney cell line HEK293 (Graham *et al*, 1977) was included in a 5-fold excess of total RNA. Neither 5′p63-p nor 3′p63-p detected any p63 expression.

p63 expression in human keratinocytes The described RPA experiments demonstrated the tissue distribution of individual p63 splice modules. Proliferating human primary keratinocytes in cell culture expressed ΔNp63α as the major splice variant. In order to investigate the p63 expression in human keratinocytes in more detail, specific PCR primers were used to amplify the entire coding

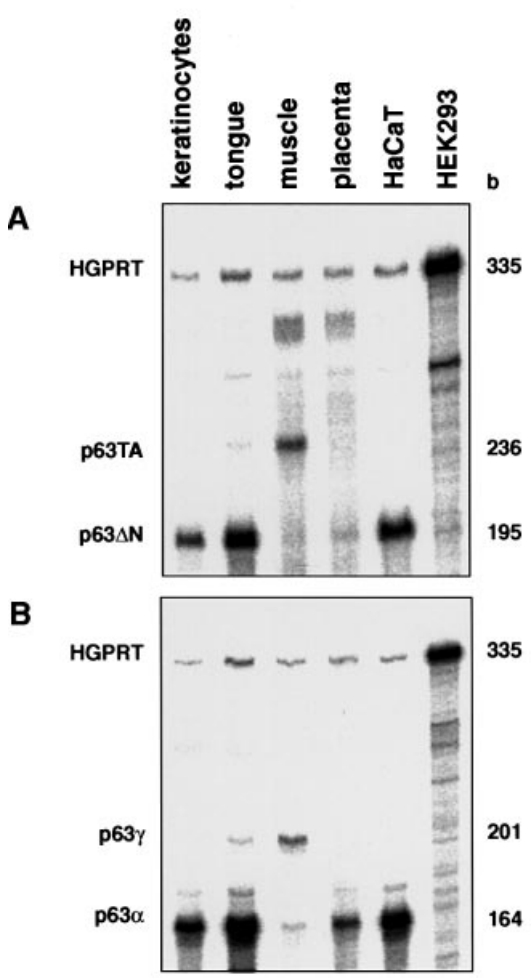

Figure 2. Expression of p63 splice variants in different human tissues and cell lines. The RPA identified 5′-end (*A*) and 3′-end (*B*) splice variants of p63 in the human tissues tongue, muscle, and placenta, and in the cell lines HaCaT and HEK and in primary keratinocyte cell culture. The quantity of RNA was monitored by HGPRT detection.

region. In normal human epidermal total RNA, ΔNp63α was verified as the major p63 splice variant (**Fig 3*A***). In addition, very weak expression of TAp63α and ΔNp63γ was detected. Southern blot analysis of 1/50 of the reverse transcription–PCR reaction volume verified the TA1p63α and TA2p63α expression in the human epithelium (not shown). TA1 and TA2 represent two alternative 5′-ends of the TA containing isoforms (Bamberger and Schmale, 2001). Reverse transcription–PCR experiments did not

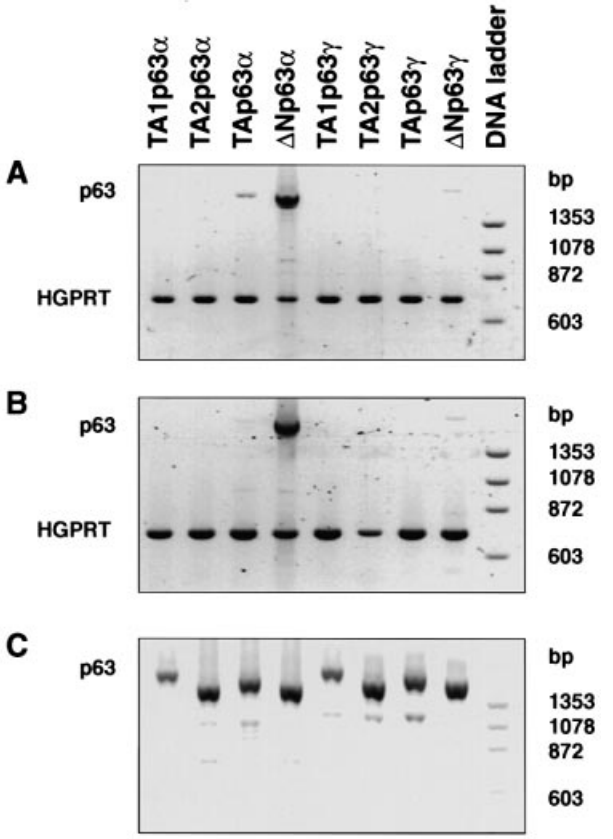

Figure 3. ΔNp63α is the major p63 splice variant expressed in keratinocytes. Different reverse and forward primer combinations were used in a reverse transcription–PCR experiment with epidermis RNA (*A*) and human primary keratinocyte RNA (*B*). Copies (1×10^4) of each cDNA splice variant were used as a template for a positive control PCR amplification (*C*).

show comparable expression intensity of TAp63α in normal human primary keratinocytes, whereas ΔNp63α and ΔNp63γ expression was still observed (**Fig 3*B***). Nonquantitative reverse transcription–PCR results suggest a ratio of ΔNp63β to ΔNp63α of 1:10. HGPRT amplification was included in the same reverse transcription–PCR reaction as positive control in both experiments. In order to demonstrate successful PCR of the p63 variants, about 10,000 cDNA copies of every splice variant served as the template in a positive PCR reaction (**Fig 3*C***). Amplification failed in a negative control reaction (not shown).

p63 expression during keratinocyte differentiation One major question concerning p63 in skin is its change in expression during early differentiation of epidermal keratinocytes. We followed p63 expression in keratinocytes grown under differentiation promoting conditions with emphasis on the quantity and the pattern of the observed p63 splice variants. Northern blot analysis indicated a 5-fold reduction in the ΔNp63α mRNA level after 11 d of culture under differentiation promoting conditions when normalized to β-actin (**Fig 4*A***). The process of ongoing differentiation of the keratinocytes was shown by the 4-fold induction of the involucrin mRNA expression, used as a positive marker for early differentiation. The observed reduction of ΔNp63α mRNA results in a 50-fold decrease in protein as shown by western blot analysis (**Fig 4*B***). In addition, RPA experiments indicated no upregulation of any of the two other p63 splice variants TA and γ at the time points investigated (**Fig 4*C*, *D***). A second protein detected by the monoclonal 4A4 antibody, which migrates faster than the 61 kDa marker protein, might result from the translation of ΔNp63γ mRNA because very weak expression of the γ isoform was also

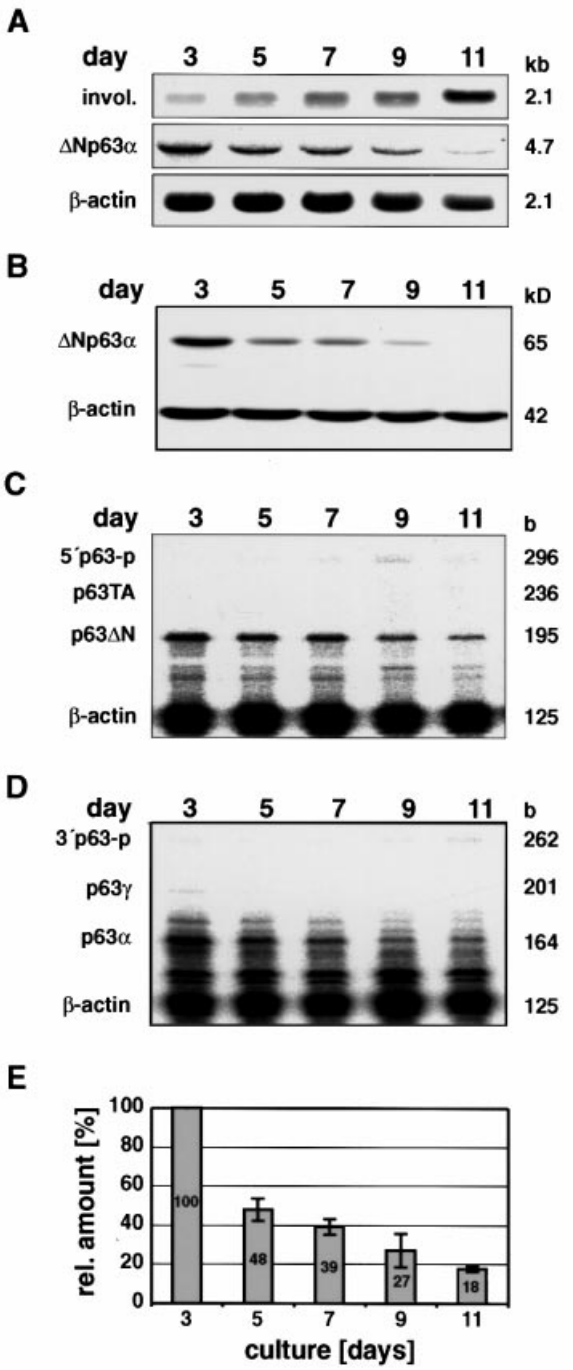

Figure 4. ΔNp63α expression drops during differentiation promoting cultivation of human primary keratinocytes. The involucrin, p63 and β-actin mRNA were detected in a northern blot assay (*A*). A western blot assay verified the change of p63 protein (*B*). In addition, RPA determined the splice variant expression of the p63 5′-end (*C*) and 3′-end (*D*). The p63 mRNA expression relative to β-actin was quantified in three independent experiments (*E*). Error bars: ± SD.

detected with RPA (**Fig 4*D***). The change in p63 mRNA expression relative to β-actin expression was quantified in three independent experiments using RPA (**Fig 4*E***).

The influence of retinoic acid on p63 expression Addition of retinoic acid to the cell culture medium prevents terminal differentiation of human primary keratinocytes. When compared with control cell cultures, p63 mRNA and protein levels remained high in keratinocytes after 10 d of treatment with 0.1 μM all-*trans*

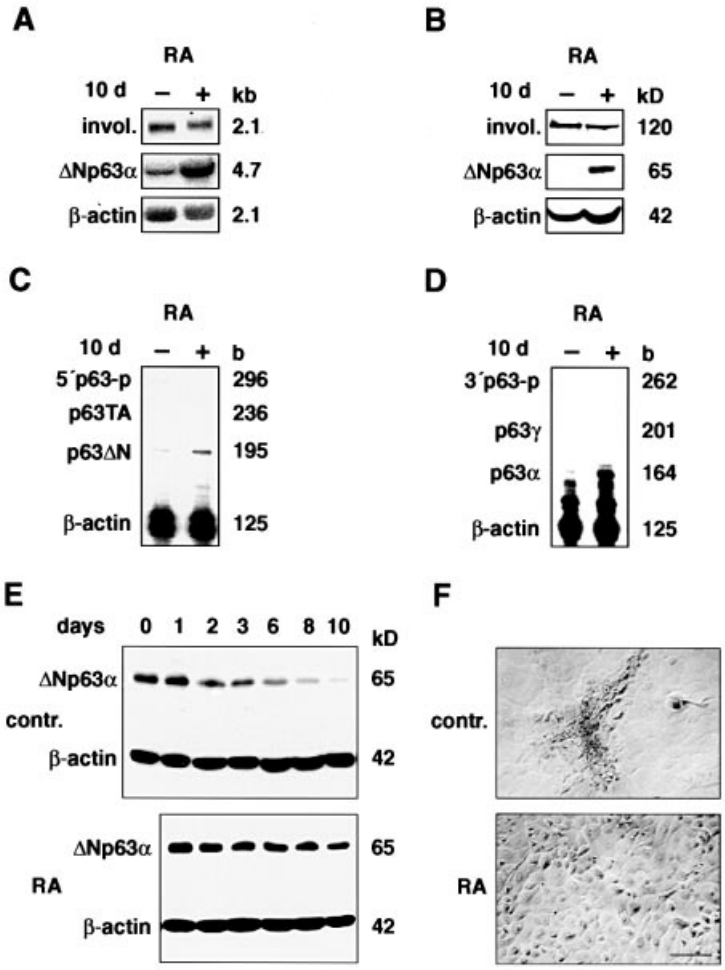

Figure 5. Retinoic acid blocks the ΔNp63α reduction observed during differentiation of human primary keratinocytes. After 10 d exposure to 0.1 μM retinoic acid, northern blot (*A*) and western blot (*B*) assays indicated continuous ΔNp63α expression in comparison with untreated keratinocytes. The splice variant expression was investigated with RPA for different p63 5′-ends (*C*) and 3′-ends (*D*). The development of ΔNp63α expression during cultivation in control and retinoic acid treated human primary keratinocytes was monitored on protein level using the western blot technique (*E*). Phase-contrast photographs indicate the morphologic changes of human primary keratinocytes after 11 d of culture with or without retinoic acid (*F*). *Scale bar*: 50 μm.

retinoic acid (**Fig 5**). Northern blot analysis (**Fig 5*A***) and western blot analysis (**Fig 5*B***) indicated high expression of both ΔNp63α mRNA and protein. RPA investigations verified these results and further indicated unchanged expression of the two other p63 splice variants TA and γ (**Fig 5*C*, *D***). Upregulation of involucrin mRNA and protein was not influenced by retinoic acid (Green and Watt, 1982). The cornified envelope assay (Rice and Green, 1979) indicated incomplete differentiation of retinoic acid treated keratinocytes as expected (not shown). The expression of ΔNp63α in the presence of retinoic acid was constant when studied at different time points (**Fig 5*E***). In the keratinocyte control culture ΔNp63α was downregulated during the process of differentiation.

DISCUSSION

Expression of a predominant p63 splice variant might be characteristic for a specific cell type. In a proliferating keratinocyte primary cell culture ΔNp63α is the major isoform found. In contrast, TAp63γ is the isoform predominantly observed in skeletal muscle. Different tissue components might contribute to the detection of all major p63 isoforms in the human tongue; however, the p63 positive cell types in tongue and placenta were not determined.

Very low expression of TAp63α was observed in normal human epidermis. Nonquantitative reverse transcription–PCR results suggest a reduction in TAp63α expression in keratinocytes upon *in vitro* cultivation. Interestingly, TAp63α was detected in HaCaT cells on protein level (Hall *et al*, 2000) and in SCC25 cells by reverse transcription–PCR (Nylander *et al*, 2000). Expression of TAp63α is the major difference between epidermis and cultured epidermal keratinocytes. Although contamination of the human epidermis with another cell type expressing this p63 isoform cannot be excluded, these data might imply that TAp63α is the critical splice variant for the long-term survival of keratinocytes. Alternatively, the existence of both minor expressed splice variants, TAp63α and ΔNp63γ, might be explained by leaky transcription and accidental splicing events.

In the cell culture model used here, keratinocytes began to differentiate and started to express the early differentiation marker involucrin. We showed a reduction in ΔNp63α expression in primary human keratinocyte cell culture when cells escaped from the cell cycle and started to differentiate. *In situ* hybridization and immunohistochemistry revealed high p63 expression only in the basal layer, the proliferative compartment of the skin (Parsa *et al*, 1999). This expression profile correlates with the high p63 expression observed in the proliferating keratinocyte cell culture and subsequent downregulation during differentiation. The change in ΔNp63α expression is in agreement with the previously reported cell size dependent expression of ΔNp63α (Parsa *et al*, 1999). Parsa *et al* (1999) found that small keratinocytes with high colony forming potential expressed high amounts of ΔNp63α, whereas large differentiated keratinocytes showed only marginal expression. In accordance with our results, normal human epidermal keratinocytes induced to differentiate *in vitro* by high calcium exposure downregulated all p63 isoforms (De Laurenzi *et al*, 2000). Results obtained by different experimental paradigms – the p63 downregulation after calcium induced differentiation (De Laurenzi *et al*, 2000), the cell size dependent expression observed by Parsa *et al* (1999) and the differentiation time dependent expression

described here – argue for high ΔNp63α levels in proliferating keratinocytes and a downregulation during differentiation. No change in the relative p63 splice variant pattern was observed at the investigated time points. Whether the p63 expression changes its pattern in keratinocytes in the early response phase to growth factor withdrawal was not investigated.

Retinoic acid added to a keratinocyte cell culture is known to inhibit the expression of the differentiated phenotype (Fisher and Voorhees, 1996). Retinoids allow keratinocytes to proceed through the early steps of differentiation but block the subsequent cornified envelope formation (Green and Watt, 1982). *In vivo*, retinoic acid stimulates in the skin the division of the basal keratinocytes that migrate upward and differentiate (Fisher *et al*, 1991). Sustained expression of ΔNp63α in retinoic acid treated keratinocytes argues for this p63 isoform as a marker for the keratinocyte proliferation capacity. In addition, other signals provided in the basal compartment of normal human epidermis may be needed for keratinocytes in order to proceed through the cell cycle.

Is downregulation of ΔNp63α essential for the onset of differentiation or rather required to allow other processes that are specific for the differentiated phenotype in suprabasal layers of the epidermis? Transgenic mice overexpressing ΔNp63α in the skin under the control of the basal loricrin promoter show unaltered proliferation and differentiation of keratinocytes (Liefer *et al*, 2000). This finding and our data suggest that ΔNp63α downregulation is not the driving force for differentiation. Absence of the dominant-negative ΔNp63α isoform may release specific DNA-binding sites in responsive genes and allow transcriptional activity of p53 and p73, the other members of the p53 family.

Despite a decrease in p53 mRNA and protein in human keratinocytes during differentiation, p53-mediated transcription activity increases (Weinberg *et al*, 1995). The downregulation of the dominant negative ΔNp63α isoform might explain the observed increase of p53 activity because both proteins may compete for the same responsive elements in target gene promoters. Recently it has been shown that downregulation of p63 is required for epidermal Ultraviolet B induced p53-dependent apoptosis (Liefer *et al*, 2000). Along these lines, the transcriptionally active isoform of p73, TAp73δ, is induced in keratinocytes exposed to high calcium, whereas the expression of ΔNp63α is decreased. TAp73δ was reported to be effective in inducing transactivation of the epidermal differentiation markers, involucrin and loricrin (De Laurenzi *et al*, 2000). p53- and p73-null mice, however, exhibit histologically normal skin (Yang *et al*, 2000) suggesting a highly redundant and complex regulatory protein network for keratinocyte differentiation.

Vitamin A deficiency and loss of p63 result in malformation of distal limb structures. Mice without a functional p63 gene show major defects in their limb, craniofacial, and epithelial development (Mills *et al*, 1999; Yang *et al*, 1999). Their hindlimbs are absent and the forelimbs are truncated because the apical ectodermal ridge, an epithelium essential for limb development, is not maintained. Interestingly, vitamin A deficiency during early mammalian development results in malformations that include cleft face, palate, and lip, and abnormalities of the forelimbs (Morriss-Kay and Ward, 1999). A similar phenotype is caused by heterozygous germline mutations in p63 observed in patients who exhibit a syndrome of ectrodactyly, ectodermal dysplasia and cleft lip with or without cleft palate (Celli *et al*, 1999).

Hence p63 expression maintained by retinoic acid seems to be necessary for normal limb development.

The excellent technical assistance of Bernhard Oltrogge and Michael Brandt in keratinocyte cell culture handling and of Heidje Christiansen in molecular biology techniques is gratefully acknowledged. The data presented here form part of a Ph.D. thesis (CB).

REFERENCES

Bamberger C, Schmale H: Identification and tissue distribution of novel KET/p63 splice variants. *FEBS Lett* 501:121–126, 2001

Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE: Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* 106:761–771, 1988

Celli J, Duijf P, Hamel BC, *et al:* Heterozygous germline mutations in the p53 homolog p63 are the cause of EEC syndrome. *Cell* 99:143–153, 1999

Cline PR, Rice RH: Modulation of involucrin and envelope competence in human keratinocytes by hydrocortisone, retinyl acetate, and growth arrest. *Cancer Res* 43:3203–3207, 1983

De Laurenzi V, Rossi A, Terrinoni A, *et al:* p63 and p73 transactivate differentiation gene promoters in human keratinocytes. *Biochem Biophys Res Commun* 273:342–346, 2000

Fisher GJ, Voorhees JJ: Molecular mechanisms of retinoid actions in skin. *FASEB J* 10:1002–1013, 1996

Fisher GJ, Esmann J, Griffiths CE, *et al:* Cellular immunologic and biochemical characterization of topical retinoic acid-treated human skin. *J Invest Dermatol* 96:699–707, 1991

Graham FL, Smiley J, Russell WC, Nairn R: Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 36:59–74, 1977

Green H, Watt FM: Regulation by vitamin A of envelope cross-linking in cultured keratinocytes derived from different human epithelia. *Mol Cell Biol* 2:1115–1117, 1982

Hall PA, Campbell SJ, O'Neill M, Royston DJ, Nylander K, Carey FA, Kernohan NM: Expression of the p53 homologue p63α and ΔNp63α in normal and neoplastic cells. *Carcinogenesis* 21:153–160, 2000

Liefer KM, Koster MI, Wang XJ, Yang A, McKeon F, Roop DR: Down-regulation of p63 is required for epidermal UV-B-induced apoptosis. *Cancer Res* 60:4016–4020, 2000

Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A: p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* 398:708–713, 1999

Morriss-Kay GM, Ward SJ: Retinoids and mammalian development. *Int Rev Cytol* 188:73–131, 1999

Nylander K, Coates PJ, Hall PA: Characterization of the expression pattern of p63α and ΔNp63α in benign and malignant oral epithelial lesions. *Int J Cancer* 87:368–372, 2000

Parsa R, Yang A, McKeon F, Green H: Association of p63 with proliferative potential in normal and neoplastic human keratinocytes. *J Invest Dermatol* 113:1099–1105, 1999

Poumay Y, Herphelin F, Smits P, De Potter IY, Pittelkow MR: High-cell-density phorbol ester and retinoic acid upregulate involucrin and downregulate suprabasal keratin 10 in autocrine cultures of human epidermal keratinocytes. *Mol Cell Biol Res Commun* 2:138–144, 1999

Rice RH, Green H: Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. *Cell* 18:681–694, 1979

Strano S, Rossi M, Fontemaggi G, Munarriz E, Soddu S, Sacchi A, Blandino G: From p63 to p53 across p73. *FEBS Lett* 490:163–170, 2001

Weinberg WC, Azzoli CG, Chapman K, Levine AJ, Yuspa SH: p53-mediated transcriptional activity increases in differentiating epidermal keratinocytes in association with decreased p53 protein. *Oncogene* 10:2271–2279, 1995

Yang A, McKeon F: p63 and p73: p53 mimics, menaces and more. *Nat Rev Mol Cell Biol* 1:199–207, 2000

Yang A, Schweitzer R, Sun D, *et al:* p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 398:714–718, 1999

Yang A, Walker N, Bronson R, *et al:* p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature* 404:99–103, 2000