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p63 and p73, members of the p53 gene family, transactivate PKCδ

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PKC, protein kinase C
TA, transactivation domain
ΔN, amino terminal truncated protein
Dox, doxycycline
BSA, bovine serum albumin
RE, responsive element
NHEK, normal human epidermal keratinocytes

ABSTRACT

The p53 family comprises three genes that encode for p53, p63 and p73. These genes have a significant degree of sequence homology, especially in the central sequence-specific DNA-binding domain. The high homology among the three DNA-binding domains indicates that these transcription factors have identical residues interacting with DNA, and thus potentially can recognize the same transcriptional targets. In this study, we demonstrate that PKCδ is induced by p63 and p73 in Saos2 cells. The putative human PKCδ promoter harbours three p53-like binding sites (RE I, RE II, RE III). In order to confirm the transactivation of PKCδ by p53 family members, we performed transcription assays using the entire or selected regions of the promoter upstream of a luciferase reporter gene. The results obtained demonstrated that, at least *in vitro*, the p53 family members tested (TAp63α, TAp73α, ΔNp63α, but not ΔNp73α) were able to drive transcription from the PKCδ promoter.

1. Introduction

The p53 family comprises three genes that encode for p53, p63 and p73. These genes have a significant degree of sequence homology, especially in the central sequence-specific DNA-binding domain (DBD), the amino-terminal activation (TA) domain and the carboxy-terminal oligomerization domain [1–3]. All p53 family genes give rise to different proteins by the use of two different promoters, generating the TA and ΔN isoforms, and

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alternative splicing, generating C-terminal truncated isoforms.

The TA domain is encoded by exons 2 and 3 of the p63 gene. Utilization of the alternative promoter for ΔNp63 expression, results in the generation of proteins with a shorter amino-terminus, thus lacking the TA domain [4–6]. In addition, the α isoforms of p63 contain a sterile α-motif (SAM domain) at the carboxy-terminal, which is a protein–protein interaction domain that is absent in p53. This domain is absent in the beta and gamma isoforms due to alternative splicing.

Considering the primary sequence, only the TA isotypes would appear to have transcriptional activity. In contrast, the ΔN isotypes, lacking the TA domain, would be expected to be transcriptionally silent or, alternatively, act as dominant-negative inhibitors of the TA isoforms. Indeed this is the case for p73; however, recent studies indicate that the ΔNp63β isoform is a potent transactivator, stronger than ΔNp63γ, thus suggesting that a second activation domain is present in the p63 sequence. Indeed, deletion experiments have shown that a second TA domain is located between residues 410 and 512, corresponding to exons 11 and 12 [7]. The presence of a second transactivation domain has been further confirmed by studies on ΔNp63 mutations, which lead to gain of function in the Adult syndrome [8]. Together, these studies indicate that the ΔNp63 isoforms, in which the N-terminus domain is missing, are also transcriptionally active.

The high homology (63% amino acid identity) between the DBDs of the three proteins suggests that these transcription factors have identical residues interacting with DNA, thus potentially recognising the same transcriptional targets; although recent findings suggest that the target specificities of p53, p63 and p73 are determined by differential recognition of response elements in the promoters [9,10].

Like p53, p63 and p73 also play a role in human cancer, although their precise role in tumorigenesis is not completely understood [11]. Although they do not act as classic tumour suppressor genes, co-operation between the three p53 family members has recently been demonstrated. In transitional cell carcinomas, expression of p63/p73 and wild-type p53 was mostly seen in superficial non-invasive lesions, while invasive tumours showed mutant p53 with loss of p63/p73 expression [12]. In E1A-expressing mouse embryo fibroblasts, loss of p63 and p73 impaired the induction of p53-dependent apoptosis in response to doxorubicin [13], thus suggesting that p63 and/or p73 are required for p53 activity.

Despite their molecular homology and possession of at least some overlapping functions, p53, p63 and p73 also appear to have distinct individual activities. From gene deletion studies, p53 is mainly involved in cell cycle arrest and apoptosis, while p73 and p63 are more related to development [14,15]. In particular, data from p63 null mice suggest that p63 is important in epithelial, craniofacial and limb development. Indeed, p63 null mice die within a few hours of birth, probably due to dehydratation. The skin, limbs, urothelium and secretory epithelia are absent probably because of failure to maintain regenerative epithelia. All the affected organs are characterized by profound hypocellularity and denuded surfaces [16,17].

To characterise how downstream functions of the p53 family members are mediated, we performed a microarray analysis to identify genes that are differentially regulated by the TA and ΔN isoforms of p63 [18]. Among the 171 genes that are upregulated by TAp63α, 163 are unique, and 8 are also activated by ΔNp63α. We identified protein kinase C delta (PKCδ) as one of the differential transcriptional targets of TA and ΔNp63. PKCδ is part of a large family of serine/threonine kinases that are activated by extracellular stimuli. PKCδ is ubiquitously expressed and is involved in a variety of cellular signalling pathways involving cell growth, differentiation, apoptosis, tumour promotion and carcinogenesis. Within the PKC isoenzyme family, PKCα and ε have been implicated in cell proliferation [19,20] while PKCδ and η have been associated with apoptosis and terminal differentiation [21,22]. PKCα, δ, ε, η, ζ, and μ, have been localized in epidermis [23–25], and keratinocytes express the PKCα, δ, ε, ζ, η isoforms [26] where they act as regulators of keratinocyte differentiation-dependent gene expression [19,26–31]. Transgenic mice over expressing PKCδ and ε in the skin develop fewer benign tumours induced by chemical carcinogens while PKCα over expression does not alter the tumour yield [32]. Interestingly, during the apoptotic response to DNA-damage, PKCδ is activated by c-Abl tyrosine kinase [33] and cleaved by caspase-3 to form a kinase-active catalytic fragment; this fragment contributes to the induction of apoptosis by activating p73 [34]. Indeed, PKCδ, both full length and activated fragment, physically interact with p73 and phosphorylates p73β at the Ser-289 residue located in the DNA-binding domain resulting in p73β protein accumulation and activation [25].

In the present study, we focus on PKCδ regulation at the transcriptional level by the p53 family members.

2. Materials and methods

2.1. Cell lines

The p63 and p73 Saos-2 clones, stably transfected with the pTet-On regulator plasmid, and the p53 overexpressing Saos-2 inducible clone were a generous gift of Dr. Karen Vousden (Tumor Suppression Laboratory, Beatson Institute for Cancer Research, Cancer Research UK Beatson Laboratories, Glasgow, United Kingdom). HaCaT cells were kindly provided by Prof. N.E. Fusenig (German Cancer Research Center, Heidelberg, Germany). Cells were grown in a 1:1 mixture of minimal essential medium and Ham's F-12 medium supplemented with 10% (v/v) heat-inactivated FCS, 1.2 g/l Na-bicarbonate, 1% (v/v) non-essential amino acids and 15 mM HEPES. Normal human epidermal keratinocytes (NHEKs) were obtained from Clonetics and grown in calf skin collagen coated dishes in serum-free keratinocyte growth medium (SFM, Gibco) at 0.05 mM calcium, supplemented with 60 mg/ml of bovine pituitary extract. Third passage NHEKs were used for each experiment. Human small lung adenocarcinoma cells H1299 were maintained in RPMI (GibcoBRL) supplemented with 10% fetal bovine serum (GibcoBRL). All cells were grown at 37 °C with 5% CO_2 in a humidified atmosphere.

2.2. Immunoblot analysis

Cells were resuspended in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail

(Sigma)) and sonicated. 100 μg of total protein was loaded to 10% SDS-PAGE and transferred to PVDF membrane (Amersham). The membranes were incubated with the blocking solution (5% non-fat dry milk in Tris buffered saline (TBS)) and then incubated with the specific primary antibody. The antibodies against p53 (FL393), p63 (H179) and actin were from Santa Cruz. The PKCδ antibody was from R&D. The anti-HA antibody was from Sigma. Following incubation with the relevant horseradish peroxidase-conjugated secondary antibodies, detection was carried out with ECL (Amersham).

2.3. Cell transfection and luciferase assay

Transient transfections were performed using Lipofectamine 2000 reagent (Invitrogen) following the protocol of the manufacturer. H1299 cells were transfected in 96-well plates (Corning Costar) with the HA-tagged p53, TAp63α, ΔNp63α; TAp73α or ΔNp73α plasmids (75 ng/well), PKCδ luciferase reporter plasmid (75 ng/well) and Renilla luciferase reporter (6 ng/well). Twenty-four hours later, the luciferase activity was quantified by using a dual-luciferase reporter assay system (Promega, Madison, USA) with a Perkin-Elmer Victor2 luminometer.

2.4. Real-time PCR assay

RNA was extracted from cells, using the Trizol reagent (Invitrogen). One microgram of RNA was used for reverse transcription using the InPromII kit (Promega, Madison, USA) and 1/5 of the reaction was used for PCR. Real-time PCR was performed using the Platinum SYBR Green qPCR SuperMix UDG with Rox (Invitrogen), with an amplification program as follows: 1 cycle of 95 ° for 3′ and 40 cycles of 94 ° for 15″ and 59° for 50″. The reaction was followed by a melting curve protocol according to the specification of the ABI 7000 instrument (Applied Biosystem). The primers used were: PKCδ (+GCAA-GGCTGAGTTCTGGCTG, −GGT GCA CAT TCA TGC CGC AG), beta actin (+AAA GAC CTG TAC GCC AAC A, −CGG AGT ACT TGC GCT CAG). Beta-actin was used as housekeeping gene for quantity normalization; relative quantization of gene expression was performed according the method described in ABI User Bulletin #2 (updated 10/2001).

2.5. Cloning the putative PKCδ promoter region

A region 2 kb upstream of the transcription-start site of the human PKCδ gene was analysed using Math-Inspector Professional software and TRANSFAC database [35]. The analysis revealed the presence of three p53-like responsive elements located respectively at −314 (RE-I), −156 (RE-II), and −61 (RE-III) upstream of the transcription-start site. The region from −564, up to +428, including the three p53-like binding sites and the untranslated exon 1, was selected as a minimal promoter of PKCδ. The region was amplified by PCR from genomic DNA, using primers (+TAG GGG CAA CTG CCA GTC CGA GTA G, −TGT CTA GCG GAG GGA CAC ACA GACG), and cloned into *Kpn*I and *Hin*dIII site of pGL3 basic luciferase reporter (Promega, Madison, USA), using adapter primers (+*Kpn*I CCG AGT AGG GGT ACC TCC CAG AAC CGC C, −*Hin*dIII GGG ACA CAC AAG CTT ACA GGG GGA CGG). Three PKCδ mutant putative promoters were also generated by selective deletion of single p53-like binding sites. The deletions were generated by PCR and the products were cloned into *Kpn*I and *Hin*dIII sites in the same reporter vector. The primers used for the deletions were: RE-I (+GGG GTA CCT CAG CGT CCT CCT CTG TA, −GGG ACA CAC AAG CTT ACA GGG GGA CGG); RE-II (+GGG GTA CCT CCC CGA TTG CAG CTG G, −GGG ACA CAC AAG CTT ACA GGG GGA CGG), and RE-III (+GGG GTA CCT CGC ACT TCC GTG TG, −GGG ACA CAC AAG CTT ACA GGG GGA CGG). The vectors containing the wild type putative minimal promoter and the deletion mutants were used in luciferase assay experiments using the dual luciferase reporter assay system according the manufacturers instructions.

2.6. Site directed mutagenesis of the PKCδ promoter

Site directed mutagenesis of single p53-like responsive elements was performed by PCR in a two-step cloning strategy. Three PCR fragments containing the single p53 core sequences mutated were generated using the following primers and cloned into *Nhe*I and *Hin*dIII sites of pGL3 basic luciferase reporter vector (Promega). Forward primers: RE I-*Nhe*IF CC**GCTAGC**CAGTGGGGAGTCCCGGGTTAAGGTGTG; RE II-*Nhe*IF GGGAG**GCTAGC**GAAGAGGGCTTAACAGGGCAG; RE III-*Nhe*IF CA**GCTAGC**GGGGCGAGGCCTGGTTAAGCGAGGC. The reverse primer was the same for all PCR reactions: *Hin*dIIIR GGGACA-CAC**AAGCTT**ACAGGGGGACGG. The three constructs obtained as described were used for a second step cloning into *Kpn*I and *Nhe*I sites of three PCR fragments generated using the following primers. The forward primer was the same for all PCR reactions:

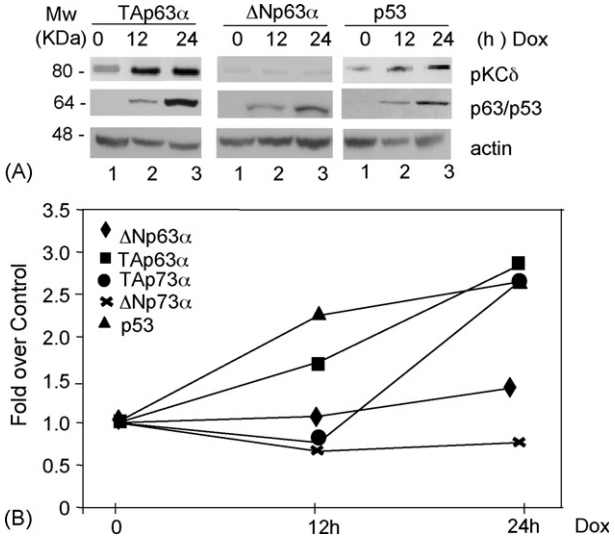

Fig. 1 – Induction of PKCδ by p63 and p53. (A) Induction of PKCδ expression by p63 and p53. Saos-2 inducible cells were treated with doxocyclin (2 μg/ml) for 12 and 24 h, and p63, p53 and PKCδ levels were analyzed by Western blotting. Actin was used as a loading control. (B) Real-time PCR analysis of PKCδ expression following induction of p53, TA or ΔNp63 and p73 isoform expression in inducible Saos-2 cells revealed that PCKδ messenger RNA is induced by TAp63α, TAp73α and p53 in a time-dependent manner. A representative experiment of two is shown.

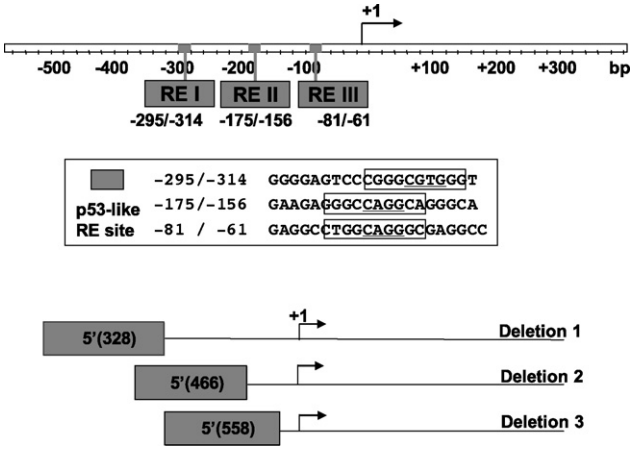

Fig. 2 – Map of the human PKCδ promoter region. The PKCδ promoter has three putative p53-like responsive elements located between −295/−314 (RE I), −175/−156 (RE II) and −81/−61 (RE III), indicated by the boxes; corresponding base pairs and sequences are indicated. The deletion constructs of the PKCδ promoter plasmid are indicated in the lower panel.

*Kpn*IF CCGAGTAGG**GGTACC**TCCCAGAACCGCC. Reverse primers: RE I-*Nhe*IR ccccact**ggctagc**tggcccgc; RE II-*Nhe*IR CCC-TCTTC**GCTAGC**CTCCCGCC; RE III-*Nhe*IR GCCCC**GCTAGC**TG-CAATCGGGG. *Kpn*I, *Nhe*I, *Hind*III restriction sites and p53 core sequence mutations are indicated in bold while p53 putative responsive elements are underlined.

3. Results and discussion

The data obtained from the microarray analysis showed that TAp63α and ΔNp63α can promote specific transcriptional gene profiles. We identified PKCδ as a target that is regulated differentially by the two p63 isoforms.

In order to verify the data obtained by the array, we induced expression of TAp63α, ΔNp63α and p53 in inducible-Saos-2 cells by doxocyclin treatment. 12 and 24 h following induction, PKCδ levels were upregulated in TAp63α and p53 expressing cells (Fig. 1A). No induction of PKCδ was seen in ΔNp63α expressing cells. We also confirmed these results by real-time PCR. Expression of TAp63α and p53, but not ΔNp63α, resulted in induction of PKCδ expression, in a time-dependent manner (Fig. 1B). As the DNA binding domain of the p53 family members are highly homologous, we asked whether p73 expression will cause the same effect on PKCδ levels. As shown in Fig. 1B, similar to p63, TAp73α, but not ΔNp73α induced PKCδ expression.

By using MatInspector Professional software and the TRANSFAC database [35], we searched the promoter region of human PKCδ for potential p53-like binding sites. We identified three p53-like consensus motifs (matrix V$P53F/P5301), which contained the core sequence CWWG (Fig. 2). This promoter region was cloned upstream of a luciferase reporter gene, and luciferase assays were performed in H1299 cells by co-transfecting the reporter plasmid, with plasmids expressing different p63/p73 isoforms, or p53 (Fig. 3). Human TAp63α

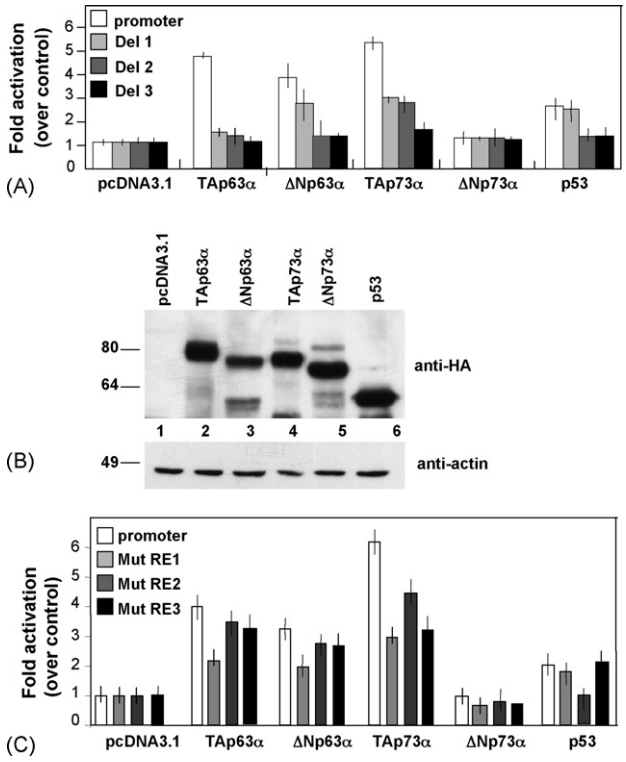

Fig. 3 – TAp63α-dependent transactivation of PKCδ depends on the RE I element. H1299 cells were transfected with constructs containing the entire promoter region (promoter) or with selective deletion/mutation of the individual p53 consensus motifs in the PKCδ promoter (deletion 1, 2 and 3, Mut RE 1, Mut RE 2, Mut RE 3, respectively). (A) All p53 family members tested (except ΔNp73α) transactivated the entire PKCδ promoter containing three p53-like binding sites (RE I–III). Deletion of the p53-like RE I (deletion 1: −295/−314) caused loss of the TAp63α transcriptional activity, suggesting that the TAp63α-dependent transactivation strictly depends on the RE I element. Deletion 1 did not change the luciferase activity of p53, but decreased by about 50% the luciferase activity triggered by TAp73α. Deletion of the p53-like RE II (deletion 2: −175/−156) resulted in the loss of p53 transcriptional activity, suggesting that p53-dependent transactivation strictly depends on the RE II element. Deletion 2 again reduced the transcriptional activity of TAp73α by about 50%. Deletion of the p53-like RE III (deletion 3: −81/−61) abolished the activity of every transcription factor tested. Results shown are the average of three independent experiments. (B) Following transfection, H1299 cells were lysed and Western blot was performed using an anti-HA antibody. Anti-actin Western blot was performed as loading control. The figure shows a representative experiment. (C) Mutation in the first p53-like binding site (Mut RE I) did not affect p53 activity, but significantly reduced that of TAp63α and partially inhibited the activity of the ΔNp63α and TAp73α isoforms. Mut RE II abolished the luciferase activity of p53 and reduced TAp73α activity. Mut RE III did not changed the promoter activity of any transcription factors tested, except for reduction in TAp73α luciferase activity, suggesting that the third site is not used by p53 and TAp63α.

significantly increased luciferase activity (4.7-fold activation over control). Unexpectedly, although we did not detect induction of PKCδ expression following ΔNp63α overexpression in the microarray, or by Western blotting and real-time PCR, ΔNp63α also produced similar enhancements of PKCδ promoter activity in the luciferase assay (Fig. 3A). One possible explanation could be the presence of a ΔNp63 responsive negative regulatory region, upstream of the cloned PKCδ promoter sequence or epigenetic regulations (DNA methylation and other modifications in the native promoter that were not present in the promoter of the luciferase reporter plasmid).

We also performed luciferase-assays after co-transfection of the PKCδ promoter with TAp73α and ΔNp73α. Although ΔNp73α had no effect, TAp73α significantly increased luciferase activity (4.9-fold over control) (Fig. 3A).

In order to identify which of the p53 consensus motifs are responsible for the increased promoter activity, we performed deletions on the PKCδ promoter (Figs. 2 and 3). Deletion of RE III (−81 to −61) closest to the transcription-start site abolished promoter activity of p53 and all p63 and p73 isoforms (Fig. 3A). However, deletion of the most 5′ p53 consensus site (RE I: −314 to −295) did not affect p53 activity, but totally abrogated that of TAp63α and partially inhibited that of the ΔNp63α and TAp73α isoforms. Total loss of promoter activity by p53 and both α isoforms was produced by deletion of the intermediate RE II −175 to −156 element. To further validate the results obtained with the PKCδ deleted-promoter constructs, we also performed site directed mutation approaches in the three p53-like binding sites. Mutation of the p53 consensus site RE I did not affect p53 activity, but significantly reduced that of TAp63α and partially inhibited the activity of the ΔNp63α and TAp73α isoforms (Fig. 3C). Mutation of the second p53 consensus site, RE II, abolished luciferase activity of p53 and reduced TAp73α activity (Fig. 3C). Finally, mutation of RE III did not change the promoter activity of any transcription factors tested, except for reduction in TAp73α luciferase activity (Fig. 3C), suggesting that the third site is not used by p53 and TAp63α. The site-directed mutation studies showed a decrease of the luciferase activity and not a complete abolishment upon mutation of RE I and RE II sites indicating that, as also suggested by others [36], other transcription factors are regulating PKCδ expression. As control, using the same cell extracts of the luc-assay, we performed Western blot to show that the absence of lucifierase is not due to different level of expression of the transcription factors (Fig. 3B).

These data presented indicate that not all p53 family members necessarily use the same consensus promoter elements for transactivating specific downstream genes and may reflect a preference for a CGTG motif with a downstream GC-rich sequence by p63 as opposed to CATG by p53 as has been suggested for other promoters [9].

4. Conclusions

The discovery of two p53 homologues, p73 and p63, has opened new questions on p53 family signalling, as distinct and overlapping functions have been identified for the different family members. Here, we identify PKCδ as a target gene of p53, p63 and p73. We demonstrate that, at least *in vitro*, p53, TAp63α and TAp73α are able to contribute to the transcription of the PKCδ promoter, although transcript abundance and PKCδ protein levels are not increased by ΔN isomeric forms. These effects of p53 family members on the PKCδ promoter appear to result at least in part, from selective recognition of different consensus p53 promoter elements. Furthermore, these studies suggest a possible positive regulatory loop between p73 and PKCδ in the apoptotic response induced by DNA-damage.

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