Distinct regulation of p53-mediated apoptosis by protein kinase Cα, δ, ε and ζ: Evidence in yeast for transcription-dependent and -independent p53 apoptotic mechanisms

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CHAPTER 2

Differential regulation of p53 function by protein kinase C isoforms revealed by a yeast cell system

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DIFFERENTIAL REGULATION OF P53 FUNCTION BY PROTEIN KINASE C ISOFORMS REVEALED BY A YEAST CELL SYSTEM

2.1. ABSTRACT

The complexity of the mammalian p53 pathway and protein kinase C (PKC) family has hampered the discrimination of the effect of PKC isoforms on p53 activity. Using yeast co-expressing the human wild-type p53 and a mammalian PKC–α, –δ, –ε or –ζ, we showed a differential regulation of p53 activity and phosphorylation state by PKC isoforms. Whereas PKC-α reduced the p53-induced yeast growth inhibition and cell cycle arrest, PKC–δ, –ε enhanced the p53 activity through p53 phosphorylation, and PKC–ζ had no effect on p53. This work identified positive and negative p53 regulators which represent promising pharmacological targets in anti-cancer therapy.

Keywords: p53, PKC isoforms, Cell growth, Cell cycle, p53 phosphorylation, Yeast

2.2. INTRODUCTION

The p53 tumour suppressor protein is mutated in about half of all human tumours and in many others that retain a wild-type (WT) p53, p53-dependent pathways leading to cell cycle arrest or cell death are often deficient. Restoring p53 function represents therefore a promising approach for cancer therapeutics. Among the different mechanisms of p53 regulation, post-translational modification by phosphorylation has been shown to play a critical role in the stabilization and activation of WT p53 (1).

One of the key enzymes involved in p53 phosphorylation is protein kinase C (PKC) (2,3). PKC is a family of serine/threonine kinases with at least 10 isoforms grouped into three subfamilies based on their primary structure and cofactors required for activation: classical (α, βI, βII and γ), novel (δ, ε, η and θ) and atypical (ζ and λ/ι). PKC isoforms are important regulators of several cellular processes, such as cell proliferation and death, and a striking feature is that individual isoforms can exert either similar or opposite effects in these processes (4,5).

Due to the high complexity of the mammalian p53 pathway and PKC family, particularly the coexistence of several PKC isoforms in the same cell, discrimination of the role of each PKC isoform in the regulation of p53 activity has been hampered. To circumvent this limitation, in this work, we exploited yeast cells co-expressing the human WT p53 and an individual mammalian PKC isoform.

The remarkably high degree of conservation of many pathways and cellular processes among yeast and human has allowed transposing the knowledge obtained in yeast to mammalian cells and vice versa. Additionally, the data obtained with the expression of human proteins that lack a yeast orthologue have been crucial to elucidate the role of proteins and the molecular mechanisms of complex human disorders, such as cancer and neurodegenerative diseases (6). Since the yeast PKC (Pkc1p in *Saccharomyces cerevisiae*) is a structural but not a functional homologue of mammalian PKC isoforms, yeast was considered a well-suited organism to study individual mammalian PKC isoforms (7). Indeed, the yeast PKC assay has been used by us to search for isoform-selective PKC modulators (8) and to study the regulation of human apoptotic proteins by PKC isoforms (9). Similarly, though no orthologues of human p53 have been identified in yeast, *S. cerevisiae* has been extensively used to score several functional properties of human p53 (10).

The yeast expression systems established in this study allowed investigating the existence in yeast of a molecular mechanism underlying the regulation of cell proliferation through specific PKC isoforms and WT p53. The results obtained underscore a differential regulation of p53 activity by PKC isoforms with the identification of kinases responsible for both a positive and negative regulation of p53.

2.3. MATERIAL AND METHODS

2.3.1. Plasmids

Constructed yeast expression plasmids YEplac181-LEU2 encoding bovine PKC–α, rat PKC–δ, mouse PKC–ε or PKC–ζ and the empty vector, and pLS89-TRP1 encoding human WT p53 and the empty vector, were kindly provided by Dr. Nigel Goode (The Royal Veterinary College, UK) and Dr. Richard Iggo (Swiss Institute for Experimental Cancer Research, Switzerland), respectively. Plasmids used have a galactose-inducible *GAL1-10* promoter.

2.3.2. Yeast strain, transformation and growth conditions

S. cerevisiae CG379 was co-transformed by the lithium acetate method, as reported (9). For selection of co-transformed yeast, cells were routinely grown in a minimal selective medium, with 2% glucose, 0.67% yeast nitrogen base without amino acids and all the amino acids required for yeast growth (50 μg/ml) except leucine and tryptophan, to approximately 1 optical density $(OD)_{600}$. To induce expression of mammalian proteins, yeast were diluted to 0.05 OD_{600} in selective medium with 2% galactose and raffinose instead of glucose and incubated at 30 ºC with shake (200 r.p.m.) for up to 60 hours in growth curves experiments, or for approximately 45 hours (time required by control yeast, co-transformed with the empty vectors pLS89 and YEplac181, to achieve 0.5 OD_{600}) in all other experiments. Yeast growth was analysed by counting the number of colony-forming units per ml (CFU/ml) after 2 days incubation at 30 ºC on Sabouraud Dextrose Agar plates.

2.3.3. Effect of the selective PKC inhibitor Ro 32-0432 on yeast growth

To analyse the effect of Bisindolylmaleimide XI hydrochloride (Ro 32-0432; ALX-270-058, Alexis Biochemicals) on yeast growth, co-transformed cells were incubated in galactose selective medium with 1 μM Ro 32-0432 or solvent only (0.1% DMSO) for approximately 45 h, at 30 ºC with shake (200 r.p.m.). Yeast growth was analysed as described above.

2.3.4. Cell death markers

Plasma membrane integrity and DNA fragmentation were analysed by fluorescence microscopy using propidium iodide (PI) and *In Situ Cell Death Detection Kit*, Fluorescein, respectively, whereas yeast metacaspase (Yca1p) activation and reactive oxygen species (ROS) accumulation were analysed by flow cytometry using FITC-VAD-fmk and dihydroethidium (DHE), respectively, as described (8).

2.3.5. Cell cycle

Flow cytometric analysis of DNA content was performed using Sytox Green Nucleic Acid, as described (8). Yeast cell cycle phases were identified and quantified using ModFit LT software (Verity Software House Inc., Topsham, USA).

2.3.6. Western blot

For human WT p53 and mammalian PKC isoforms detection, anti-p53 (DO-1) and anti-PKC$-\alpha$/PKC$-\delta$/PKC$-\varepsilon$/PKC$-\zeta$ mouse monoclonal antibodies (Santa Cruz Biotechnology) were used. p53 phosphorylation was analysed using phospho-p53(Ser15) mouse monoclonal antibody, phospho-p53(Ser20) and phosphop53(Ser46) rabbit polyclonal antibodies (Cell Signalling Technology), and PAb421 mouse monoclonal antibody (Calbiochem). The etoposite-treated MCF7 cell lysate (sc-2281; Santa Cruz Biotechnology) was used as positive control. Immunoblots were developed by enhanced chemiluminescence, as described (9). Band intensities were quantified using Bio-Profil Bio-1D++ software.

2.3.7. Statistical analysis

Data were analysed statistically using *SigmaStat 3.5 programme*. Differences between means were tested for significance using the unpaired Student's *t*-test ($P < 0.05$).

2.4. RESULTS

2.4.1 Differential regulation of WT p53-induced yeast growth inhibition by PKC isoforms

In accordance with other authors (11,12), we verified that expression of human WT p53 in *S. cerevisiae* inhibited cell growth (Fig. 2.1A,B). Instead, and as previously reported by us (8,9), in our experimental conditions expression of a mammalian PKC isoform did not significantly interfere with yeast growth (Fig. 2.1A,B). However, when PKC$-\alpha$, $-\delta$, $-\varepsilon$ or $-\zeta$ was co-expressed with p53, a differential regulation of p53-induced growth inhibition by PKC isoforms was obtained (Fig. 2.1A,B). This was particularly evident for 45 hours incubation (Fig. 2.1B). For this time, whereas PKC$-\alpha$ significantly reduced the p53-induced growth inhibition, PKC$-\delta$ and $-\varepsilon$ significantly increased the p53 growth-inhibitory effect and PKC$-\zeta$ did not interfere with the p53 effect.

The direct effect of PKC isoforms on p53 was evidenced using the selective PKC inhibitor Ro 32-0432. Though 1 μM Ro 32-0432 did not significantly interfere with the growth of yeast expressing p53 or a PKC isoform only, it significantly reduced the effects exhibited by PKC$-\alpha$, $-\delta$ and $-\varepsilon$ on p53 activity (Fig. 2.1C).

As confirmed by Western blot analysis, this distinct influence of each PKC isoform on p53 effect was not a consequence of significant changes in the expression levels of p53 or a PKC isoform in yeast co-expressing both proteins (Fig. 2.2).

2.4.2. WT p53 yeast growth-inhibitory effect and its stimulation by PKC$-\delta$ and $-\varepsilon$ are not associated with cell death

A recent work associated the human WT p53 growth-inhibitory effect to the induction of an apoptotic cell death in *S. cerevisiae* (11). Hence, typical necrotic and apoptotic markers, as loss of plasma membrane integrity, DNA fragmentation, ROS accumulation and Yca1p activation, were investigated.

However, in our experimental conditions, expression of human WT p53 in yeast did not significantly increase the PI and TUNEL positive cells (Fig. 2.3A,B), Yca1p activation and ROS levels (Fig. 2.3C,D). Even in the presence of a PKC isoform, particularly PKC$-\delta$ or $-\varepsilon$, characteristic features of cell death were not detected (Fig. 2.3B–D). Indeed, though yeast co-expressing p53 and PKC$-\varepsilon$ presented a significant increase in ROS levels, this was not accompanied by an increase of PI and TUNEL positive cells and Yca1p activation (Fig. 2.3B–D).

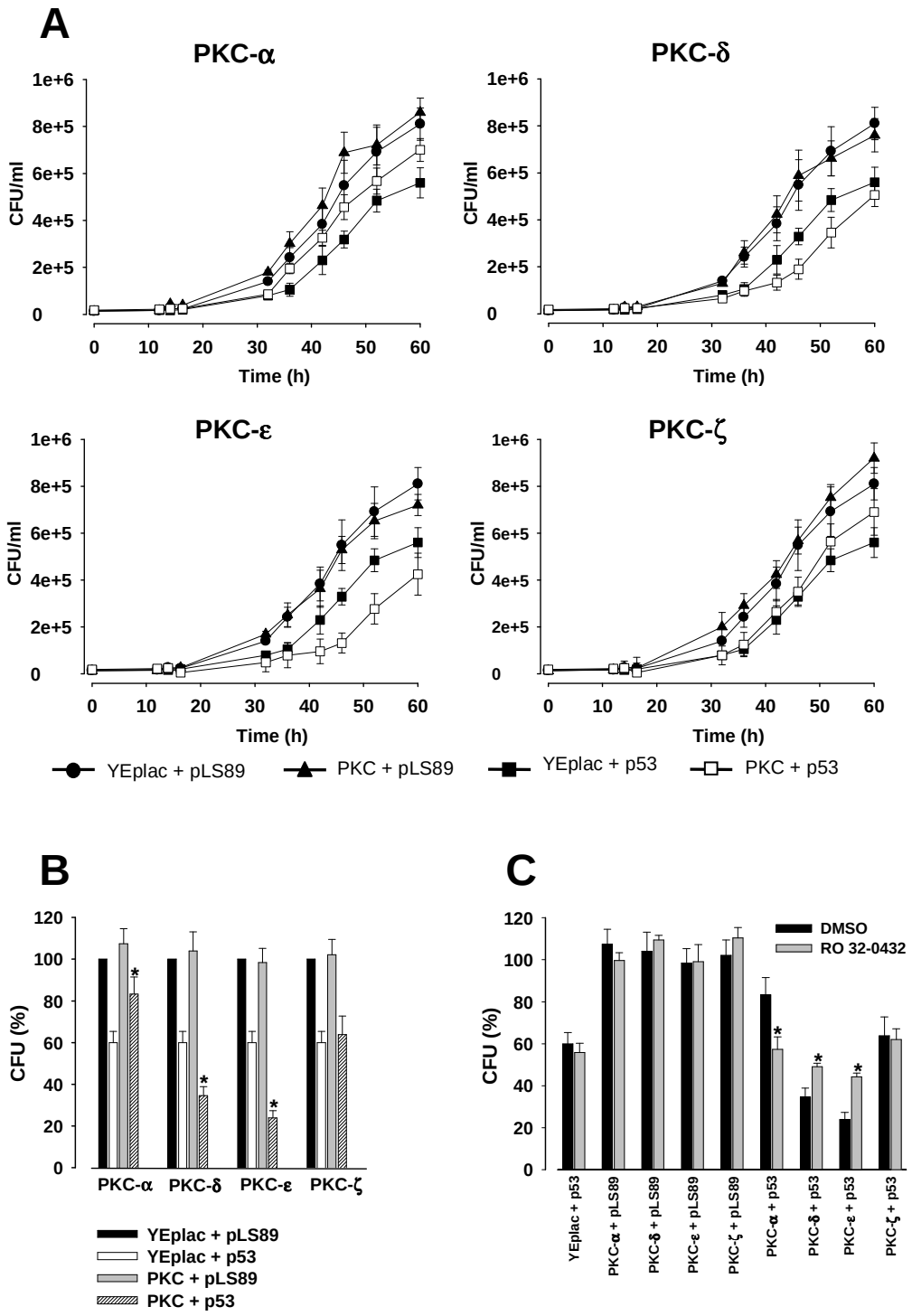

Fig. 2.1. WT p53-induced yeast growth inhibition is differently regulated by PKC isoforms. (**A**) Growth curves were obtained by CFU counts. (**B**) Percentage of growth obtained for 45 hours incubation; value of yeast co-expressing p53 and a PKC isoform significantly different from that of yeast expressing p53 only: **P*<0.05. (**C**) Effect of 1 μM RO 32-0432 on the growth of co-transformed cells for 45 hours incubation; value obtained with RO 32-0432 significantly different from that obtained with DMSO only: **P*<0.05. In **B** and **C**, values were obtained considering the growth achieved with control yeast (YEplac181+pLS89) as 100%. Data represent mean ± S.E.M. (n=4).

According to Amor et al. (11), a complete abolishment of yeast growth associated with cell death can be achieved using a multicopy vector for p53 expression and minimal medium culture conditions that rendered yeast more responsive to target gene regulation by p53. In fact, similarly to that obtained by us, Nigro et al. (12) only observed a modest yeast growth inhibition using a centromeric low-copy-number vector for p53 expression. However, considerable levels of p53 expression were also achieved in our work using a pLS89 centromeric vector (Fig. 2.2A,B). Additionally, a minimal medium similar to that described by Amor et al. (11) was used in our study. In order to understand the different results obtained, co-transformed yeast expressing WT p53 only (YEplac181 + p53) were treated with different concentrations of hydrogen peroxide (a known yeast apoptotic inducer, see (13)). In accordance with the high-level resistance to cell death detected in a previous work performed by us with co-transformed yeast (9), a significant percentage of TUNEL positive cells (21.3 ± 5.4%; n = 3), with a low percentage of IP positive cells (9.2 ± 4.1%; n = 3), was only achieved at high concentrations of hydrogen peroxide (10 mM H_2O_2, 1 hour treatment). Thus, the use of co-transformed yeast strains with high-level resistance to cell death may be a possible explanation for the results obtained by us.

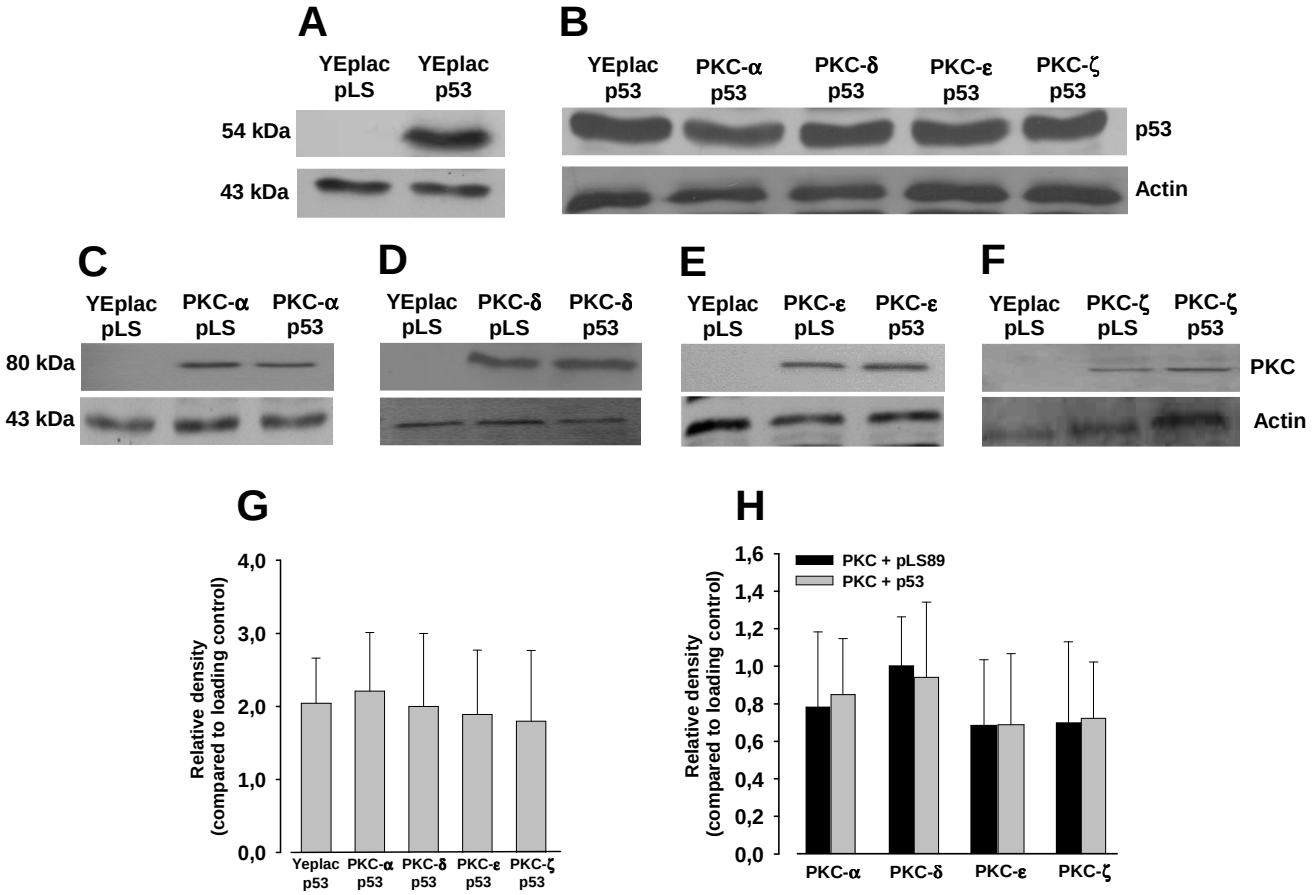

Fig. 2.2. Expression of human WT p53 or a mammalian PKC isoform is not affected by co-expression of both proteins in yeast. Western blot analysis of (**A**, **B**) p53, (**C**) PKC-α (**D**) PKC-δ (**E**) PKC-ε and (**F**) PKC-ζ

represent 1 of duplicate experiments; β-actin was used as loading control. (**G**, **H**) Quantification of band intensities of Western blots obtained for (**G**) p53 and (**H**) PKC isoforms; data represent mean ± S.E.M. (n=2).

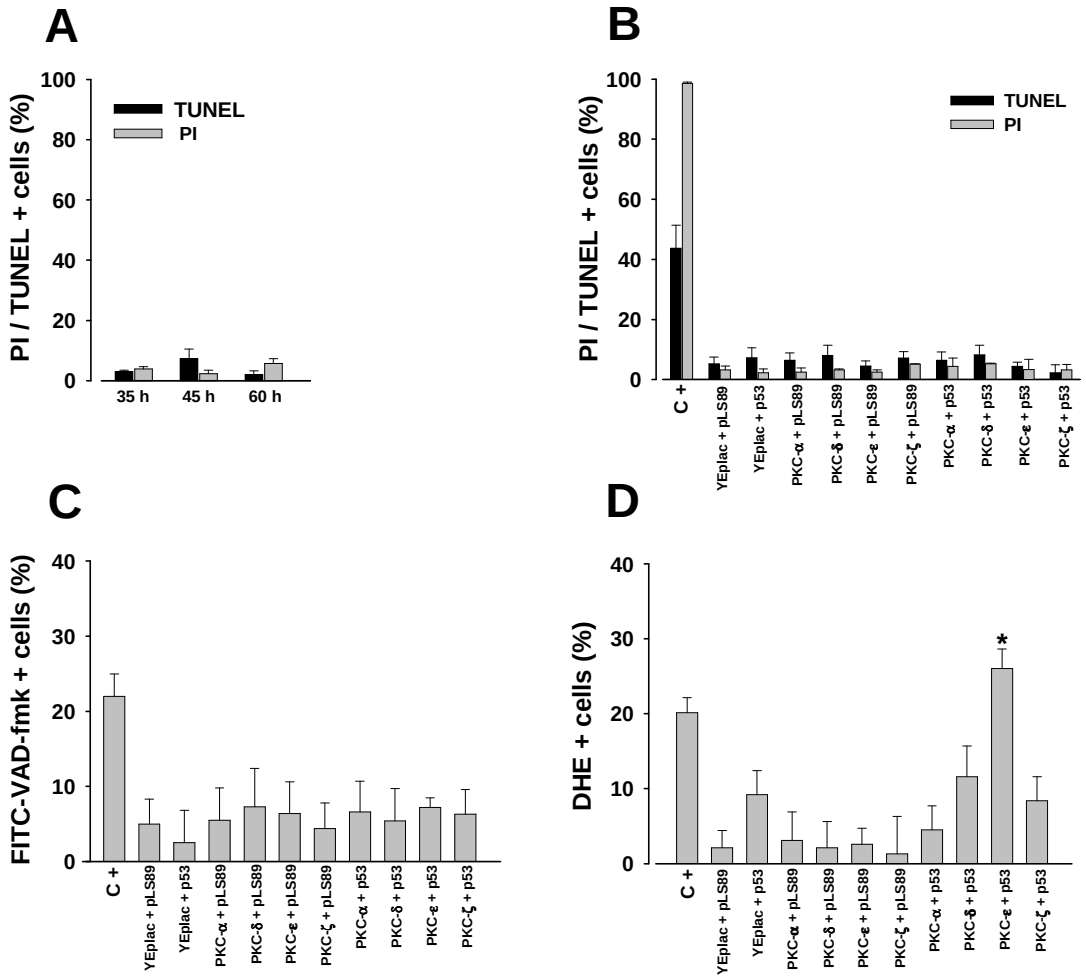

Fig. 2.3. Effects of WT p53 and/or a PKC isoform on yeast growth are not associated with cell death. (**A**) Cells with DNA fragmentation (TUNEL +) and necrotic cells (PI +) obtained from yeast expressing WT p53 for 35, 45 and 60 hours incubation; (**B**) TUNEL and PI positive cells, (**C**) Yca1p activation and (**D**) ROS accumulation obtained from yeast expressing WT p53 and/or a PKC isoforms for 45 hours incubation. In **B**, **C** and **D**, yeast expressing PKC-ε treated with 1 μM coleon U (see (8)) were used as positive control (C+). Data represent mean ± S.E.M. (n=2); values significantly different from control yeast (YEplac181+pLS89): * P<0.05.

2.4.3. WT p53 yeast growth-inhibitory effect is associated with S-phase cell cycle arrest that is differently regulated by PKC isoforms

As in mammalian cells, where p53 controls cell cycle mainly through the G1/S checkpoint (2), we detected that p53-induced yeast growth inhibition was associated with S-phase cell cycle arrest (Fig. 2.4). Instead, and in agreement with the absence of effect on yeast growth, PKC isoforms only slightly interfered with the yeast cell cycle progression (Fig. 2.4). However, when PKC–α, –δ, –ε or –ζ was co-expressed with p53, a specific PKC isoform-dependent modulation of cell cycle through p53 was obtained (Fig. 2.4).

Whereas PKC–α decreased the percentage of cells in S-phase and increased the percentage of cells in G2/M, PKC–δ and –ε markedly increased the percentage of cells arrested in S-phase. Consistently, PKC–ζ that did not affect the p53-induced growth inhibition also did not interfere with the p53-induced S-phase arrest.

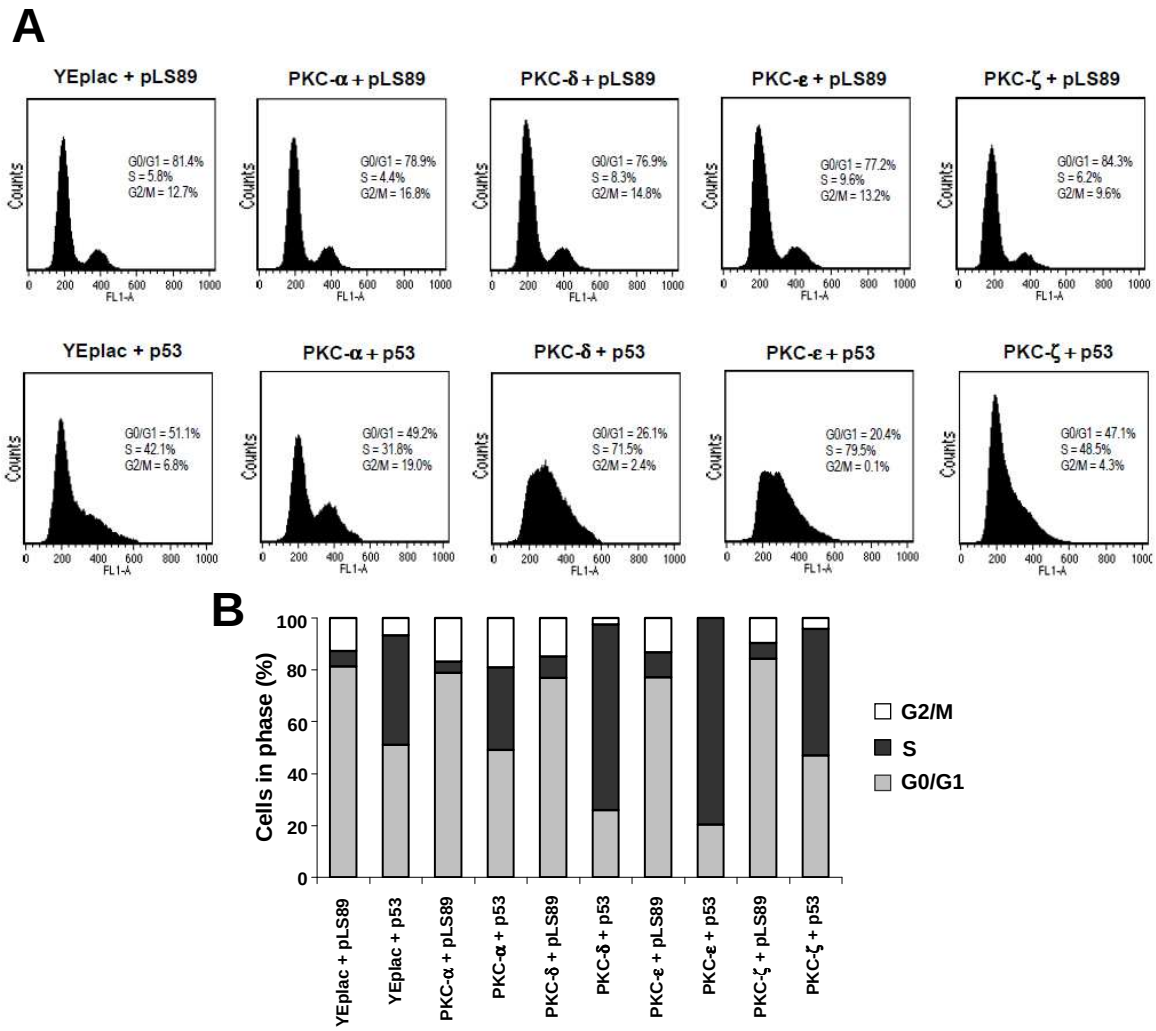

Fig. 2.4. WT p53-induced S-phase cell cycle arrest is differently regulated by PKC isoforms. (**A**) Histograms represent 1 of triplicate experiments. (**B**) Quantification of yeast cell cycle phases; data represent mean values (n=3).

2.4.4. Differential regulation of p53 effects by PKC isoforms is associated with distinct patterns of p53 phosphorylation in yeast

Phosphorylation of WT p53 at commonly reported p53 phosphorylation sites, Ser15, Ser20, Ser46 and Ser376-378 (2,3,14), was checked by Western blot analysis. p53 phosphorylation at Ser15, Ser20 and Ser46, analysed using a phospho-p53 antibody specific for each serine, was not detected neither in yeast expressing p53 only nor in yeast co-expressing p53 and a PKC isoform (Fig. 2.5A–C).

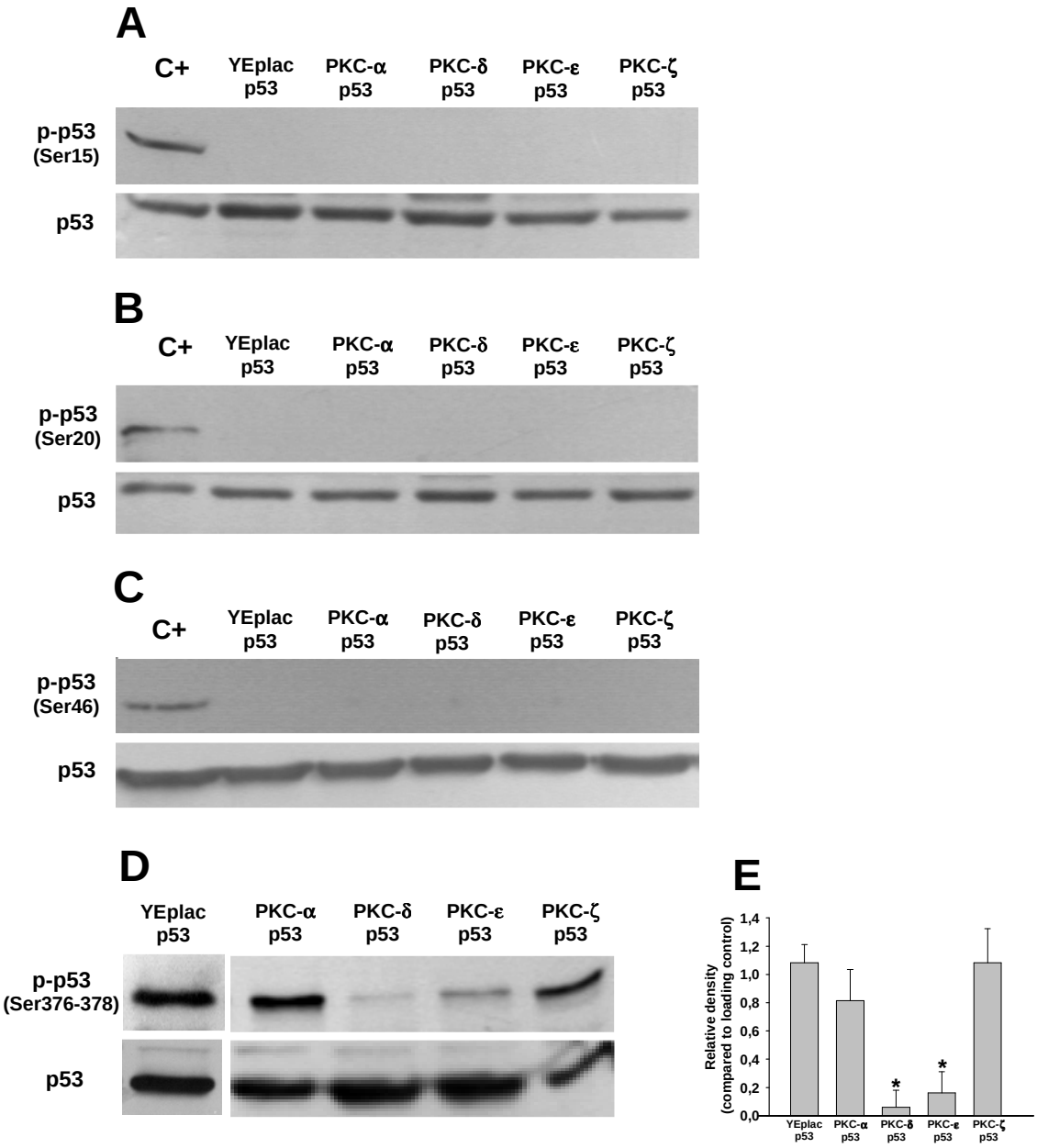

Fig. 2.5. PKC isoforms cause distinct patterns of p53 phosphorylation in yeast. p53 phosphorylation was analysed using (**A**) phospo-p53(Ser15), (**B**) phospo-p53(Ser20), (**C**) phospo-p53(Ser46) and (**D**) PAb421 (Ser376-378) antibodies. Detection of p53 with DO-1 antibody was used as a loading control. In **A**, **B** and **C**, the etoposite-treated MCF7 cell lysate was used as positive control (C+); in **D**, the positive control corresponds to p53 from yeast cells expressing p53 only (YEplac181+p53), which expression was confirmed using the anti-p53 DO-1 antibody. Immunoblots represent 1 of duplicate experiments. In **D**, YEplac181/p53 lane was located in a different part of the same gel. (**E**) Quantification of band intensities of **D**; data represent mean ± S.E.M. (n=2); values significantly different from control band (YEplac181+p53): **P*<0.05.

p53 phosphorylation at Ser376-378 was analysed using the PAb421 antibody which preferentially recognises unphosphorylated p53 residues at Ser376-378. Indeed, it was demonstrated that when p53 was phosphorylated at Ser376-378 by PKC the PAb421 band almost disappeared (3). In our case, a high intensity PAb421 band was obtained for

yeast expressing p53 only (Fig. 2.5D,E), indicating the absence of p53 phosphorylation at Ser376–378 by endogenous yeast kinases. This band was therefore used as control. Interesting, when compared to control band, whereas PKC–α slightly decreased and PKC–ζ did not interfere with the PAb421 band intensity, PKC–δ and –ε markedly decreased the intensity of this band (Fig. 2.5D,E). This indicated that, while PKC–α and –ζ did not significantly interfere with the degree of p53 phosphorylation, PKC–δ and –ε markedly increased p53 phosphorylation.

Overall, despite phosphorylation of human WT p53 by endogenous kinases was already reported for *S. cerevisiae* (12), to our knowledge the sites of p53 phosphorylation were still unclear. The results obtained indicated that p53 is not phosphorylated at Ser15, Ser20, Ser46 and Ser376-378 by yeast kinases. Importantly, though p53 phosphorylation by PKC has been described at distinct serine residues (2,3,14), in yeast we only detected phosphorylation at Ser376–378, which is the most commonly reported PKC phosphorylation site in mammalian cells (2,3). For this p53 residue, a PKC isoform-dependent phosphorylation pattern was identified.

2.5. DISCUSSION

The involvement of PKC family in the regulation of cell proliferation was early recognised. While PKC–α and –ζ are frequently associated to the proliferation of human cancers and PKC–δ is often linked to an anti-proliferative effect (4,5), PKC–ε has been associated both to proliferative (4,5) and anti-proliferative (15) effects. In spite of this, the molecular mechanism of induction or suppression of cell proliferation by PKC isoforms remains unclear. Another open issue is the role of PKC isoforms in the regulation of p53, a key player in cell proliferation and death. The PKC-mediated effects are in fact largely tissue and cell-type-specific, thus it has been difficult and controversial to extrapolate conclusions from one mammalian cell type to another. This study represents the first attempt to reconstitute in yeast key parts of the intricate mammalian p53-PKC network. With this yeast approach, it was possible to ascertain the effect of individual PKC isoforms, representative of the classical (PKC–α), novel (PKC–δ and –ε) and atypical (PKC–ζ) PKC subfamilies and considered major isoforms in carcinogenesis, cell proliferation and human WT p53 activity. The results obtained revealed that even though the PKC isoforms per se had no effect on yeast growth and cell cycle, they differentially interfered with the p53-induced yeast growth inhibition and cell cycle arrest and p53 phosphorylation. They underscored an anti-proliferative effect of PKC–δ and –ε through phosphorylation and consequent activation of p53. In opposition, they evidenced a proliferative effect of PKC–α through inhibition of p53 function. Further studies are underway to better elucidate the molecular mechanism of this PKC–α-inhibitory effect. Finally, the absence of effect of PKC–ζ on the activity and phosphorylation state of p53 indicated that p53 was not a target of this kinase. As a whole, this study established a unifying mechanism between the modulation of cell proliferation by PKC–α, –δ and –ε and the regulation of p53. For PKC–ζ, a p53-independent effect on cell proliferation was suggested. Additionally, it underscored a differential regulation of p53 activity and phosphorylation state by PKC isoforms, with the identification of kinases responsible for both a positive and negative regulation of p53. These kinases represent therefore promising molecular and pharmacological targets in anti-cancer therapy. As reported by others, the distinct roles of individual PKC isoforms in cancer progression suggests that either PKC activators or inhibitors may serve as anti-tumour agents if target specific members of the PKC family (5). Accordingly, our data support that selective PKC–α inhibitors as well as selective PKC–δ and –ε activators may be beneficial in the therapy of several cancers by inhibiting the proliferation particularly of those tumours where PKC–α

is up-regulated and PKC$-\delta$ or $-\varepsilon$ are down-regulated. This study will certainly contribute to the elucidation of the effect of PKC activators and inhibitors on tumour cells proliferation, providing new perspectives for PKC modulators as anti-cancer agents.

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CHAPTER 3

Distinct regulation of p53-mediated apoptosis by protein kinase Cα, δ, ε and ζ: evidence in yeast for transcription-dependent and -independent p53 apoptotic mechanisms

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DISTINCT REGULATION OF P53-MEDIATED APOPTOSIS BY PROTEIN KINASE Cα, δ, ε AND ζ: EVIDENCE IN YEAST FOR TRANSCRIPTION-DEPENDENT AND -INDEPENDENT P53 APOPTOTIC MECHANISMS

3.1. ABSTRACT

The role of individual protein kinase C (PKC) isoforms in the regulation of p53-mediated apoptosis is still uncertain. Using yeast cells co-expressing the human wild-type p53 and a single mammalian PKCα, δ, ε or ζ, we showed a differential regulation of p53-mediated apoptosis by these PKC isoforms. Whereas PKCα and ζ had no effect on p53 activity, PKCδ and ε stimulated a p53-mediated mitochondria-dependent apoptosis. Moreover, using pifithrin-α and -μ, selective inhibitors of p53 transcriptional activity and mitochondrial p53 translocation, respectively, we showed the activation of a transcription-dependent and -independent p53-mediated apoptosis by PKCδ and ε. The activation of mitochondrial p53 translocation by PKCδ and ε was further confirmed by immunofluorescence and Western blot analysis.

Together, this work reveals the conservation in yeast of functional transcription-dependent and -independent p53 apoptotic mechanisms. Furthermore, it gives mechanistic insights about the regulation of p53-mediated apoptosis by PKCδ and ε through modulation of p53 transcriptional activity and of its translocation to mitochondria. Finally, it underscores a major role of PKCδ and ε as positive regulators of p53-mediated apoptosis, and therefore as promising therapeutic targets in cancer.

Keywords: p53; PKC isoforms; Apoptosis; Transcription; Mitochondria; Yeast

3.2. INTRODUCTION

The major tumour suppressor protein, p53, is a sequence-specific transcription factor that determines the fate of a cell. p53 regulates the expression of an assortment of genes involved in cell cycle regulation, apoptosis and numerous other processes. In large part, the potent tumour suppressing function of p53 has been attributed to its ability to induce apoptosis. Despite the prominence of p53 nuclear transcriptional activity in the induction of apoptosis, a transcription-independent mechanism involving mitochondrial p53 translocation, is receiving increasing attention. Indeed, recent findings provide encouragement to further explore the potential of mitochondrial p53-based cancer therapeutics (1-3).

Inactivating mutations of the p53 gene are found in approximately half of all human cancers. In most of the remaining cancers that retain a wild-type (WT) p53, the p53 pathway is deactivated by an increase in its inhibitors, a reduction in its activators or by inactivation of downstream targets (1). In these cases, restoring WT p53 apoptotic function has been recognised as a promising strategy for cancer therapy. Hence, a detailed understanding of the mechanisms of regulation of p53-mediated apoptosis has been an important research objective with significant clinical impact (3).

One of the key enzymes involved in the regulation of WT p53 function is the protein kinase C (PKC). PKC is a family of serine/threonine kinases with at least 10 isoforms grouped into three major subfamilies according to their primary structure and cofactors required for activation: classical (α, βI, βII and γ), novel (δ, ε, η and θ) and atypical (ζ and λ\ι). PKC isoforms are important regulators of several cellular processes, such as cell proliferation and death, and a striking feature is that individual isoforms can exert either similar or opposite effects in these processes (4). In fact, several studies underscore a major role of PKC, particularly of PKCδ, in the regulation of p53 apoptotic activity. It was demonstrated that PKCδ leads to p53 accumulation and phosphorylation with consequent activation of a p53-mediated apoptosis (5-10).

However, due to the high complexity of the mammalian p53 pathway and PKC family, namely the coexistence of several PKC isoforms in the same cell, the discrimination of the role of PKC isoforms in the regulation of p53 apoptotic mechanisms, particularly of its transcription-dependent and -independent activity, is still uncertain. To address this issue, yeast cells co-expressing the human WT p53 and an individual mammalian PKC isoform of the classical (PKCα), novel (PKCδ and ε) and atypical (PKCζ)

PKC subfamilies, and considered the isoforms most commonly involved in carcinogenesis, were used. In fact, using this yeast co-expression system, a differential modulation of p53-induced yeast growth inhibition and cell cycle arrest by distinct PKC isoforms was shown in our previous work (11). Moreover, other authors have confirmed the versatility of the yeast cell model to study different aspects of p53 function (12). For instance, a recent work showed that p53 could also function as a sequence-specific transcription factor in yeast. This study revealed several remarkable similarities between the transcription-dependent p53 activity in yeast and mammalian cells (13).

With the yeast approach used, it was possible to ascertain the role of PKCα, δ, ε and ζ in the regulation of p53-mediated apoptosis. In contrast to PKCα and ζ, PKCδ and ε activated transcription-dependent and -independent p53 mechanisms that cooperated to ultimately cause an apoptotic cell death. This study therefore identified PKCδ and ε as major positive regulators of p53-mediated apoptosis, and consequently as promising therapeutic targets for cancer treatment. Another relevant point arising from this study was to provide the first evidence for the conservation in yeast of a transcription-dependent and -independent p53-mediated apoptosis, with the validation of the yeast cell model to further understand the regulation of these p53 apoptotic mechanisms.

3.3. MATERIAL AND METHODS

3.3.1. Plasmids

The following yeast expression plasmids were used: pLS89-*TRP1* encoding human WT p53 and the respective empty vector (kindly provided by Dr. Richard Iggo, Swiss Institute for Experimental Cancer Research, Switzerland); YEplac181-*LEU2* encoding bovine PKCα, rat PKCδ, mouse PKCε or PKCζ and the respective empty vector (kindly provided by Dr. Nigel Goode, The Royal Veterinary College, Hawkshead Lane, Hertfordshire, UK). All plasmids have a galactose-inducible *GAL1-10* promoter.

3.3.2. Yeast strain, transformation and growth conditions

Co-transformants of *Saccharomyces cerevisiae* strain CG379 were prepared in previous work (11). To ensure selection of co-transformed yeast, cells were routinely grown in a minimal selective medium with 2% (w/v) glucose, 0.67% (w/v) yeast nitrogen base without amino acids, and all the amino acids required for yeast growth (50 μg/ml) except leucine and tryptophan, at 30ºC with mechanical shaking (200 r.p.m.) to approximately 1 optical density measured at 600 nm (OD_{600}; Jenway 6310 Spectrophotometer, Jenway). To induce expression of mammalian proteins, yeast cultures were diluted to 0.05 OD_{600} in selective medium with 2% (w/v) galactose and 1% (w/v) raffinose, instead of glucose, and incubated at 30ºC with mechanical shaking (200 r.p.m.) to 0.45 OD_{600}. Expression of human WT p53 and/or a mammalian PKC isoform (α, δ, ε or ζ in *S. cerevisiae* was confirmed by Western blot analysis in previous work (11).

3.3.3. Cell death assays

Co-transformed yeast cells, previously grown in galactose selective medium to 0.45 OD_{600}, were treated with 5 mM H_2O_2 for 1 hour at 30ºC with mechanical shaking (200 r.p.m.). Cell death was assessed by counting the number of colony-forming units (CFU) after 2 days incubation at 30ºC on Sabouraud Dextrose Agar plates. For each co-transformant, the percentage of dead cells was estimated considering 100% survival (0% death) as the number of CFU obtained with cells incubated in the same conditions but without H_2O_2.