Short Communication

PKC δ requires p53 for suppression of the transformed phenotype in human colon cancer cells

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Abstract

We have previously demonstrated that the delta isoform of Protein Kinase C (PKC δ) acts as a tumor suppressor in HCT116 human colon cancer cells, and that p21^{waf1/cip1} is an essential downstream effector of PKC δ . Our data suggested that p53 might also be involved in the suppression of the neoplastic phenotype induced by PKC δ . Here we show that homozygous knockout of p53 renders the HCT116 cell line unresponsive to PKC δ overexpression. Whereas reconstitution of p53 alone did not modify the morphology and growth properties of HCT116/p53^{null} cells, overexpression of both p53 and PKC δ induced a number of alterations indicating suppression of the transformed phenotype. Interestingly, PKC δ was ineffective when overexpressed in HT29 cells, a human colon cancer line characterized by the Arg273His dominant-negative mutation of p53. Thus, our data indicate that wild-type p53 is an essential effector of PKC δ in human colon cancer cells.

Keywords: protein kinase C delta • PKC • tumor suppressor • p53 • colon cancer

Introduction

The delta isoform of protein kinase C (PKC δ), a member of the “n”, class of PKCs, has been attracting the attention of an increasing number of research groups, including ours, due to its unique pro-apoptotic properties, and to its candidacy as a novel tumor suppressor (reviewed in: [1]).

We have previously demonstrated that PKC δ acts as a growth and tumor suppressor in *src*-trans-

formed rat colonic epithelial cells. In these cells, overexpression of PKC δ attenuated the transformed phenotype *in vitro* [2] and impaired tumorigenesis *in vivo* (unpublished results). Consistently, transfection of a dominant, kinase-defective mutant of PKC δ was sufficient to induce transformed features in non-tumorigenic, immortalized rat colonic epithelial cells [2]. In a recent study, we investigated the activity and mechanism of action of PKC δ by retrovirally-transducing a PKC δ cDNA in HCT116 human colon cancer cells [3]. PKC δ -overexpressing cells (HCT116/PKC δ) were temporarily growth-inhibited, became unable to form colonies of anchorage-independent cells in soft agar, under-

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went phenotypic changes characteristic of “mitotic catastrophe” and displayed a markedly attenuated tumorigenic profile. Moreover, when compared to mock-infected controls, HCT116/PKC δ cells established desmosome-coordinated intercellular communications, expressed cell surface microvilli, and overexpressed the colon differentiation marker intestine alkaline phosphatase (IAP). These evidences were in agreement with a report showing that overexpression of PKC δ inhibits anchorage-independent growth and induces differentiation markers in CaCo-2 human colon cancer cells [4].

A likely candidate as a downstream effector of PKC δ in HCT116 cells was p21^{waf1/cip1}, because this cyclin-kinase inhibitor was shown to be involved in colonic cell differentiation [5], mitotic catastrophe [6], and PKC-mediated cell cycle inhibition in intestinal crypt cells [7]. Consistently, we showed that p21^{waf1/cip1} was transiently induced following PKC δ overexpression in HCT116 cells. In addition, overexpression of PKC δ in a p21^{null} subline of HCT116 cells did not affect tumorigenicity or differentiation, thus indicating that p21^{waf1/cip1} is essential for the tumor-suppressive activity of PKC δ [3]. To further characterize the signalling pathways involved in the action of PKC δ , we extended our investigation to p53, a major upstream effector of p21^{waf1/cip1} which was shown to be activated by PKC [8, 9], and transcriptionally modulated by PKC δ [10]. With a pattern strikingly similar to p21^{waf1/cip1}, p53 underwent transient upregulation at the protein level following overexpression of PKC δ , and was phosphorylated at a specific PKC consensus serine residue (Ser378), known to be involved in p53 activation [9]. Interestingly, downregulation of p53 by the human papillomavirus E6 gene product rendered HCT116 cells unresponsive to PKC δ -induced tumor suppression [3]. Thus, we hypothesized that p53 might be a key effector of the antineoplastic activity of PKC δ in human colon cancer cells. The present investigation was aimed at providing experimental evidence to this hypothesis.

Materials and methods

Unless specified otherwise, chemicals and reagents were purchased from Sigma, Milan, Italy.

Expression vectors

The pBABE/GFP/PKC δ retroviral vector was constructed by directionally subcloning a 2.5 Kb cDNA encoding the human PKC δ isoform (GeneBank accession code: NM_006254) into the BamHI-SalI sites of the pBABE/GFP multicloning site, as previously described [3]. The pCMV/p53 vector, containing a human p53 cDNA, was kindly provided by Dr. Marzia Gariboldi, University of Insubria, Busto Arsizio, Italy.

Cell culture and transfection - generation of recombinant retroviruses and target cell infection

HCT116 is a human cell line derived from a colon adenocarcinoma. The HCT116/p53^{null} derivative cell line was kindly provided by Dr. Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD, USA). This p53(-/-) subline was generated by inducing a homzygous knockout of the p53 gene in HCT116 parental cells. The HT29 human colon adenocarcinoma cell line was kindly provided by Dr. Marzia Gariboldi, University of Insubria, Busto Arsizio, Italy.

For the production of retroviral particles, the ecotropic Ecopack 2-293 and the dualtropic Retropack PT-67 cell lines were used (Clontech, Milan, Italy). All cell lines were grown in standard conditions (37°C, 5% CO₂) in DMEM medium containing 10% Fetal Bovine Serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 4 mM L-glutamine and 1 mM sodium pyruvate (complete DMEM). All cell transfection procedures were performed using the Lipofectamine 2000 reagent, according to the producer's instructions (InVitrogen, Milan, Italy).

To generate replication-defective recombinant retroviral particles for gene transduction and overexpression experiments, retroviral vectors were transfected by lipofection into Retropack PT-67 cells. Viral particle harvesting was performed as previously described [3]. Briefly, following transfection of Retropack PT-67 cells with indicated vectors, viral particle-containing supernatants were used to infect directly the Ecopack 2-293 cell line. An additional cross-infection round on Retropack PT-67 cells was sufficient to obtain a suitable viral titer (~10⁶ Transducing Units/ml culture medium) for subsequent human colon cancer cell infection experiments. Recombinant viral particles were directly titrated onto target HCT116 cancer cells; infections of colon can-

cer target cells were performed at 10.0 M.O.I., as previously described [3].

Western blot analysis of protein expression.

Cells were cultured in 75 cm² flasks, harvested by trypsinization, counted with an hemocytometer, centrifuged at 1,000 g for 10 minutes at 4°C, washed in phosphate-buffered saline at 1,000 g for 10 minutes at 4°C, and resuspended in ice-cold lysis buffer (30 μ l/10⁶ cells; composition: 10 mM Tris, pH 7.5; 1 mM EDTA; 1% NONIDET P-40; 1 mM PMSF; SIGMA protease-inhibitor and phosphatase-inhibitor cocktails). Cell suspensions were sonicated for 10 seconds and centrifuged at 13,000 g for 20 minutes at 4°C. The protein content of cell lysate supernatants was assayed using a BCA Protein Assay system (Pierce, USA). Fifty micrograms protein of each lysate were combined with equal volumes of Laemmli sample buffer, boiled for 5 minutes and run onto 8% polyacrilamide gels (200 mV). Gels were blotted onto nitrocellulose membranes at 200 mA, for 2 hrs. After 1 hour incubation in blocking solution (5% MARVEL dried skimmed milk in PBS) membranes were exposed overnight at 4°C to primary antibodies, diluted as specified below with blocking solution containing 0.1% Tween. After three 10 min washes in PBS, blots were incubated for 60 minutes with peroxidase-conjugated secondary antibodies (ImmunoPure, Pierce, Rockford IL, USA) diluted 1:1000 in blocking solution. Detection on autoradiography films was performed after inducing chemiluminescence with the Supersignal WestPico reagent (Pierce, Rockford IL, USA).

Primary antibodies: antihuman PKC δ (polyclonal, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA); antihuman p53 (polyclonal, 1:500; SIGMA, Milan, Italy).

Analysis of cell growth parameters

To quantify cell proliferation, cells were trypsinized 24, 48 and 72 hours after retroviral infection and stained with the 33342 Hoechst DNA-intercalating dye as previously described [3]. The mitotic index was assessed by calculating the ratio between the number of cells in metaphase/early anaphase and the total number of cells. Data from six independent samples were analysed statistically and significance was determined using Student's t

test. For PMA exposure experiments, cells were incubated in the presence of 100 nM phorbol 12-myristate 13-acetate dissolved in DMSO. Identical volumes of DMSO were added to the media of control cells; final DMSO concentration in medium was always <0.01%. The 100 nM concentration of PMA was demonstrated, in preliminary experiments, to downregulate PKC δ in the HCT1167p53 null cell line, and to lack non-specific toxic or biological effects (data not shown).

For saturation density determinations, 4 $\cdot$ 10⁵ cells from each experimental group were plated in each well of 24 well plates. 24 hours after confluency, cells were harvested and counted. Data from six independent samples were analysed statistically by Student's t-test. To test the ability to form transformed foci, 5 $\cdot$ 10⁵ cells were plated in each well of a 12 well plate. Cells were grown beyond confluency for 6 days and foci were counted with a low-power microscope. The foci-forming capacity of each experimental group was scored as follows: (–), no foci; (+), 1-10 foci/well; (++), 10-50 foci/well; (+++), >50 foci/well. Experiments shown in Table 1 were performed in triplicate, with equivalent results.

To assay anchorage-independent growth, soft agar cultures were prepared by combining 1 ml 0.6% agar with 1 ml 2X complete DMEM containing 2 $\cdot$ 10⁴ cells. Two-milliliter aliquots were added to each well of 6-well plates pre-coated with 1 ml cell-free agar medium (0.3% agar in complete DMEM, 1X). Cells were fed weekly by addition of 1 ml cell-free agar medium. Every experiment was repeated twice. Duplicate 6-well plates were used for each cell type and for each infection experiment. Fourteen-21 days after cell plating colonies were stained by adding 1 ml PBS containing 0.5 mg/ml 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride to each well. Plates were incubated for 24 hours at 37°C; colonies were visualized using a low-power microscope.

Results and discussion

As a first step in our investigation, we assessed the responses of HCT116 and HT29 human colon cancer cell lines to the overexpression of PKC δ . Cells were retrovirally transduced with a cDNA encoding PKC δ , as previously described [3]. Compared to mock-infected controls (Figure 1, panel A), PKC δ induced in HCT116 cells a number of phenotypic and growth alterations, including the acquisition of

a flattened cell shape (Figure 1, panel B), the temporary inhibition of cell duplication, as well as the impairment of anchorage-independent and contact inhibition-independent growth (Table 1). Moreover, PKC δ -overexpressing HCT116 cells (HCT116/PKC δ in text) acquired a giant, multinucleated phenotype, characterized by micronuclei, multi-lobular nuclei, and giant polyploid nuclei (Figure 1, panel B). The presence of multiple nuclei of abnormal shape accounts for the induction of “mitotic catastrophe”, a form of cell death which develops as a consequence of the loss of coordination between elements belonging to the G1-S, G2-M and spindle checkpoints (reviewed in: [6]). In particular, it was shown that mitotic catastrophe occurs when cells, after a temporary growth-arrest involving transient overexpression of p21, re-enter prematurely into the cell cycle before key-elements of the cycle itself have been synthesized [6]. Interestingly, we have previously shown that the onset of mitotic catastrophe in HCT116/PKC δ cells parallels transient overexpression of both p21 and p53 [3]. Mitotic catastrophe generates aberrant interphase cells, which can contain giant nuclei, multiple nuclei or multi-lobular nuclei (Figure 1, panel B). Giant nuclei develop when endoreduplication occurs. This phenomenon can take place when only one centrosome is present or when the spindle checkpoint has failed and the fuse is incompletely formed. Multiple nuclei of normal size develop when endomitosis occurs, whereas multipolar mitosis, characterized by the presence of more than 2 centrosomes, leads to the formation of micronuclei containing an incomplete set of chromosomes. Micronuclei can also be due to incomplete fuse organization during the mitotic process.

Interestingly, HT29 cells were unaffected by PKC δ overexpression and no alterations of cell growth or phenotype were observed (Table 1 and Figure 1, panels C, D). Whereas HCT116 cells express wild-type p53, the HT29 line is characterized by the Arg273His substitution, caused by a p53 gene mutation commonly found in colon cancer. This dominant-negative mutation is responsible for the loss of the DNA-binding capacity of p53 homo- or heterotetramers [11]. Attempts to restore responsiveness to PKC δ by reconstituting wild-type p53 in HT29 cells failed in our hands, thus confirming the dominant characteristics of the Arg273His p53 mutant (data not shown). Although our data suggest that lack of responsiveness of HT29 cells to PKC δ could be due to the presence of negative-mutant p53, it should be stressed that this cell line is characterized by a genetic makeup involving several activated oncogenes, such as *myc*, *ras*, *fos* and *sis* [12] which may cooperate to resistance to PKC δ by activating alternative, p53-independent oncogenic signalling pathways. This issue is under current investigation.

To assess the involvement of p53 in the tumor-suppressing activity of PKC δ , we have extended our studies to a p53(-/-) subline of HCT116 cells (HCT116/p53null). This cell line was transduced with a PKC δ cDNA using retroviral particles prepared with a pBABE/GFP/PKC δ vector. Compared to the HCT116/PKC δ cell line (Figure 1, panel B), PKC δ -overexpressing HCT116/p53null cells (HCT116/p53null/PKC δ in text) did not undergo morphological changes (Figure 1, panel F) and were not temporarily growth-arrested (Table 1). Moreover, HCT116/p53null/PKC δ cells retained the power to grow in an anchorage-independent fashion in 0.3% agar, did not show decreased saturation density, and

Fig. 1 A, Morphology of mock-transduced HCT116 cells. B, HCT116 cells overexpressing PKC δ ; several giant cells displaying micronuclei, multiple nuclei and giant, polyploid nuclei are visible; this picture was taken 96 hours after PKC δ retroviral transduction. C, mock-transduced HT29 cells; cell morphology is indistinguishable from that of PKC δ -overexpressing cells (panel D). E, morphology of mock-infected HCT116/p53null cells. F, PKC δ -overexpressing HCT116/p53null cells retain a transformed morphology and can form foci when grown beyond confluency. G, mock-infected HCT116/p53null cells, previously transfected with p53 (HCT116/p53null/p53/MOCK), display an irregular, transformed shape, very similar to that of cells shown in panel F. Reconstitution of p53, followed by PKC δ overexpression in the HCT116/p53null line (HCT116/p53null/p53/PKC δ), induces marked cell flattening and formation of giant cells (circled) containing large, polyploid nuclei (arrow), a marker of mitotic catastrophe (panel H).

A, B, F: 150 X magnification; C, D: 100x magnification; E, G, H: 200 X magnification, with slight differences due to figure composition. Insets show the PKC δ /p53/IAP protein profiles of each cell line.

Table 1 Cell growth parameters.

CELL LINE		Mitotic Index (x 100)			Cell density at confluency (cell no. x 10 ⁴ /cm ² mean ±SD)	Colony forma- tion efficiency in 0.3% agar (%) ^g	Formation of transformed foci on cell monolayers ^p
		24h ^f	48h ^f	72h ^f			
HCT116	MOCK^a	3.3±0.54	3.2±0.19	3.4±0.66	72.6±7.17	15.37	+++
	PKCδ^b	0.7±0.38 ^h	1.8±0.41 ⁱ	2.9±0.65	51.2±5.35 ^j	0.25	+
HCT116/ p53null	MOCK^a	2.6±0.61	2.7±0.77	2.7±0.49	51.9±11.69	+ ⁿ	++
	PKCδ^b	2.4±0.43	2.7±0.55	2.6±0.68	48.5±6.21	+ ⁿ	++
	p53^c	2.2±0.74	2.5±0.23	2.4±0.61	47.3±5.33	+ ⁿ	++
	p53/MOCK^d	1.9±0.72	1.9±0.68	2.0±0.88	47.2±10.04	+ ⁿ	++
	p53/PKCδ^e	0.5±0.12 ^k	1.1±0.35	1.9±0.79	35.6±4.73 ^m	- ⁿ	-
	p53/PKCδ^e (+100 nM PMA)	2.1±0.71	2.1±0.96	2.0±0.48	45.1±8.63	+ ⁿ	+
HT29	MOCK^a	2.1±0.53	2.1±0.59	2.19±0.44	64.4±12.23	17.97	++
	PKCδ^b	2.3±0.12	2.3±0.78	2.60±0.87	69.4±6.76	16.52	++

a, MOCK: mock-transduced with an empty BABE/GFP retroviral vector

b, PKCδ: transduced with a BABE/GFP/PKCδ retroviral vector

c, stably transfected with a pCMV/p53 vector

d, p53/MOCK: stably transfected with a pCMV/p53 vector and mock-transduced with an “empty” BABE/GFP retroviral vector

e, p53/PKCδ: stably transfected with a pCMV/p53 vector and transduced with a BABE/GFP/PKCδ retroviral vector

f, time after infection with indicated retroviral vector (p53/MOCK and p53/PKCδ groups were stably trans-

duced with the pCMV/p53 expression vector before-hands)

g, measured at day 14 after plating

h, p<0.01 vs. HCT116/MOCK, 24h

i, p<0.05 vs. HCT116/MOCK, 48h

j, p<0.05 vs. HCT116/MOCK

k, p<0.01 vs. HCT116/p53null/p53/MOCK, 24h

m, p<0.05 vs. HCT116/p53null/p53/MOCK

n, due to small colony size, colonies were not quantitated, but scored as follows: (+), presence of proliferating colonies; (-), absence of colonies

p, (-), no foci; (+), 1-10 foci/well; (++) , 10-50 foci/well; (+++) , >50 foci/well

did not lose the capacity to form transformed foci on cell monolayers grown beyond confluency (Table 1 and Figure 1, panel F). These data show that HCT116/p53null cells are resistant to the suppressing activity of PKCδ and retain a transformed phenotype. To confirm that p53 is essential for the transformation-suppressing activity of PKCδ, we have reconstituted p53 in the HCT116/p53null line by stable transfection with a mammalian expression vector containing a human *p53* cDNA under the transcriptional control of a CMV promoter. Transfection experiments were followed by selection of a cell clone (HCT116/p53null/p53 in text) stably expressing p53 at levels comparable to HCT116 parental cells (Figure 1, panels G, H, blot inset). It should be stressed that expression of *p53* did not alter the mor-

phology and/or growth properties of transfected HCT116/p53null cells (Table 1). As a subsequent step, we overexpressed PKCδ in HCT116/p53null/p53 cells by retroviral vector transduction. Interestingly, p53-reconstituted, PKCδ-overexpressing cells (HCT116/p53null/p53/PKCδ in text) underwent marked morphologic and phenotypic alterations. Compared to mock-infected HCT116/p53null/p53 cells (HCT116/p53null/p53/MOCK in text), displaying a highly transformed morphology (Figure 1, panel G), PKCδ transfectants became more flattened and the culture was characterized by the presence of giant cells (Figure 1, panel H) containing polyploid, giant nuclei, a marker of endoreduplication and mitotic catastrophe [3]. Moreover, cells were temporarily growth-inhibited, as shown by a highly significant

decrease of the mitotic index 24 hours after PKC δ transduction (Table 1). This response was identical to the behaviour shown by PKC δ -overexpressing HCT116 parental cells (Table 1). Phenotypic alterations also included a marked, significant decrease of cell saturation density (Table 1). Compared to parental HCT116 cells, the HCT116/p53null subline grows at a lower rate *in vitro*, and the quantitation of anchorage-independent growth is cumbersome, due to the small size of proliferating colonies. Nevertheless, qualitative assessment of colony formation showed the absence of cell colonies in plates containing the HCT116/p53null/p53/PKC δ line (Table 1). The ability to form transformed, contact inhibition-independent foci was also suppressed in HCT116/p53null/p53/PKC δ cells, as shown in Table 1. Moreover, PKC δ -overexpressing cells expressed the differentiation marker IAP, which was undetectable in mock-infected controls (Figure 1, panels G, H, blot inset). Interestingly, PKC δ -induced alterations of cell growth described above were not observed when HCT116/p53null/p53/PKC δ cells were continuously exposed to PKC-downregulating concentrations of PMA (Table 1), thus showing that all observed phenotypical alterations were PKC-dependent. In summary, reconstitution of p53 restored responsiveness of the HCT116/p53null cell line to PKC δ : these data confirm our hypothesis that PKC δ can suppress the transformed phenotype of HCT116 human colon cancer cells *via* p53.

In a previous report we have demonstrated that overexpression of PKC δ in the HCT116 cells line induced transient overexpression of both p21^{Waf1/Cip1} and p53, and phosphorylation of p53 at a specific PKC consensus site [3]. We showed as well that p21 is essential for the antineoplastic activity of PKC δ , and that the human papillomavirus E6 gene product, causing proteasome-mediated disruption of the p53 protein, rendered HCT116 cells unresponsive to PKC δ [3]. The experiments presented in this report add important data to our previous results, showing that p53 is an essential effector of PKC δ in our colon cancer model. Since p53 is a major transcription factor for p21, we hypothesize that a "classic" p53-p21 transduction pathway might be directly or indirectly activated by PKC δ , leading to suppression of the transformed phenotype in human colon cancer cells.

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