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Polycystin-1 promotes PKCα-mediated NF-κB activation in kidney cells

Manuela Banzi [a], Gianluca Aguiari [a], Viky Trimi [a], Alessandra Mangolini [a], Paolo Pinton [b], Ralph Witzgall [c], Rosario Rizzuto [b], Laura del Senno [a,*]

[a] *Department of Biochemistry and Molecular Biology, University of Ferrara, Ferrara, Italy*
[b] *General Pathology, Department of Experimental and Diagnostic Medicine, University of Ferrara, Ferrara, Italy*
[c] *Institute for Molecular and Cellular Anatomy, University of Regensburg, Regensburg, Germany*

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Abstract

Polycystin-1 (PC1), the *PKD1* gene product, is a membrane receptor which regulates many cell functions, including cell proliferation and apoptosis, both typically increased in cyst lining cells in autosomal dominant polycystic kidney disease. Here we show that PC1 upregulates the NF-κB signalling pathway in kidney cells to prevent cell death. Human embryonic kidney cell lines ($HEK293^{CTT}$), stably expressing a PC1 cytoplasmic terminal tail (CTT), presented increased NF-κB nuclear levels and NF-κB-mediated luciferase promoter activity. This, consistently, was reduced in HEK293 cells in which the endogenous PC1 was depleted by RNA interference. CTT-dependent NF-κB promoter activation was mediated by PKCα because it was blocked by its specific inhibitor Ro-320432. Furthermore, it was observed that apoptosis, which was increased in PC1-depleted cells, was reduced in $HEK293^{CTT}$ cells and in porcine kidney LtTA cells expressing a doxycycline-regulated CTT. Staurosporine, a PKC inhibitor, and parthenolide, a NF-κB inhibitor, significantly reduced the CTT-dependent antiapoptotic effect. These data reveal, therefore, a novel pathway by which polycystin-1 activates a PKCα-mediated NF-κB signalling and cell survival.

Keywords: Polycystin-1; NF-κB; Promoter activity; siRNA; Apoptosis; PKCα

The main pathogenic mechanism for autosomal dominant polycystic kidney disease (ADPKD), a common inherited kidney disorder, is the total or even partial loss of function of either polycystin-1 (PC1) or polycystin-2 (PC2) [1]. PC1 is a 500 kDa protein with a large extracellular N-terminus, 11 transmembrane regions, and a small cytoplasmic C-terminal tail involved in signal transduction pathways [1]. Recently, it has been shown that PC1 acts as a mechano-fluid stress sensor in primary cilium of kidney tubular cells [2] by interacting with the intracellular C-terminus of PC2, a Ca^{2+}-permeable cation channel [3], and transducing the cilial deflection as a transient increase in cytoplasmic Ca^{2+} levels [4]. This observation provided the most convincing evidence for the functional interaction of the two proteins in the regulation of intracellular Ca^{2+} homeostasis and Ca^{2+}-dependent signal transduction pathways [4], mainly regulating epithelial cell proliferation and apoptosis [5,6], both abnormally increased in ADPKD cyst lining cells [7]. However, the molecular bases underlying the PC1 effects on Ca^{2+} signalling pathways leading to cystogenesis are incomplete. In particular, the relation between PC1 expression, Ca^{2+} levels, and cell proliferation/apoptosis still remains unclear.

In earlier studies we found that the overexpression of the cytoplasmic PC1 C-terminal tail (CTT) in HEK293 kidney epithelial cells increased the ATP-evoked intracellular Ca^{2+} levels, providing evidence for a PC1 role in modulating Ca^{2+} release and/or capacitative calcium entry (CCE) [8]. In these cells there was an increase in serum-induced cell

* Corresponding author. Fax: +39 532 202723.
E-mail address: sen@unife.it (L.del Senno).

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proliferation that was dependent on both Ca^{2+}-activated PKCα and ERK1/2 signalling pathways [9], indicating that the PC1 tail amplifies and strengthens the Ca^{2+} response after exposure to extracellular factors. We, therefore, proposed that the unbalance of the PC1/PC2 complex by the overexpression of the PC1 tail caused a Ca^{2+}-dependent PKCα activation inducing a cell proliferation increase, thus counteracting the putative growth-suppression activity of the endogenous full length PC1 [10].

Since PKC- and Erk-dependent pathways are critical components of the cell survival cascade in epithelial cells by inhibiting apoptosis [11], we predicted that the CTT of PC1 might increase cell survival by suppressing the apoptosis. Various extracellular signals, often converging in common intracellular pathways, can induce apoptosis in a cell-type-specific fashion [12]. Rel/NF-κB transcription factors have been demonstrated to regulate apoptosis in many cell types, including kidney cells [13].

The present study, carried out in kidney cells either overexpressing CTT or depleted of PC1 by RNA interference, demonstrates the positive role of PC1 on NF-κB activity and provides evidence for the PC1-mediated increase in cell survival by NF-κB activation.

Materials and methods

Reagents. G418, staurosporine, doxycycline, and tumor necrosis factor α (TNFα) were purchased from Sigma–Aldrich (Milano, Italy); Ro-320432 and parthenolide were from Calbiochem (La Jolla, CA, USA); the TdT-mediated dUTP nick end labelling (TUNEL) kit was from Promega (Milano, Italy); commercial antibodies were from Santa Cruz (DBA Italia Srl, Segrate, Italy), pSUPER RNAi System from OligoEngene (Seattle, WA, USA) and pNFκB-TA-Luc from Clontech (Celbio Srl, Italy).

Cells, DNA constructs, and transfections. Human embryonic kidney (HEK293), baby hamster kidney (BHK), and porcine kidney LtTA cells (LLC-PK_1 cells, producing a tetracycline-controlled Trans-Activator) were cultured as previously reported [9,14].

pCDNA3/TrkPC1 construct contains human PC1 CTT fused to Trk-A transmembrane and N-terminal cDNA sequences [8]. This and the pCDNA3/Trk0 (containing the Trk-A domains) were transiently transfected in HEK293 or BHK cells, or stably transfected in HEK293 cells [9]. From pCDNA3 constructs, CTT and Trk0 sequences were excised, cloned in pUHD3-10, and used to generate $LtTA^{CTT}$ and $LtTA^{Trk0}$ clones with CTT or Trk0 expression negatively controlled by doxycycline. Positive clones were screened by Western blotting with anti-TrkA antibody on extracts of G418-resistant cells cultured for three days in absence of doxycycline [14].

A PKD1 siRNA was constructed as previously reported [15], by using the forward PKD1 primer sequence: 5′GATCCCCCGACAAGCAGTCCCTGACCTTCAAGAGAGGTCAGGGACTGCTTGTCGTTTTTAAA3′ and the reverse primer sequence: 5′AGCTTTTCCAAAAACGACAAGCAGTCCCTGACCTCTCTTGAAGGTCAGGGACTGCTTGTCGGGG3′. The annealed double-stranded DNA was cloned into the pSUPER plasmid. HEK293 cells were co-transfected with either wild type (pSuper) or recombinant construct (pSsiPKD1) and with pCDNA3 by calcium phosphate [9], and G418-resistant positive clones ($HEK293^{pSuper}$ and $HEK293^{pSsiPKD1}$) were screened by immunocytochemistry with a previously described anti-PC1rabbit polyclonal antibody [16].

Immunoblot and immunocytochemistry analysis. Cell fractions (20–100 μg) from stably transfected clones were loaded on 8% SDS–PAGE, blotted to nitrocellulose membrane, and analyzed as already described [8].

Cells, plated in 8-well chamber slides, were allowed to grow in appropriate medium until 85–90% confluence. After fixation and permeabilization, antibody specific binding was revealed with peroxidase-coupled secondary antibodies and diaminobenzidine reaction.

Luciferase reporter assay. Cells were seeded in 6-well plates, cultured to 80% confluence in 2% FBS supplemented medium, and co-transfected, using polyethylenimine, with 250 ng/well pNFκB-TA-Luc reporter construct (containing four NF-κB consensus sequences upstream the firefly luciferase reporter gene), and with 750 ng/well of recombinant plasmids. After 8 h of incubation, an equal volume of medium without FBS was added. Forty-eight hours later, cells were treated for 16 h with medium alone or with TNFα (16 ng/ml). Cell extracts were assayed, in triplicate, with a luciferase assay kit (Promega). Data were expressed as relative firefly luciferase units (RLU_S) normalized by the *Renilla* luciferase units and as fold increase with respect to control cells.

Identification of apoptosis. Cells were cultured on glass coverslips in serum-deprived conditions (0.4% BSA) for 24 h, exposed to 70 J/m^2 UV radiation or treated with appropriate molecules and times as indicated in Fig. 3 legend. After 48 h from UV radiation, apoptotic cells were determined by TUNEL assay and staining with diaminobenzidine. Apoptosis was also measured by fluorescence after staining with Hoechst 33258 (10 μg/ml). Images were acquired with a LSM 510 Zeiss confocal microscope, and fluorescence was quantified by Z Scan series analysis.

Statistical analysis. The statistical significance of results were calculated by the unpaired *t* test.

Results

NF-κB is activated in kidney cells expressing CTT

The effect of PC1 CTT on NF-κB expression was initially investigated in kidney epithelial HEK293 cells stably expressing the TrkA-fused CTT [9]. NF-κB p65 levels did not significantly differ in $HEK293^{CTT}$ cells and $HEK293^{Trk0}$ control cells; however, in the latter cells NF-κB p65 was only detected in the cytoplasm, while in $HEK293^{CTT}$ cells it was also detected in nuclei (Fig. 1A). Furthermore, after exposure to TNFα, a potent activator of NF-κB signalling [17], nuclear positivity to NF-κB p65 was also found in some $HEK293^{Trk0}$ nuclei (data not shown), whereas that to NF-κB p50 was found in all $HEK293^{CTT}$ nuclei. Overall these results suggest a positive effect of CTT on NF-κB function, which was highlighted by TNFα.

We then evaluated the capacity of CTT to modulate the NF-κB DNA binding. Luciferase activity was measured in HEK293 cells which were transiently co-transfected with either CTT or Trk0 plasmid and with the pNFκB-TA-Luc luciferase reporter plasmid, and cultured in serum-deprived medium in absence and in presence of TNFα. Under basal conditions, luciferase promoter activity of Trk0 transfected cells was similar to that of cells transfected with the pCDNA3 empty vector, while that of CTT transfected cells was approximately 3-fold increased (2.994 ± 0.253, the mean ± SEM compared to Trk0 transfected cells, $p < 0.05$) (Fig. 1B). Moreover, TNFα treatment increased the CTT-dependent luciferase activity both in transiently transfected HEK and BHK cells. Taken together, these data show that CTT expression in kidney cells potentiates basal and TNFα-induced promoter activity mediated by NF-κB.

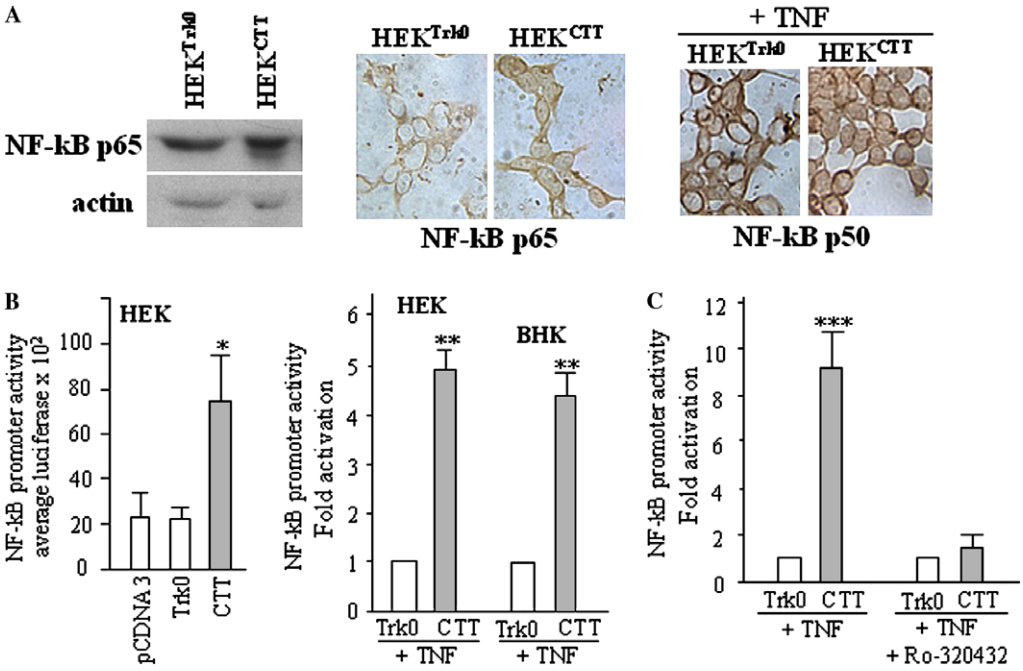

Fig. 1. CTT expression results in increased NF-κB nuclear translocation and NF-κB-dependent promoter activation in kidney cells. (A) Representative Western blot showing NF-κB p65 levels in HEK293^{CTT} and control HEK293^{Trk0} cells, cell images showing the presence of NF-κB p65 in nuclei of HEK293^{CTT}, but not of HEK293^{Trk0} cells, and the TNFα-induced nuclear translocation of NF-κB p50 higher in HEK^{CTT} than in HEK^{Trk0} cells. (B) Luciferase activity in HEK293 and BHK cells transiently co-transfected with the pNFκB-TA-Luc reporter plasmid together with either the empty vector pCDNA3 (pCDNA3), or the recombinant control (Trk0) and the CTT (CTT) constructs. Luciferase activity was measured as described in Materials and methods. TNFα (16 ng/ml) was added for 16 h. The bars represent average luciferase ± SD of a representative experiment carried out in triplicate, and fold activation ± SD of at least three independent experiments. (C) Luciferase activity in HEK293^{Trk0} (Trk0) and HEK293^{CTT} (CTT) cells transfected with the NF-κB-responsive promoter reporter construct and analyzed as described in (B). One, two, and three asterisks represent data significant at $p < 0.05$, 0.01, and 0.001, respectively.

CTT-dependent NF-κB promoter activity is mediated by PKCα

As expected from transient transfection results, in stably transfected HEK293^{CTT} cells the NF-κB promoter activity induced by TNFα was approximately 8-fold higher than in HEK293^{Trk0} control cells (7.728 ± 1.94, $p < 0.001$) (Fig. 1C). Interestingly, this increase was markedly blunted by the presence of a PKCα specific inhibitor (Ro-320432), thus indicating that the CTT-dependent NF-κB activation was mediated by PKCα.

NF-κB activation is reduced in PKD1-siRNA expressing HEK293 cells

If CTT expression is involved in NF-κB activation, we could expect a loss of its activation by loss of PC1 expression. Therefore, NF-κB activation was investigated in HEK293^{pSsiPKD1} cells in which the endogenous PC1 was reduced by stable transfection with a plasmid expressing the PKD1 specific siRNA, as demonstrated by the loss of anti-PC1 positivity in two stably transfected clones (a and b, inset of Fig. 2). Luciferase assay analysis in PKD1 suppressed clones showed that PC1 depletion reduced approximately to half the NF-κB binding compared to control HEK293^{pSuper} cells ($p < 0.05$). These results confirmed the positive role of PC1 on NF-κB activation.

Expression of CTT induces resistance to apoptosis

In order to test whether the CTT-dependent increase in NF-κB promoter activity could protect from apoptosis, we looked for TUNEL-positive cells in HEK293^{CTT} and HEK293^{Trk0} cells where apoptosis was induced by serum starvation and exposure to UV radiation. Apoptotic cells resulted significantly ($p < 0.05$) reduced in CTT expressing cells (Fig. 3A). This finding was supported by data obtained measuring apoptotic nuclei after Hoechst staining. HEK293^{CTT} and HEK293^{Trk0} cells were serum starved and cultured in absence or presence of TNFα in the conditions used for measuring NF-κB-dependent promoter activity. In absence of TNFα, the fluorescence intensity, mainly due to apoptotic nuclei (Fig. 3B), was significantly higher in HEK293^{Trk0} than in HEK293^{CTT} cells (489.7 ± 47.9 vs 293 ± 21.8, mean ± SEM, $p < 0.01$); the fluorescence in latter cells was 0.59-fold lower than in Trk0 expressing cells. After TNFα treatment, HEK293^{CTT} cell fluorescence was still reduced with respect to HEK293^{Trk0} cells. On the other hand, treatment with 0.5 μM staurosporine (STS), which is known to trigger intrinsic pathway of apoptosis [18],

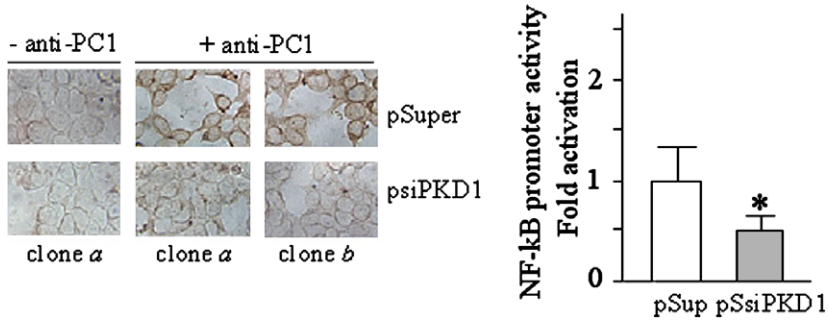

Fig. 2. PC1 deletion by RNA interference results in reduced NF-κB-dependent promoter activation. HEK293^{pSsiPKD1} cells stably expressing the PKD1 siRNA (pSsiPKD1) and HEK293^{pSuper} control cells (pSup) were transfected with the pNFκB-TA-Luc reporter plasmid and analyzed as described in this Fig. 1. Inset: strong reduction in the faint, but consistent, positivity to anti-PC1 antibody in two PKD1 siRNA expressing clones (a and b) (see Materials and methods for technical details). The PC1 depletion was associated to a significant reduction in the NF-κB-responsive promoter activation in HEK293^{pSsiPKD1} cells (*, $p < 0.05$ compared with cells expressing the empty vector).

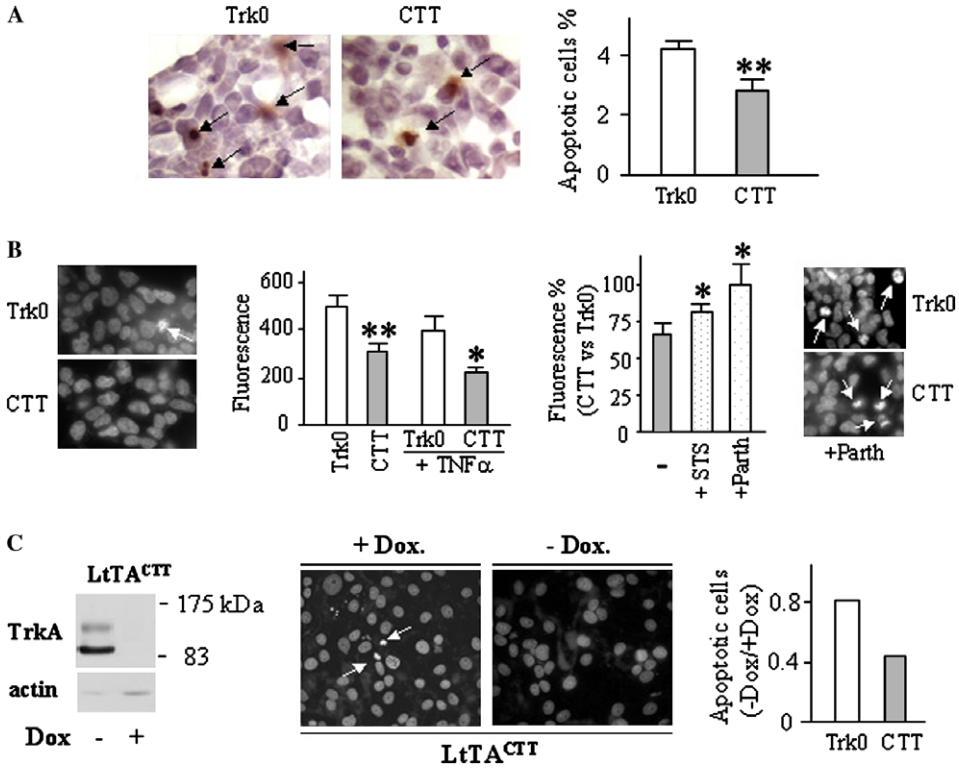

Fig. 3. CTT expression protects from apoptosis. (A) Representative images of HEK293^{Trk0} (Trk0) and HEK293^{CTT} (CTT) cells that were stained by TUNEL assay to detect apoptosis triggered by serum deprivation and UV irradiation (see Materials and methods for technical details). The percentage of apoptotic cells was calculated from the ratio of apoptotic nuclei to total cells counted. Bars represent the percent of apoptotic cells in two different experiments (average ± SEM). (B) Representative images of HEK293^{Trk0} and HEK293^{CTT} cells that were stained with Hoechst 33258 to detect apoptosis triggered by 40 h serum starvation. CTT stable expression reduces fluorescence intensity caused by typical changes in apoptosis (i.e. nuclei fragmentation with condensed chromatine indicated by arrows), as detected by fluorescent microscopy. Basal and TNFα-induced apoptosis (24 h starvation and further 16 h in absence or presence of 16 ng/ml TNFα) are reduced by CTT expression compared to Trk0 expressing control cells. Bars represent the average fluorescence in a representative experiment, in two different Trk0 and CTT expressing clones (average ± SEM). Staurosporine (STS, 0.5 μM for 16 h) reduces the CTT-dependent protective effect. Bars represent fluorescence in HEK293^{CTT} related to that in HEK293^{Trk0} (average ± SD) in at least two experiments performed in triplicate. The pre-treatment with the NF-κB inhibitor Parthenolide (Parth, 10 mM for 1 h, images on the right) blunts the CTT-dependent antiapoptotic effect. (C) Representative images of LtTA^{CTT} porcine kidney cells stained with the Hoechst 33258. Apoptosis is lower in cells that expressed a CTT under the negative control of doxycycline, as shown in the blot: TrkA-positive bands were detected in CTT expressing cells cultured in absence of doxycycline (−Dox), but not in its presence (+Dox). Bars represent fluorescence ratios between the percentage of apoptotic cells in untreated and Dox treated cells in HEK293^{CTT} and in HEK293^{Trk0} cells, in a representative experiment. One and two asterisks represent data significant at $p < 0.05$ and 0.01, respectively.

significantly reduced the CTT-dependent anti-apoptotic effect in HEK293^{CTT}, the mean fluorescence ratio of HEK293^{CTT} to HEK293^{Trk0} cells increasing to 0.85 ± 0.05 from 0.66 ± 0.08 basal value, $p < 0.05$. Furthermore, parthenolide, an NF-κB inhibitor targeting the IκB kinase, markedly decreased the anti-apoptotic effect, the

mean fluorescence ratio of HEK293^{CTT} to HEK293^{Trk0} cells increasing to 0.98 ± 0.16, $p < 0.05$. These data show, therefore, that CTT is able to confer a resistance to apoptosis, which is reduced in presence of the apoptogenic factor staurosporine or blunted in presence of the NF-κB inhibitor.

The role of CTT on apoptosis was further investigated in a polarized and ciliated tubule cell line: the porcine kidney LtTA cells [14], expressing heterologous CTT under the doxycycline control (LtTA^{CTT} clones). Only in absence of doxycycline (Dox) did plasma membrane proteins become positive to the anti-TrkA antibody (Fig. 3C). While the majority of LtTA^{CTT} cells grown in presence of Dox had some irregular and chromatine-condensed apoptotic nuclei, those grown in Dox absence (expressing CTT) did not. Moreover, apoptosis in cells grown without Dox was higher in control LtTA^{Trk0} than in LtTA^{TRKPC1} cells, as shown by the higher ratio in fluorescence between cells grown with and without Dox (0.79 in LtTA^{Trk0} and 0.41 in LtTA^{TRKPC1}, the ratios between apoptotic index in treated and untreated cells).

Apoptosis is increased in PKD1-siRNA expressing HEK293 cells

Consistent with the protective effect of CTT, apoptosis was increased in PC1-depleted cells by PKD1-specific siRNA: TUNEL-positive nuclei were 1.3-fold increased ($p < 0.05$) in serum starved HEK293^{pSsiPKD1} compared to control HEK293^{pSuper} cells (Fig. 4A). Moreover, after 20 h treatment with 10 μM Ro-320432, the PKCα inhibitor, TUNEL-positive nuclei increased in both cell lines, but those in HEK293^{pSsiPKD1} were 1.7-fold higher (Fig. 4B), thus providing evidence for an involvement of PKCα on the PC1-dependent cell survival.

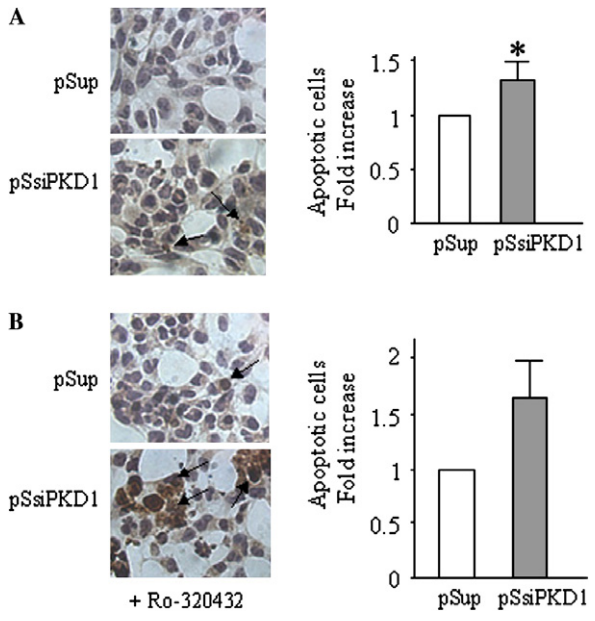

Fig. 4. Apoptosis increases in PC1-depleted cells. (A) Representative images of HEK293^{pSuper} (pSup) and HEK293^{pSsiPKD1} (pSsiPKD1) cells that were assayed with TUNEL assay to detect apoptosis triggered by 40 h serum deprivation. The percentage of apoptotic cells was calculated as in Fig. 3A. Bars represent the average increase (±SEM) in apoptotic cells in PC1-depleted cells (*, $p < 0.05$, compared to control cells). This increase becomes much more evident after starvation in presence of the PKCα specific inhibitor Ro-320432, as shown by the representative images in (B).

Discussion

We have recently reported that the expression of the PC1 C-terminal tail CTT as a TrkPC1 construct in kidney HEK293 cells increases cell proliferation which is mediated by activation of the calcium-dependent PKCα [9]. The data here reported show that in kidney epithelial cells CTT, as well as the endogenous PC1, also controls apoptosis through a PKCα-dependent NF-κB activation. In particular, the heterologous expression of CTT in kidney cells induces a constitutive activation of NF-κB, which is demonstrated by an increase in its nuclear localization and in the NF-κB-dependent promoter activation, which is highlighted by the NF-κB signalling activator TNFα, in both human and hamster cells. Moreover, the observation that PC1 depletion by siRNA interference causes a reduction in NF-κB-dependent promoter activation strongly indicates that NF-κB signalling is not only activated by CTT exogenous expression, but can be intrinsically modulated by the endogenous PC1. Interestingly, since the CTT-dependent NF-κB binding is markedly reduced by the treatment with a specific inhibitor of the calcium-dependent PKCα, calcium may presumed to be an important upstream effector of this NF-κB signalling. With regard to this, we have previously shown that CTT expression increases both basal and induced cytoplasmic calcium levels [8,9].

Numerous studies have demonstrated increased sensitivity to apoptosis associated with inhibition of NF-κB activation, and, consistently, we found an increase in apoptosis in PC1-depleted HEK293^{pSsiPKD1} cells which showed reduced NF-κB promoter activation. In line with this finding, we observed that CTT expression, which increases NF-κB activation, reduces apoptosis in both human and porcine kidney cells. These data lead us to hypothesize that the PC1-dependent NF-κB activation contributes to the PC1-dependent resistance to apoptosis. This hypothesis is supported by the observations that in HEK293^{CTT} cells this resistance is maintained in presence of the NF-κB activator TNFα, is decreased by Staurosporine, which is known to induce apoptosis through a mitochondria-mediated pathway [19], and, even more important, is blunted by the NF-κB inhibitor parthenolide.

The observation that PC1 protects from apoptosis is in line with previous findings, obtained by overexpressing the human full length PKD1 cDNA in MDCK canine kidney cells, showing that PC1 reduces apoptosis through Akt phosphorylation signalling [6]. Here we show that the PC1-dependent NF-κB activation, as well as the resistance to apoptosis, is supported by the activation of the calcium dependent PKCα. The PKCα specific inhibitor Ro-320432,

which, on the one hand, reduces NF-κB activity in CTT expressing HEK293^{CTT} cells, on the other hand does increase apoptosis in PC1-depleted HEK293^{pSsiPKD1} cells. Furthermore, the role of PKCα activation in PC1-dependent survival is also supported by the observation that, in CTT stably expressing HEK293^{CTT} cells, the CTT-dependent protection from apoptosis is reduced by STS, a potent protein kinase C inhibitor with a broad spectrum of activity [18]. The PKCα activation, therefore, represents an additional signalling pathway involved in the PC1-mediated increase in cell survival via NF-κB activation.

The calcium-dependent anti-apoptotic effect of PC1 may seem paradoxical because calcium release from endoplasmic reticulum may result in a mitochondrial overload of calcium and cell death [20]. The latter observation combined with the PC1-dependent NF-κB activation suggests that the PC1-dependent anti-apoptotic effect may be caused by a controlled calcium release, possibly constant repetitive calcium transients, like those observed in ouabain-treated rat proximal tubule cells [21]. With regard to this, it is noteworthy that PC1 can regulate the calcium channel activity of its interacting partner PC2 [2]. Furthermore, it was recently reported that low doses of ouabain abolished the apoptotic effect of serum starvation and that the protective effect depends on IP_3R-mediated calcium release and, subsequently, activation of NF-κB [13].

In conclusion, the present study, carried out in kidney cell lines of different species and tubule origin, either overexpressing CTT or depleted of PC1 by RNA interference, demonstrates the positive role of PC1 on NF-κB activation and provides evidence for the PC1-mediated cell survival by NF-κB via a PKCα.

Acknowledgments

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