Available online at www.sciencedirect.com

SCIENCE DIRECT

Experimental Cell Research 304 (2005) 391–406

Experimental Cell Research

www.elsevier.com/locate/yexcr

The cytoplasmic C-terminus of polycystin-1 increases cell proliferation in kidney epithelial cells through serum-activated and Ca^{2+}-dependent pathway(s)

Elisa Manzati[a,b], Gianluca Aguiari[a,b], Manuela Banzi[a,b], Michele Manzati[a,b], Rita Selvatici[c], Sofia Falzarano[c], Iva Maestri[d], Paolo Pinton[e], Rosario Rizzuto[e], Laura del Senno[a,b,*]

[a]*Department of Biochemistry and Molecular Biology, University of Ferrara, Via Luigi Borsari 46, I-44100 Ferrara, Italy*
[b]*Department of Experimental and Diagnostic Medicine, University of Ferrara, Via Luigi Borsari 46, I-44100 Ferrara, Italy*
[c]*Section of Medical Genetics, University of Ferrara, Via Luigi Borsari 46, I-44100 Ferrara, Italy*
[d]*Section of Clinical Pathology, University of Ferrara, Via Luigi Borsari 46, I-44100 Ferrara, Italy*
[e]*Section of General Pathology, University of Ferrara, Via Luigi Borsari 46, I-44100 Ferrara, Italy*

Received 21 February 2004, revised version received 9 October 2004
Available online 15 December 2004

Abstract

Polycystin-1 (PC1) is a large transmembrane protein important in renal differentiation and defective in most cases of autosomal dominant polycystic kidney disease (ADPKD), a common cause of renal failure in adults. Although the genetic basis of ADPKD has been elucidated, molecular and cellular mechanisms responsible for the dysregulation of epithelial cell growth in ADPKD cysts are still not well defined. We approached this issue by investigating the role of the carboxyl cytoplasmic domain of PC1 involved in signal transduction on the control of kidney cell proliferation. Therefore, we generated human HEK293 cells stably expressing the PC1 cytoplasmic tail as a membrane targeted TrkA-PC1 chimeric receptor protein (TrkPC1). We found that TrkPC1 increased cell proliferation through an increase in cytoplasmic Ca^{2+} levels and activation of PKCα, thereby upregulating D1 and D3 cyclin, downregulating $p21^{waf1}$ and $p27^{kip1}$ cyclin inhibitors, and thus inducing cell cycle progression from G0/G1 to the S phase. Interestingly, TrkPC1-dependent Ca^{2+} increase and PKCα activation are not constitutive, but require serum factor(s) as parallel component. In agreement with this observation, a significant increase in ERK1/2 phosphorylation was observed. Consistently, inhibitors specifically blocking either PKCα or ERK1/2 prevented the TrkPC1-dependent proliferation increase. NGF, the TrkA ligand, blocked this increase. We propose that in kidney epithelial cells the overexpression of PC1 C-terminus upregulates serum-evoked intracellular Ca^{2+} by counteracting the growth-suppression activity of endogenous PC1 and leading to an increase in cell proliferation.

Keywords: ADPKD; Polycystin-1; TrkA chimeric receptor; Signal transduction; Cell proliferation; Calcium homeostasis; Ca2+-aequorin; PKC-GFP isoforms; PKCα; ERK1/2

Introduction

The hallmark of autosomal-dominant polycystic kidney disease (ADPKD), a common renal disorder in adults, is the progressive expansion of multiple fluid-filled cysts derived from tubular epithelia in kidney and other organs [1]. Several lines of evidence suggest that the dysregulation of epithelial cell growth is a key step in this process. In fact, proliferation-associated genes, such as c-*myc*, Ki-67, and PCDNA, are

Abbreviations: ADPKD, Autosomal dominant polycystic kidney disease; PC, Polycystin; HEK, Human embryonal kidney; HEK^{pCDNA3}, HEK^{TrkPC1} and HEK^{Trk0} cells, Stably transfected cell lines; CDK, Cyclin-dependent kinase; CKI, Cyclin-dependent kinase inhibitor; ERK, Extracellular signal-regulated kinase; MEK, Mitogen activated protein kinase; PBS-T, PBS-0.05% Tween-20; GFP, Green fluorescent protein.

* Corresponding author. Department of Biochemistry and Molecular Biology, University of Ferrara, Via Luigi Borsari 46, I-44100 Ferrara, Italy. Fax: +39 532 2027.
E-mail address: sen@unife.it (L. del Senno).

0014-4827/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2004.10.023

significantly overexpressed in cyst epithelia from ADPKD subjects [2–4].

ADPKD is basically caused by mutations in the PKD1 gene in most cases (85%), while mutations in the PKD2 gene account for the remaining approximately 15% of cases [5,6].

The PKD1 gene product, polycystin-1 (PC1), is an integral glycoprotein composed of a large extracellular amino-terminal domain including a combination of protein–protein interaction motifs and a G-protein-coupled receptor proteolytic site (GPS), 11 transmembrane domains and a short cytoplasmic C-terminus involved in signal transduction [6–9]. The cytoplasmic tail contains a coiled-coil motif able to bind polycystin-2 (PC2) [10,11], the PKD2 gene product, which is a non-specific Ca^{2+}-permeable cation channel [12,14].

Based on comparative analysis of the primary structure and functional data, PC1 is predicted to be a plasma membrane non-kinase type receptor that, in association with PC2 as part of a multiprotein membrane signaling complex [15,16], is involved in cell–cell/–matrix interactions and in Ca^{2+} dependent signal transduction pathways mainly regulating epithelial cell differentiation and proliferation [6,17,18].

Although there are few direct functional in vitro studies in support of this model [19–21], a number of studies have provided direct evidence connecting PC1 function to known signaling pathways. In particular, the C-terminal cytosolic domain of human PC1 is able to stimulate AP-1 promoter activity in transiently transfected mammalian cells by a PKC- and c-Jun N-terminal kinase (JNK)-dependent mechanism [22]. The same PC1 peptide also stimulates the Wnt signaling pathway through the inhibition of glycogen synthase kinase-3β (GSK-3β) and subsequent stabilization of β-catenin [23]. In addition, the PC1 intracellular tail has been demonstrated to bind and activate heterotrimeric G-proteins [24,25], to bind RGS7, a regulator of heterotrimeric G-protein signaling [26], and to be a phosphorylation target by protein kinase A (PKA) and a Src-like tyrosine kinase [27,28].

It has also been reported that overexpression of the C-terminal tail of mouse PC1 can transform the cAMP-growth responsive phenotype of mouse cortical (M-1) collecting duct cell lines from a cAMP-inhibited to cAMP-stimulated cell proliferation [29]. Furthermore, the overexpression of the human PKD1 transgene in transgenic mice causes the appearance of polycystic kidneys [30], indicating that an overexpression of full-length PC1 could cause ADPKD. On the contrary, overexpression of full-length PC1 in stably transfected Madin-Darby canine kidney cells results in decreased proliferation [31], suggesting that the increased proliferation in ADPKD may be a consequence of the loss of PC1 function. Taken together, these results suggest that PC1 plays a role in the control of renal epithelial cell growth and differentiation for which the molecular determinants and effects are still not completely understood.

We have recently found that in kidney HEK293 cells the transient expression of a membrane targeted PC1 carboxy-terminal construct (TrkPC1), containing the PC1 cytoplasmic C-terminus fused to TrkA transmembrane and extracellular sequences, increases the cell response to ATP-stimulated calcium levels, demonstrating that PC1 pathways are involved in the regulation of intracellular calcium homeostasis [32]. Furthermore, in human lymphoblastoid cell lines, we have recently demonstrated a direct relation between cell proliferation, polycystin expression and calcium levels [33]. Since one function of calcium signals is to promote cell proliferation [34,35], we investigated whether the stable expression of TrkPC1 in epithelial HEK293 cell lines would affect their growth.

Our findings show that the overexpression of TrkPC1 induces an increase in cell proliferation through a serum-evoked cytoplasmic Ca^{2+} rise, and activation of PKCα and Erk1/2. Therefore, the abnormal and uncontrolled cell proliferation observed in epithelial cyst-lining cells could be caused by a growth factor-dependent signal transduction pathway upregulated by the C-terminal tail of PC1.

Materials and methods

Reagents

D-MEM/F12 medium, G418 antibiotic, Bovine Serum Albumin (BSA), Nerve Growth Factor (NGF), and β-octyl glucoside were obtained from Sigma-Aldrich (Milano, Italy). Phenol-free MEM medium was from Invitrogen (Invitrogen Srl, Italy). FBS was obtained from BioWhittaker (BioWhittaker Italia Srl). Selective cell-permeable inhibitors of PKCα, PKCβ1, and MEK kinases (PD 98059, hispidin and Ro-320432, respectively), were purchased from Calbiochem (La Jolla, CA, USA), and protease inhibitors from Roche Diagnostics (Monza, Italy).

Rabbit polyclonal anti-N-ter-TrkA antibody was obtained from Upstate (DBA. Italia Srl, Segrate, Italy); mouse monoclonal anti-p21, rabbit polyclonal anti-p27, anti-D1 cyclin, anti-D3 cyclin, anti-cdk4, anti-phospho-TrkA (Tyr490) were obtained from Cell Signaling Technology (Celbio Srl, Italy); mouse monoclonal anti-actin, rabbit polyclonal anti-calnexin, anti-ERK1/2, and phospho-ERK1/2 were obtained from Santa Cruz (DBA Italia Srl, Segrate, Italy). Monoclonal anti-N-ter-TrkA (H10) was kindly provided from Dr. Natali (Istituto Regina Elena, Rome, Italy). Enhanced chemiluminescent substrates for Western blotting (SuperSignalDura or SuperSignalFemto) and HRP-conjugated goat anti-rabbit and anti-mouse antibodies were purchased from Pierce (Celbio Srl, Italy).

Constructs and stable transfection

The Trk-$PC1_{26–226}$ construct (TrkPC1) used to generate the cells stably expressing the PC1 cytoplasmic domain

has been described elsewhere [32]. A PKD1 cDNA sequence encoding the last 200 amino acids of the human PC-1 cytoplasmic domain (226 aa) was fused to a TrkA cDNA sequence encoding transmembrane and N-terminal domains of the NGF tyrosine receptor (Trk-A) (Fig. 1A) and inserted into pCDNA3 expression vector. PKD1 cDNA sequence was replaced by an unrelated random 18 aa sequence in the control truncated Trk0 expressing construct [32].

Human embryonic kidney (HEK-293) cells were cultured to 60% confluence in 10 cm^2 culture dish in DMEM/F12 medium supplemented with 10% FBS. One hour before transfection, the medium was refreshed and cells were transfected with 10 μg of either plasmid DNA, using the calcium phosphate method [32]. After 24 h, cells were collected by scraping and, after dilution at 1:10, 1:50, and 1:100 in selection medium containing G418 (500 μg/ml), they were cultured for selection in G418 for at least 15 days by refreshing the medium every 3 days. Stable cell lines (HEK^{TrkPC1}, HEK^{Trk0} and HEK^{pCDNA3} clones) were cultured in D-MEM/F12 supplemented with 10% of FBS, in the presence of G418.

Cell proliferation and cell cycle analysis

Cells were seeded at low density (30,000 cell/ml) in 24-well plates. After 24 h starvation in serum- and phenol-free medium, cells were cultured for 3 and 5 days in phenol-free MEM medium supplemented with 1% FBS (unless otherwise specified). After trypan blue staining, cells were directly counted in a Burker chamber. Growth curves were generated performing three different cell counts in at least three separate cell cultures. Mean and SEM of each time point were calculated, and levels of significance ($P < 0.05\%$) were determined by the unpaired t test. In experiments with specific inhibitors, cells were plated, synchronized as described, and treated for 2 days with the kinase-specific inhibitors (Ro-320432, 10 μM; hispidin, 5 μM; PD 98059, 30 μM). Cell cycle was analyzed by flow cytometry of propidium iodide-treated cells, using the FACSCalibur Becton Dickinson Immunocytometry System.

Western blotting

Cells were collected, lysed and processed for immunoblots as previously described [32]. For the protein phosphorylation analysis, cell lysates were supplemented with 10 mM sodium pyrophosphate and 1 mM sodium vanadate. One hundred or 50 μg of total proteins were subjected to SDS-polyacrylamide gel electrophoresis (4–12% gradient mini gel, ICN). Blocked membranes were probed with primary antibody in 5% powdered milk in PBS-T for 2 h at room temperature. Membranes were then washed three times with PBS-T and incubated with the secondary antibody in 5% milk in PBS-T for 1 h. Finally, membranes were rinsed three times with PBS-T, and proteins visualized by using the enhanced chemiluminescence system. Band intensity was detected by X-ray film scanning and quantitative analysis by Model GS-700 Imaging Densitometer (BIORAD).

Isolation of cell membranes and subcellular fractionation

Cells were cultured and processed as previously described [32]. For differential centrifugation, cell lysates were homogenized in hypotonic lysis buffer [32], and the homogenate was centrifuged at 8000 × g (20 min, 4°C). The pellet was solubilized in β-octyl glycoside buffer (HEPES 10 mM, NaCl 118 mM, KCl 4,7 mM, $MgCl_2$ 5 mM, EGTA 1 mM, pH 7.4 supplemented with cocktail of inhibitors (P8 fraction, containing whole cells, plasma membranes, debris and nuclei), while the supernatant was subsequently centrifuged at 100,000 × g (45 min, 4°C). The pellet obtained was resuspended in single detergent lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 8.0, 1% v/v Triton X-100, 0.25 mM PMSF, protease inhibitors) (P100 membrane fraction). An aliquot (100 μg) of ultracentrifugation supernatants (S100 fraction), of P100 and P8 fractions was analyzed by Western blot using the anti-TrkA antibody.

For subcellular fractionation, homogenized cells were centrifuged at 1000 × g, and the supernatant was layered on top of a 12.5 ml discontinuous density gradient, as previously reported [36]. Fifteen microliters from each gradient fraction were loaded on a 4–12% polyacrylamide mini gel, electrophorized, and blotted to a nitrocellulose filter. Filters were analyzed with anti-Trk-A polyclonal antibody and with antibodies recognizing organelle membrane protein markers: β_1-integrin for plasma membrane, actin for cytoskeleton, and calnexin for endoplasmic reticulum.

FACS analysis

Cells in T25 flasks were cultured up to 80% confluence, washed twice in 10 ml PBS, scraped and collected by centrifugation at 1000 × g. Cells were incubated in 3 ml PBS with 5 μg of H10 anti-TrkA for 1 h at room temperature. After three washes in PBS solution, cells were incubated in 3 ml PBS with the FITC-conjugate anti-mouse secondary antibody (1:1000 dilution) for 30 min at 4°C in the dark, then centrifuged for 5 min at 1000 × g. After four washes in PBS, cells were analyzed with a FACscan flow cytometer (Becton Dickinson, Oxford, UK), as previously described [37].

Fluorescence microscope analysis

Cells were cultured to 30% confluence on 24 mm circular glass coverslips in DMEM/F12 medium with 10% FBS. Cells were starved and, 1 h before transfection, were

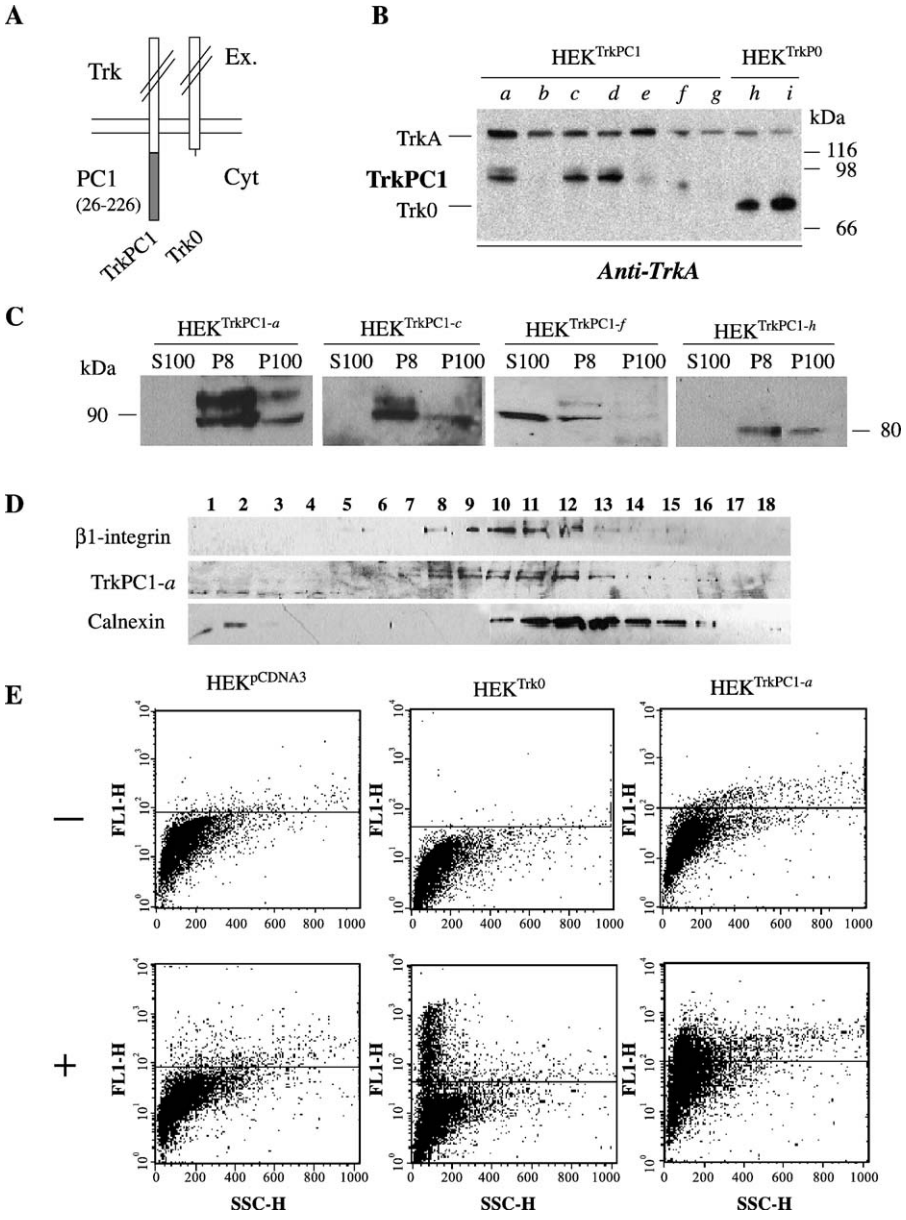

Fig. 1. Characterization of TrkPC1 expression in stably transfected clones. (A) Schematic representation of TrkPC1 and Trk0 control constructs. The $TrkPC1_{26\text{-}226}$ construct encodes extracellular, transmembrane, and the 32 amino acid intracellular domains of the tyrosine receptor Trk-A, fused to the C-terminal 200 amino acids of the human PC1 (grey box), as previously reported [32]. The Trk0 control construct contains the N-terminal, transmembrane, and cytoplasmic TrkA sequence followed by a random 18 amino acid tail. (B) Western blot showing TrkPC1 and Trk0 proteins positive to the anti-N-ter-TrkA polyclonal antibody, in whole cell lysates from G-418 selected stably transfected HEK293 cells (HEK^{TrkPC1} *a-g* and HEK^{Trk0} *h*, *i* clones). The endogenously expressed TrkA (upper band of approximately 140 kDa), and the TrkPC1 (approximately 90 kDa) and Trk0 (approximately 80 kDa) chimeric proteins were detected. (C) Western blotting analysis of subcellular fractions obtained by differential centrifugation of HEK^{TrkPC1} and HEK^{Trk0} clones, using anti-N-ter-TrkA antibody. The bands corresponding to glycosylated TrkPC1 and Trk0 chimeric proteins were detected mainly in the P8 fraction (plasma membrane with whole cells, debris and nuclei) and less in the P100 fraction (microsomes). A TrkPC1 band in the S100 (cytosol) fraction was detected only in the HEK^{TrkPC1} clone *f*. (D) Western blot analysis of fractions from a sucrose gradient centrifugation of total cell extract of HEK^{TrkPC1} clone *a*. Five hundred-microliter fractions were collected from the gradient top (0.2 M) to the bottom (1.43 M) and equal volumes from each fraction were loaded on 8% mini-acrylamide gel. Samples were analyzed by immunoblot with antibodies against Nter-Trk-A, β1-integrin, and calnexin. TrkPC1 and Trk0 mainly co-migrate with the β1-integrin, a plasma membrane marker. (E) FACS analysis of a HEK^{pCDNA3}, HEK^{Trk0} and HEK^{TrkPC1} clone using the monoclonal anti-N-ter-Trk-A antibody. Plots on the top represent cells treated with anti-mouse Ig FITC-conjugated secondary antibody (−), while plots on the bottom represent cells treated with both primary anti-Nter-Trk-A and secondary antibodies (+). Cells expressing TrkPC1 and Trk0 fusion proteins showed an increased fluorescence compared to the pCDNA3 stable transfected cells.

supplemented with fresh phenol-free MEM medium with 10% FBS. Cells were co-transfected with GFP-PKCs and pCDNA3 constructs (ratio, 1:5) by using the calcium phosphate procedure. PKC membrane translocation was visualized by digital fluorescence microscope and images were captured and recorded using a digital imaging system.

This is based on a Zeiss Axiovert 200 fluorescence microscope equipped with a back-illuminated CCD camera (Roper Scientific, USA), excitation and emission filter wheels (Sutter Instrument Company, USA) and piezoelectric motoring of the z stage (Physik Instrumente, GmbH and Co., Germany). The data were acquired and processed using the MetaFluor analyzing program (Universal Imaging Corporation, USA).

Calcium measurements

Calcium measurements were performed in cells that were cultured on 24 mm circular glass coverslips and transfected with recombinant cytoplasmic aequorin cDNA as previously reported [32]. After 72-h transfection, 5 μM coelenterazine (the prosthetic group of aequorin) was added to the medium to reconstitute the active form of this protein. Two hours after reconstitution, cells on coverslips were transferred into a perfusion chamber of measuring instrument (luminometer) and perfused at 37°C with KRB buffer (125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, and 20 mM HEPES, pH 7.4, supplemented with 1 mM $CaCl_2$) (KRB/Ca^{2+}). After approximately 1 min of perfusion, HEK293 cells were stimulated with 1% serum and, subsequently, with 100 μM ATP. Calcium ions, released into the cellular cytoplasm, bind aequorin molecules which emit photons that are captured by the luminometer. Using a computer algorithm, the relationship between photon emission (L/L_{max}) and Ca^{2+} concentration allows the conversion of the light release into calcium concentration. L and L_{max} are the instant and the maximal rate light emission. L is obtained after cell stimulation, while L_{max} is produced by cell lysis with 100 μM digitonin as previously described [38]. The calibration procedure of aequorin (based on the calculation of the fractional rate of consumption, and its correlation with the [Ca^{2+}] to which the photoprotein is exposed) renders this Ca^{2+} probe essentially insensitive to variations in probe loading (that can occur in independent transfections, e.g., of control and TrkPC1 stably transfected cells) [38].

Basal levels of the cytoplasmic Ca^{2+} concentration were measured in Fura-2AM-loaded cells as previously described [39]. Cells were grown on 24 mm coverslips in DMEM/F12 medium supplemented with 1% FBS and loaded with 4 μM Fura-2AM (added to KRB/Ca^{2+} solution + 1% FBS) at 37°C for 30 min. After a brief washout, the coverslip was transferred to the thermostated stage of a Zeiss Axiovert 200 inverted microscope, equipped with a Sutter filterweel and 340/380 excitation filters. The sample was excited at 340 and 380 nm using a random access monochromator (Photon Technology International, NJ, USA) and emission was detected using 505/30 emission filter. Images at 2 frames were acquired by the BFT512 camera (Princeton Instruments, AZ, USA). Image acquisition was controlled by the MetaFluor 5.0 software (Universal Imaging Corporation, PA, USA). Calibration was done using MetaFluor and MetaFluor Analyst software, according to the equation $[Ca^{2+}] = K_d \times (Sf2/Sb2) \times (R - R_{min})/(R_{max} - R)$, as previously described [39], using K_d = 224 nM. R_{max} and R_{min} were determined at the end of each experiment in KRB/Ca^{2+}/1 μM ionomycin, and KRB/1 mM EGTA/ionomycin, respectively.

Results

TrkPC1 is variably expressed in HEK^{TrkPC1} clones

We used immunoblot and immunocytometry to investigate the expression of chimeric proteins (Fig. 1A) in HEK293 cell lines stably expressing TrkPC1 (HEK^{TrkPC1}) and the Trk0 truncated form (HEK^{Trk0}). HEK^{TrkPC1} clones expressed different levels of TrkPC1, which was found as a prevalent band of approximately 90 kDa, while in HEK^{Trk0} clones Trk0 was found as a prevalent band of approximately 80 kDa (Fig. 1B). As previously shown in transiently transfected cells [32], PNGase and Endo H treatment markedly reduced the protein molecular weight of both TrkPC1 and Trk0 to the unglycosylated putative weight of 73 and 49 kDa, respectively (data not shown).

Differential centrifugation of HEK^{TrkPC1} and HEK^{Trk0} cell extracts showed anti TrkA-positive proteins mostly in the P8 fraction, and not in the soluble S100 fraction (Fig. 1C), except for the HEK^{TrkPC1} clone *f*, which mostly contained the fusion protein in the S100 fraction, indicating that the chimeric protein is unable to reach, or to remain in, membrane compartments.

The TrkPC1 membrane localization was further analyzed by sucrose gradient fractionation (Fig. 1D) showing that the chimeric protein co-migrated with β1-integrin, a plasma membrane protein marker, and in part with calnexin, an endoplasmic reticulum membrane marker.

Furthermore, by FACS analysis of intact and unfixed HEK^{TrkPC1} and control clones with the anti-N-ter-TrkA monoclonal antibody, we found that the fusion proteins were correctly exposed on the plasma membrane (Fig. 1E). The mean surface positivity of each clone was variable (24% in HEK^{TrkPC1} *a*, 20% in HEK^{TrkPC1} *c*, 19% in HEK^{Trk0} *h*, 3.5% in HEK^{TrkPC1} *f* and 2.5% in HEK^{pCNA3} cells, possibly due to the endogenous Trk-A). The low positivity of HEK^{TrkPC1} *f* cells confirmed the scarcity of the fused protein detected by Western blot in the P8 fraction.

The PC1 tail increases cell proliferation in HEK^{TrkPC1} clones

To determine whether the overexpression of the PC1 tail affected cell proliferation, we initially compared the proliferation rate of HEK^{TrkPC1} clones with that of HEK^{Trk0} and HEK^{pCDNA3} control clones. As shown in

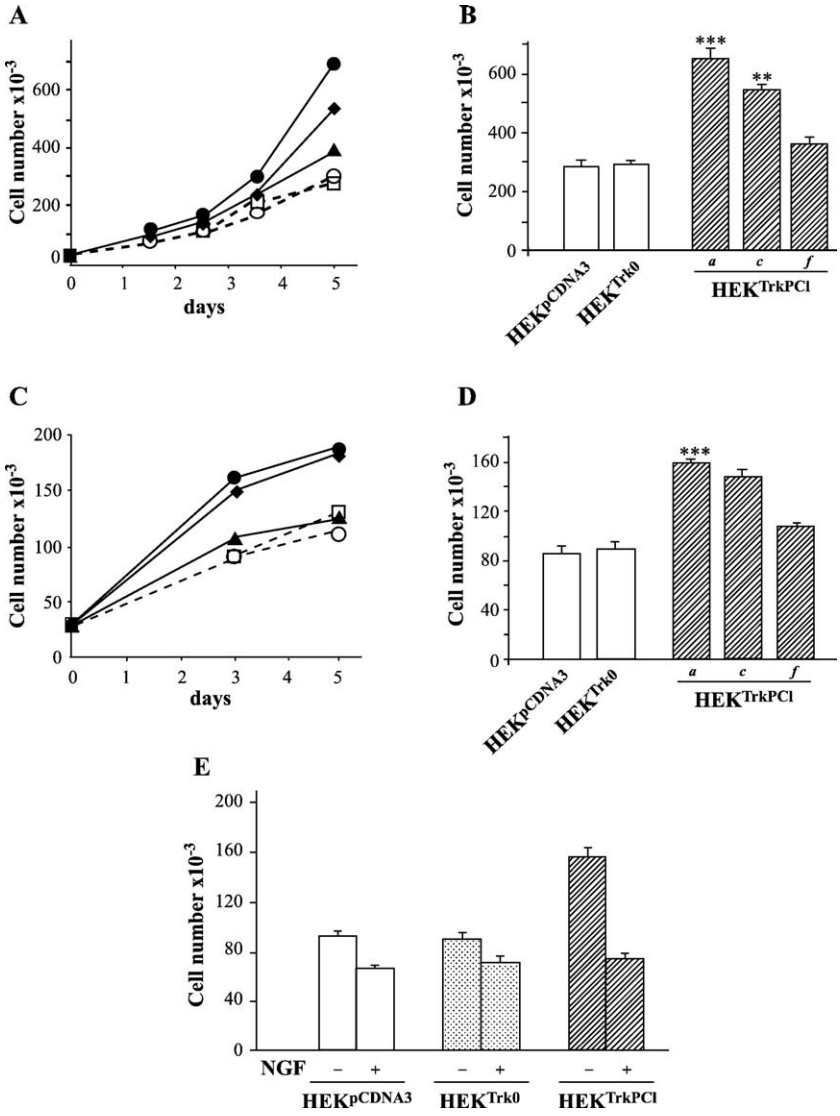

Fig. 2. The TrkPC1 expression increases HEK293 cell proliferation. (A and C) Cell growth time course of HEK^{TrkPC1} clone-*a* (●), -*c* (♦), -*f* (▲), HEK^{Trk0} (□) and HEK^{pCDNA3} (○) clones. Cells were seeded in triplicate at low density (30,000 cells/ml) in 24 well plates, and cultured for 5 days in DMEM/F12 plus 10% FBS (A) and in phenol-free medium-1% FBS (C). Cell number was evaluated by direct cell counting after trypan blue staining. Each point represents the average of three separate cultures. The clones HEK^{TrkPC1} *a*, *c*, and *f* proliferate more rapidly than the controls. The clone HEK^{TrkPC1} *f*, with a low level of TrkPC1 receptor in the plasma membrane (Fig. 1), showed a weak increase in cell proliferation both in 10% and in 1% FBS medium. Bar diagrams represent cell proliferation at day 5 of cells cultured in 10% FBS (B), and at day 3 of cells cultured in 1% FBS (D). Bars represent the means and SEM of at least three different cell cultures. Growth rates of HEK^{TrkPC1} *a* and *c* clones were significantly higher than HEK^{Trk0} cells (***$P < 0.001$ and **$P < 0.01$). (E) NGF treatment inhibits the TrkPC1 induced increase in cell proliferation. HEK^{pCDNA3} (white bars), HEK^{Trk0} (dotted bars), and HEK^{TrkPC1} (hatched bars). Cells in triplicate were synchronized by 24 h starvation, and then cultured in presence of 1% FBS, with (+) and without (−) NGF (100 ng/ml). After 3 days, cells were collected and counted. Values represent means and SEM from three different experiments.

Fig. 2A, when cultured in 10% serum, HEK^{TrkPC1} clones *a*, *c*, and *f* (black circle, diamond and triangle, respectively) grew more than control clones (open symbols). At day 5 (Fig. 2B), the average cell number in HEK^{TrkPC1} clones *a*, *c*, and *f* was 2.18, 1.82, and 1.21 times higher than that of HEK^{Trk0} control clone, and there were statistically significant differences between values of clone *a*, *c*, and controls ($P < 0.001$ and $P < 0,01$, respectively). No statistically significant difference between HEK^{TrkPC1} clone *f* and controls was found. The differences in cell number of HEK^{TrkPC1} clones, therefore, reflected the degree of TrkPC1 expression at membrane level, shown in Fig. 1C, suggesting a direct relation between cell growth and TrkPC1 surface expression in stably transfected HEK293 cells.

The difference in cell proliferation between HEK^{TrkPC1} clone *a* or *c* and control clones was also evident in 1% serum cultures (Fig. 2C), at both day 3 and day 5, while the proliferation rate of the HEK^{TrkPC1} clone *f* (black triangles) was almost similar to that of control cells at both times. After 3 day culture (Fig. 2D), the average proliferation rate of HEK^{TrkPC1} clone a was 1.8 times higher than that of HEK^{Trk0}

control clone (161,000 ± 1500 vs. 89,000 ± 10,000 $P < 0.01$). This result showed that the Trk-PC1 induced increase in cell growth may be detectable after 3 days in presence of a minimal contribution of exogenous factors.

Since similar differences were detected in other three HEK^{TrkPC1} and two HEK^{Trk0} clones (data not shown), we performed the further experiments mainly in HEK^{TrkPC1} clone *a* and HEK^{Trk0} *i*, cultured in 1% serum.

Since in HEK^{TrkPC1} cells the PC1 C-terminus is anchored to the plasma membrane via transmembrane and extracellular sequences of Trk-A, the NGF receptor, we investigated whether the TrkPC1-dependent increase in cell proliferation could be due to NGF molecules in the serum. We found, on the contrary, that the addition of 100 ng/ml of NGF to HEK^{TrkPC1} clone *a* completely blunted the TrkPC1-induced effect, as shown in Fig. 2E. This result indicated that NGF had a growth suppressive effect on control clones and that the Trk-PC1 chimeric receptor did functionally interact with its ligand. Consequently, TrkPC1, by interacting with the endogenous TrkA through the extracellular domain region, as expected in ligand-induced homotypic interactions, could have blocked the growth suppressive effect of NGF present in 1% serum, and caused a proliferation increase with respect to HEK^{Trk0} and HEK^{pCDNA3} control clones. To investigate, therefore, whether Trk-PC1 expression disrupted the TrkA signaling, we monitored the TrkA tyrosine phosphorylation through analysis of a NGF dose–response relationship using antibodies as the readout (Fig. 3A). In absence of NGF, the same phospho-TrkA levels were detected in all clones, indicating a basal activation of the endogenous TrkA (Fig. 3B). In all cells exposed for 5 min to NGF at 5 and 50 ng/ml concentrations, phospho-TrkA levels did not significantly change in comparison to untreated cells, while they increased upon exposure to 100 ng/ml for 5, 10, and 15 min. In addition, after 10 and/or 15 min exposure, phospho-TrkA levels went down to basal levels. In HEK^{TrkPC1} and HEK^{Trk0} clones TrkA phosphorylation patterns were not different, while, upon 100 ng/ml exposure, they were lower than in the HEK^{pCDNA3} control clone (Fig. 3B). These results indicated a competitive activity of both chimeric receptors on TrkA for the NGF signal at the highest dose. Moreover, the similar phosphorylation patterns observed in all clones at 5 and 50 ng/ml of NGF indicated that TrkPC1 expression did not impair TrkA receptor activation at NGF low dose. Therefore, the NGF-negative effect on TrkPC1-dependent proliferation increase might have occurred in the absence of an antagonistic role on TrkA signal transduction. Nevertheless, these results suggested that the Trk-PC1 protein underwent a ligand-dependent modification losing its stimulatory effect on cell proliferation.

TrkPC1 expression triggers cell cycle progression

As a first step in defining the mechanism by which PC1 C-terminal tail induced the increase in cell proliferation, we compared the cell cycle dependent profile of stably transfected clones. As shown in Fig. 4A, we found that HEK^{TrkPC1} clone *a* had a higher percentage of cells accumulated in S phase, compared to HEK^{Trk0} control cells (34.43 ± 0.93 and 25.9 ± 1.13 in HEK^{TrkPC1} and HEK^{Trk0}, respectively), suggesting that the expression of PC1 tail activated the transition from G0/G1 to the S

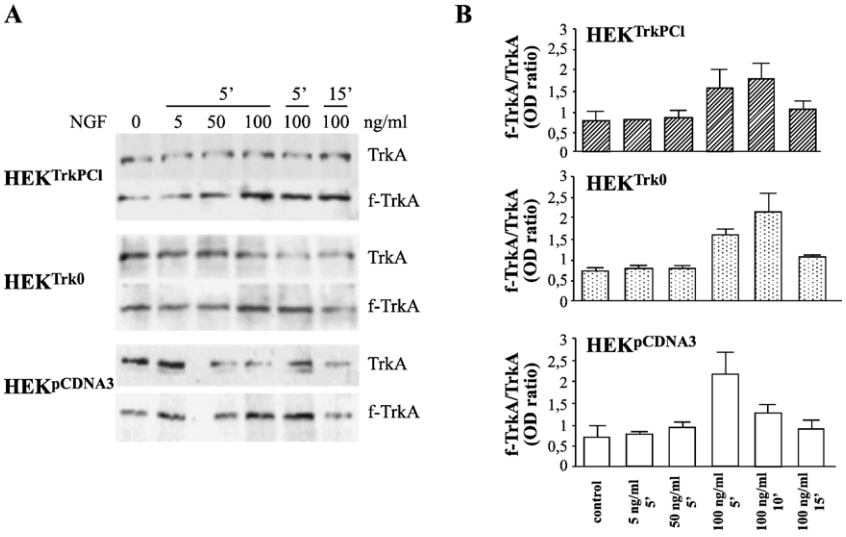

Fig. 3. NGF responsiveness of TrkA phosphorylation in transfected HEK293 clones. (A) Representative panels of TrkA protein expression (TrkA) and phosphorylation (f-TrkA) in HEK^{TrkPC1}, HEK^{Trk0} and HEK^{pCDNA3} clones. Cells were 24 h serum-starved and treated with NGF (5, 50, and 100 ng/ml, for 5 min (5′), and with 100 ng/ml for 10 and 15 min). Cell lysates were analyzed by Western blot. The phosphorylation of the receptor was detected by immunoblot using the anti-phospho-TrkA (f-TrkA) antibody (anti-Y490). The receptor expression levels were detected by reprobing the stripped membranes with the anti-TrkA polyclonal antibody (TrkA), as described in the method section. (B) Phospho-TrkA levels were normalized for the TrkA content. Histograms (mean ±SEM) show the normalized values in HEK^{TrkPC1} (hatched bars), HEK^{Trk0} (dotted bars) and HEK^{pCDNA3} (white bars), in three different experiments.

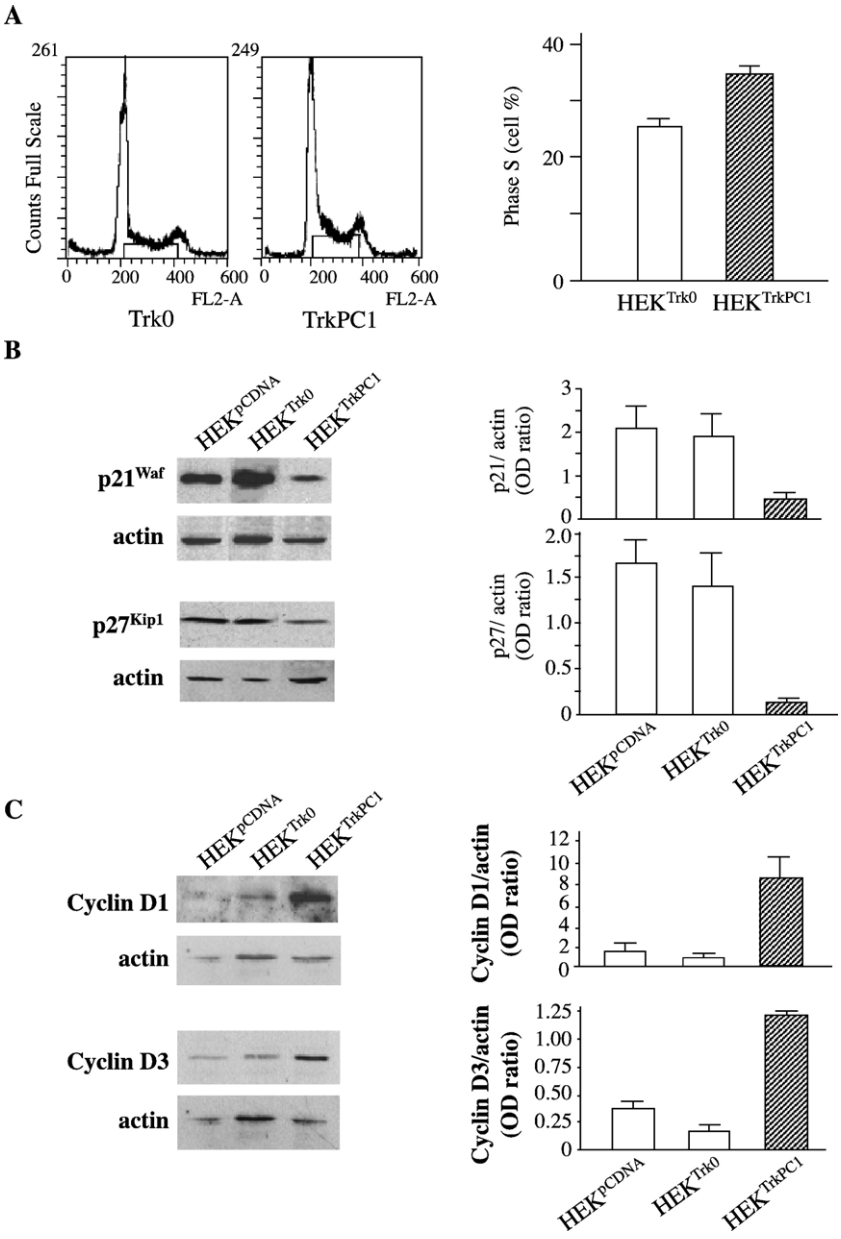

Fig. 4. TrkPC1 induces cell cycle progression, down-regulates CKIs and up-regulates D-type cyclins. (A) Cell cycle profile of HEK^{TrkPC1} and HEK^{Trk0} cells. Cells were synchronized by starvation and cultured in 1% serum for 12 h. Most of the fluorescent cells showed the GFP-PKCα and β membrane translocation (data not shown). (B) Whole cell lysates of HEK^{TrkPC1} and HEK^{Trk0} cells were analyzed by immunoblot for expression levels of $p21^{Waf1}$ and $p27^{Kip1}$ CKIs and (C) cyclins D1 and D3. Expression levels were normalized for the expression of actin housekeeping gene. Histograms show the mean values ±SEM from at least three different experiments.

phase. Similar values were found with different TrkPC1 clones (data not shown).

Since the cell cycle progression is regulated by an ordered sequence of events requiring a coordinate expression of cyclins, cyclin-dependent kinases (CDKs), and their inhibitors (CKIs), we searched for expression differences in cell cycle-related proteins between HEK^{TrkPC1} and HEK^{Trk0} or HEK^{pCDNA3} control clones.

Western blotting analysis of total lysates from synchronized cell lines, grown in 1% FBS phenol-free medium, showed a significant reduction in expression levels of $p21^{waf1}$ and, even more, of $p27^{kip1}$ in HEK^{TrkPC1} clone *a* compared to control clones (Fig. 4B). In particular, in HEK^{TrkPC1} clone *a*, there was an approximately 5 and 8 time reduction in $p21^{waf1}$ and $p27^{kip1}$, respectively, in comparison with HEK^{Trk0} cells; similar differences were found in comparison with HEK^{pCDNA3} cells. In agreement with the reduction in CKI levels, analysis of D1 and D3 cyclin expression (Fig. 4C) showed that HEK^{TrkPC1} clone *a* and *c* had higher D1 cyclin levels than those found in control

clones (there was an approximately 7 fold increase in HEK^{TrkPC1} clone *a* versus HEK^{Trk0} cells, $P < 0.05$). HEK^{TrkPC1} clone *a* cells had D3 cyclin levels approximately six times higher than HEK^{Trk0} cells. No significant modifications were observed in CDK4 and CDK6 expression levels between the different clones (data not shown).

PKCα activation is involved in TrkPC1-dependent cell proliferation

There is evidence that protein kinase C (PKC) activation is required for changes in cell cycle machinery in early stages [40]. Therefore, to determine the upstream events that modified the cell cycle in TrkPC1 expressing

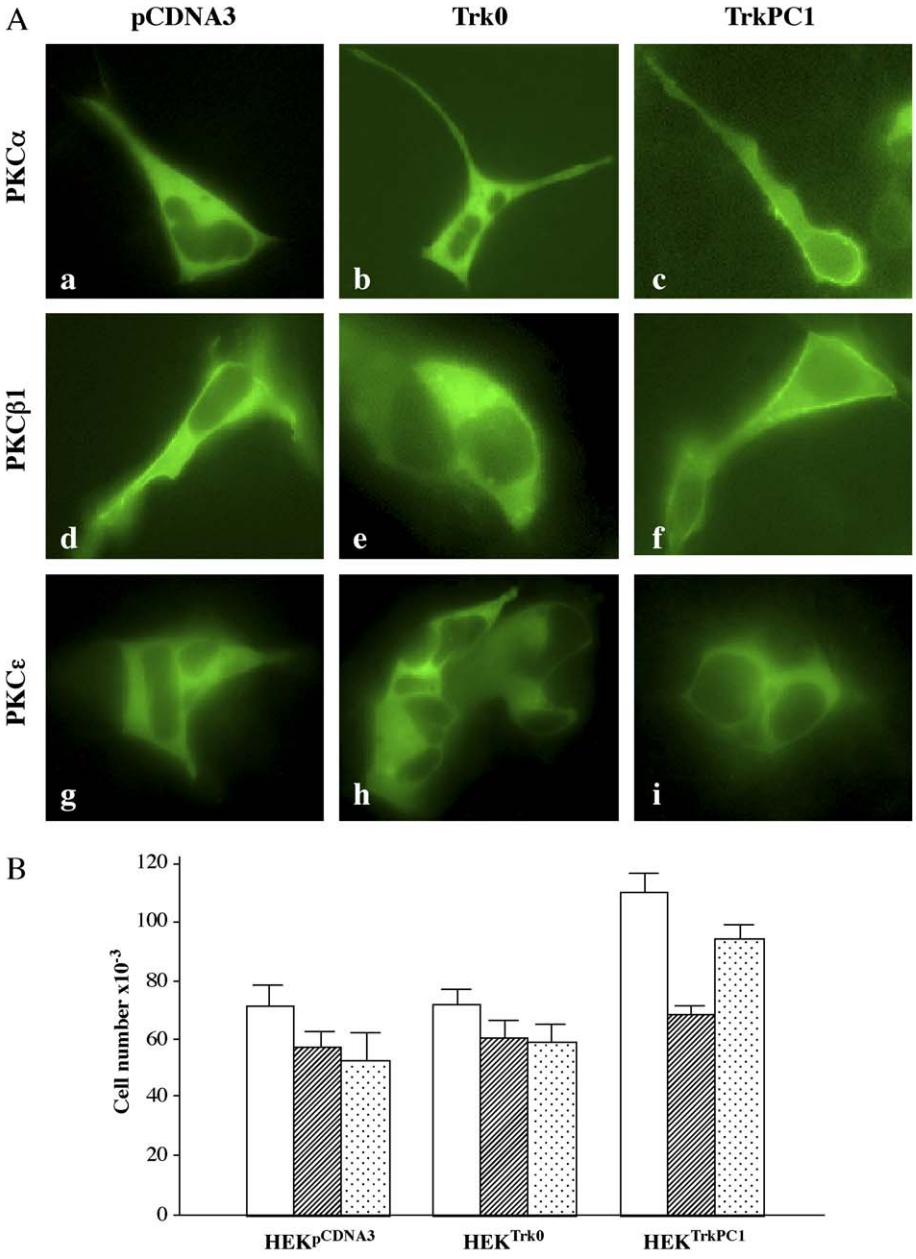

Fig. 5. The TrkPC1-induced increase in cell proliferation is dependent on PKCα activation. (A) HEK293 cells were transiently co-transfected with PKCα-GFP (*a*, *b*, *c*) or PKCβ$_1$-GFP (*d*, *e*, *f*) and PKCϵ-GFP (*g, h, i*) and with pCDNA3, Trk0 and TrkPC1 constructs, respectively. Images of GFP-positive cells were taken by digital microscope imaging. One percent FBS cultured cells showed that PKCα and β$_1$ localize to the plasma membrane in TrkPC1 transfected cells only (*c*, *f*). Most of the fluorescent cells showed the GFP-PKCα and β membrane translocation (data not shown). Control cells, and PKCϵ-GFP/TrkPC1 co-transfected cells showed a cytoplasm diffuse staining (*a*, *b*, *d*, *e*, *g*, *h*, and *i*). (B) HEK^{pCDNA3}, HEK^{Trk0}, and HEK^{TrkPC1} cells were cultured in triplicate in absence (white bars) or in presence of either the PKCα inhibitor Ro-320432 (hatched bars) or the PKCβ$_1$ inhibitor hispidin (dotted bars). After 3 days, cells were collected and counted. Values represent means from at least three different experiments ± SEM.

clones, we analyzed PKC activation by digital fluorescence microscope imaging of transiently TrkPC1 and GFP-PKCα co-transfected HEK293 cells. This analysis showed that in TrkPC1 co-transfected cells (Fig. 5A, picture c), there was a prominent recovery of GFP fluorescence in the plasma membrane. However, in pCDNA3 and Trk0 transfected cells, there was a diffuse fluorescence (Fig. 5A, pictures a and b), indicating that PKCα remained confined to the cytoplasm. This suggested that the TrkPC1 expression activated the PKCα isoform, triggering its recruitment to the plasma membrane. Similar results were obtained by co-transfecting HEK293 cells with GFP-PKCβ$_1$ isoform, which showed a marked green staining at the plasma membrane in the TrkPC1 transfected cells (Fig. 5A, picture f). When cells were co-transfected with the Ca^{2+}-insensitive PKCε isoform, no variations were observed between TrkPC1 and transfected control cells (Fig. 5A, pictures g, h, and i). A marked staining at the plasma membrane was also observed by transfecting GFP-PKCα and β$_1$ isoforms in stably transfected HEK^{TrkPC1}, but not in HEK^{Trk0} and HEK^{pCDNA3} clones (data not shown). These findings, and our previous observations demonstrating that TrkPC1 is associated to a cytoplasmic Ca^{2+} increase in transiently transfected HEK-293 cells [32], strongly indicate a Ca^{2+} involvement in the TrkPC1-dependent PKCα and PKCβ$_1$ activation.

To clarify the PKCα and PKCβ$_1$ involvement in cell proliferation increase observed in HEK^{TrkPC1} clones, we tested the effects of PKC-specific inhibitors on cell growth. In cells cultured for 48 h in presence of the PKCα-specific inhibitor Ro-320432 (hatched bars of Fig. 5B), a small reduction in cell proliferation was observed in HEK^{Trk0} and HEK^{pCDNA3} clones, while, in HEK^{TrkPC1} cells, a marked reduction in proliferation was detected (cell proliferation reached values not significantly different from that of HEK^{Trk0} control clone). Thus, PKCα inhibition appeared to abolish the stimulatory effect of TrkPC1 on proliferation to the extent of almost restoring the cell growth pattern observed in control cells. Interestingly, the treatment with hispidin, a PKCβ-specific inhibitor (dotted bars of Fig. 5B), produced a small reduction in cell proliferation both in HEK^{TrkPC1} clones, as well as in the controls. These data suggest that the Ca^{2+}-sensitive PKCα, but not the PKCβ1 isoform, is involved in the TrkPC1-dependent proliferation increase.

The TrkPC1-dependent PKCα membrane distribution is growth factor dependent

In order to discriminate in TrkPC1 transfected cells between a PKCα constitutive activation and a ligand-dependent activation, we analyzed the PKCα subcellular distribution in the presence or absence of external factors such as 1% FBS. This FBS concentration was the same used in experiments showing the TrkPC1-dependent increase in cell proliferation in stably transfected cells. In transiently PKCα co-transfected HEK293 cells cultured for 24 h in the presence of BSA instead of FBS (FBS-free), microscope analysis showed a PKCα cytoplasmic distribution in both TrkPC1 and Trk0 co-transfected cells (Fig. 6A, pictures a and e). Interestingly, the addition of 1% FBS to the medium during the microscope imaging, rapidly triggered PKCα plasma membrane translocation in TrkPC1-transfected cells (Fig. 6A, pictures f, g, and h), that was detectable after 30 s (Fig. 6A, pictures f), but not in control cells (Fig. 6A, pictures b, c, and d) in the 2-min observation (Fig. 6A, pictures d). These observations indicate that, in TrkPC1 transfected cells, the GFP-PKCα activation requires the presence of one or more factors present in FCS.

Similar results were obtained by transfecting with GFP-PKCα stably transfected HEK^{TrkPC1}, HEK^{Trk0}, and HEK^{pCDNA3} clones. After 24 h starvation, PKCα was localized in the cytoplasm of HEK^{TrkPC1}, as well as of control clones (Fig. 6B, pictures a and d). However, after 1% FBS addition, PKCα localized at the plasma membrane in HEK^{TrkPC1} clones only, and this localization persisted over the 10 min of observation (Fig. 6B, pictures e and f). Interestingly, serum addition also induced cell shape modifications compatible with cytoskeleton changes. No effect was observed in serum-treated HEK^{pCDNA3} clones either (data not shown). These data suggest that some growth factor/s in FCS are responsible for the TrkPC1-dependent PKCα activation, and therefore that TrkPC1 mediates an increase in serum-evoked intracellular Ca^{2+} levels.

TrkPC1 enhances serum -induced cytoplasmic Ca^{2+} levels

In order to assess a putative TrkPC1-dependent effect on serum-evoked intracellular Ca^{2+} levels, we co-transfected HEK-293 cells with either TrkPC1 or Trk0 chimeric construct and with the cytoplasmic aequorin cDNA. The results of a typical experiment are shown in Fig. 7A. The addition of 1% FBS increased Ca^{2+} levels to a maximal peak value of 0.52 μM in TrkPC1 and 0.21 μM in Trk0-transfected control cells; in agreement with our previous data [32], the ATP addition increased Ca^{2+} levels to a peak value of 1.32 and 0.97 μM in TrkPC1 and Trk0 transfected control cells, respectively. Serum-evoked Ca^{2+} levels from different experiments are summarized in histograms of Fig. 7B. The average elevation of Ca^{2+} levels was significantly greater in TrkPC1 transfected cells, as compared with Trk0 transfected control cells (0.4 ± 0.042 μM in TrkPC1 vs. 0.19 ± 0.026 μM in Trk0, $P < 0.01$).

Moreover, to investigate whether cell calcium was increased in TrkPC1 stably transfected cells under the condition where increased proliferation was evident, basal cytoplasmic Ca^{2+} concentrations were measured in HEK^{TrkPC1} and HEK^{Trk0} cells, grown in 1% serum and loaded with Fura-2A. In the experiment presented in Fig. 7C, the basal Ca^{2+} concentration in HEK^{TrkPC1} cells was

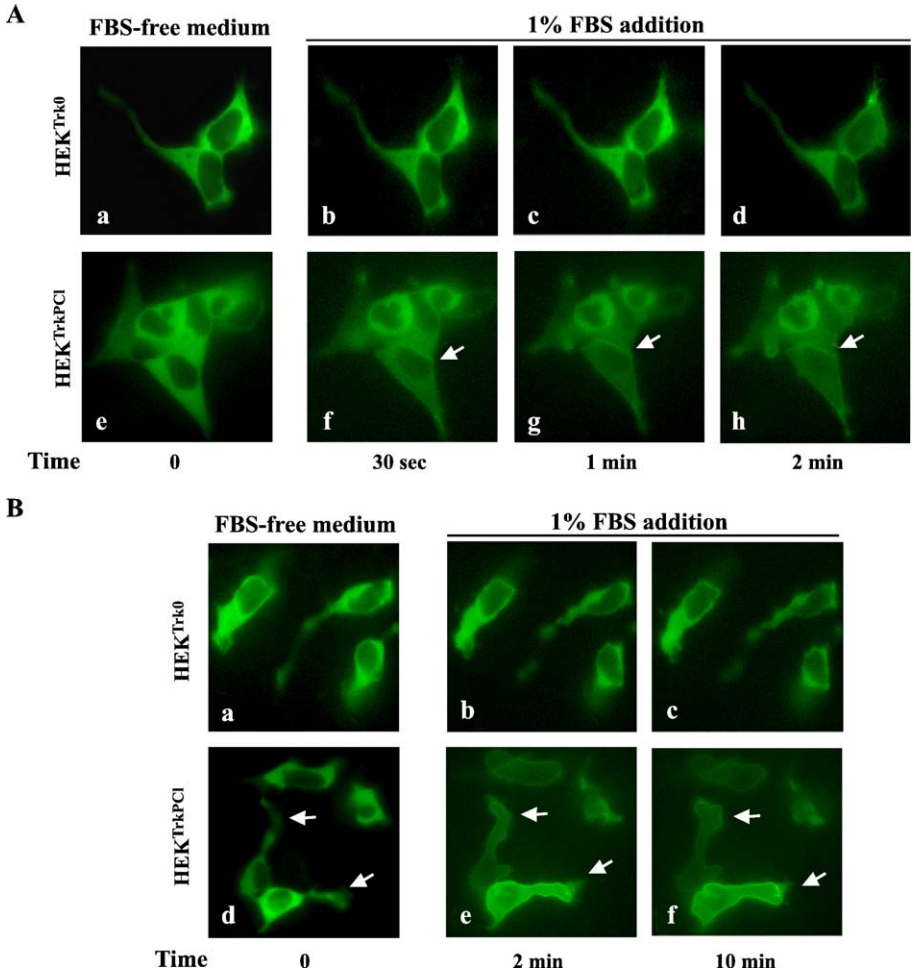

Fig. 6. The TrkPC1-induced PKCα membrane localization requires serum. HEK293 cells transiently co-transfected with PKCα-GFP and with either Trk0 or TrkPC1 constructs, respectively, before (*a*, *e*) and after the addition of 1% serum (*b*, *c*, *d*, *f*, *g*, *h*). (B) HEK^{Trk0} and HEK^{TrkPC1} cells transfected with PKCα-GFP before (time 0, *a* and *d*) and after the addition of 1% serum (5 min -*b, e*- and 10 min -*c*, *f*-, respectively). Arrows indicate membrane regions forming sprout like structures after serum addition.

higher than in HEK^{Trk0} control cells (0.162 ± 0.044 μM in HEK^{TrkPC1} vs. 0.062 ± 0.018 μM in HEK^{TrkPC1}, $P < 0.05$), and showed a slightly oscillating pattern. These findings indicate that the TrkPC1 expression induces an increase in serum-dependent intracellular Ca^{2+} levels, which are presumably appropriate to translocate to the plasma membrane and activate PKCα, as shown in Fig. 6.

TrkPC-1 triggers the ERK1/2 activation

The observation that the TrkPC1-induced increase in cell proliferation in HEK^{TrkPC1} cells was dependent on Ca^{2+}-sensitive PKCα activation, which, in its turn, was dependent on the addition of serum factor/s, led us to investigate, in these cells, the expression and possible activation of ERK1/2, the classical extracellular signal-regulated kinase. Western blot analysis (Fig. 8A) did not show significant differences in ERK1/2 expression levels in all cell lines, however, a significant increase in phosphorylated ERK1/2 was detected in HEK^{TrkPC1} cells compared to control cells (12% vs. 3% average values), indicating a major activation of ERK signaling in HEK^{TrkPC1} cells.

To further explore the role of this signaling on the TrkPC1-dependent proliferation, we examined the effect of the MEK1 inhibitor PD98059, and consequently of ERK, a MEK1 target, on the proliferation of our stably transfected clones. As shown in Fig. 8B, the PD98059 treatment abolished the difference in cell proliferation between HEK^{TrkPC1} and control clones, thus confirming that the TrkPC1 signaling requires MEK and ERKs activation as downstream effectors in HEK293 cells.

Discussion

Based on recent evidence, it has been proposed that PC1 modulates G-protein activation and PC2 channel function and, consequently, transduces signals from the extracellular environment to the cell in order to influence cell prolifer-

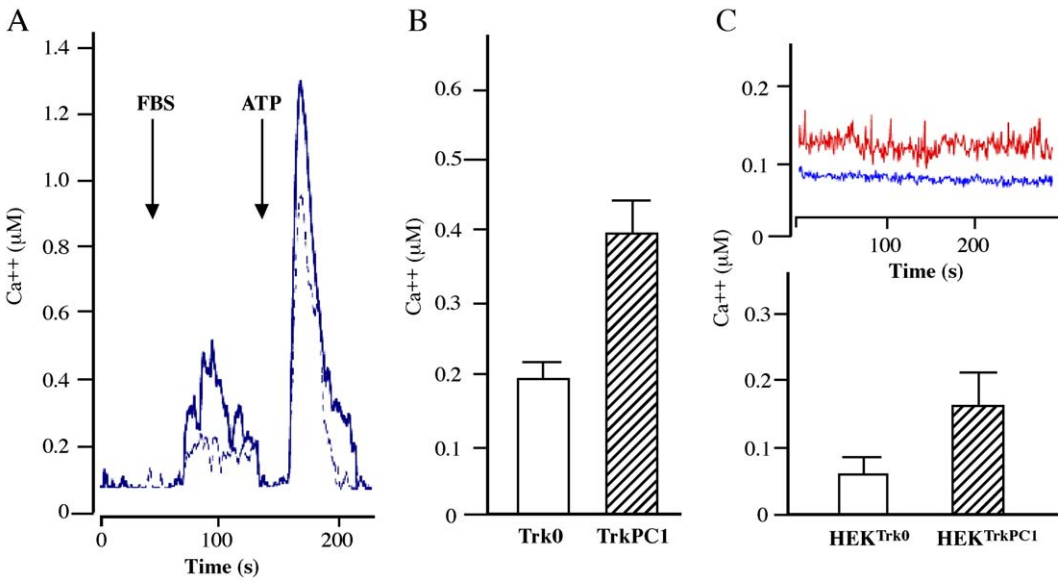

Fig. 7. TrkPC1 enhances the serum-induced cytoplasmic Ca^{2+} levels. (A) Representative traces of serum- (FBS) and ATP-evoked increases in cytoplasmic Ca^{2+} concentrations in TrkPC1 (bold line) and Trk0 (hatched line) transiently transfected cells. Cells grown on coverslips were co-transfected with either Trk0 or TrkPC1 and cytoplasmic aequorin, as described under Materials and methods. After coelenterazine reconstitution, cells were perfused in KRB buffer, stimulated with 1% FBS, and subsequently with 100 μM ATP. The Ca^{2+} concentration was determined by a luminometer. (B) Summary of response to the serum. Data (mean ±SEM) refer to serum-evoked maximal peak values in at least four different experiments. (C) Basal cytoplasmic Ca^{2+} concentrations ($[Ca^{2+}]_c$) in HEK^{Trk0} and HEK^{TrkPC1} cells stably expressing the Trk0 and TrkPC1 constructs. Cells were grown in 1% FBS on coverslips, then loaded with Fura-2AM and analyzed as reported in Materials and methods. The representative traces show an example of $[Ca^{2+}]_c$ in HEK^{Trk0} (lower line) and HEK^{TrkPC1} (upper line) cells. The $[Ca^{2+}]_c$ value relative to each trace was the average of all recorded data. Histograms show the mean ± SEM from values collected in various images in at least two experiments in duplicate.

ation, differentiation, and morphogenesis [16,24]. In the present work, we show that the membrane-targeted chimeric TrkPC1 receptor, containing the PC1 cytoplasmic tail, can induce an increase in cell proliferation in stably transfected HEK^{TrkPC1} clones, an effect which depends on PKCα and serum as a parallel and essential component. The degree of TrkPC1-dependent growth stimulation correlates with the surface expression of the fusion protein and was associated with an increase in cell cycle progression by cell transition from G0/G1 into S phase. This is confirmed by the reduction observed in the expression of $p21^{waf1}$ and $p27^{kip1}$ cyclin-dependent kinase inhibitors, and by the increase in D-type cyclins, including D3 and mainly D1cyclin. In fact, the latter is known to control the onset of cell cycle progression [41] and its promoter is one of the major targets for several growth stimulatory signaling pathways [42,43].

Since there is evidence that PKCα and PKCϵ are potent activators of D1 cyclin promoter [40], and PKCα is activated by a variety of stimuli, including binding signals to guanine-nucleotide-binding protein-coupled receptors [44,45], putatively including the PC1 protein [24], we assume that TrkPC1-induced increase in cell proliferation may depend on PKCα activation. The presence of GFP-PKCα and GFP-$PKC\beta_1$ at the plasma membrane in HEK^{TrkPC1} clones, but not in HEK^{Trk0} and HEK^{pCDNA3} clones, and in transiently TrkPC1 transfected cells, points to the activation of the α and β isoforms in these cells. It is known that upon activation, PKCα rapidly translocates from the cytoplasm to the specialized cellular compartments where it is presumed to act [46,47]. However, the suppression of the TrkPC1-induced effect on cell growth by the PKCα-specific inhibitor Ro-320432, but not by the $PKC\beta_1$-specific inhibitor hispidin, strongly supports the concept that TrkPC1 regulates cell proliferation in these clones through the PKCα isoform. Our findings on the TrkPC1-dependent increase in cell proliferation mediated by PKCα support previous studies which have shown, by different experimental procedures, that the PC1 cytoplasmic tail induces PKCα activation in HEK293 cells [32]. No information on cell proliferation was provided from these studies.

To the best of our knowledge, we are the first to demonstrate that the TrkPC1-dependent PKCα activation is not constitutive, but, interestingly, serum factor dependent. In fact, in serum-free conditions, both stably and transiently TrkPC1-transfected cells, as well as control cells, show a cytoplasmic distribution of PKCα, which, only after serum addition and only in TrkPC1-transfected cells, translocates to the plasma membrane.

That serum factor(s) are required for the TrkPC1-dependent PKCα activation is also confirmed by the association observed between TrkPC1-induced cellular effects and the activation of the classical extracellular signal-regulated kinase, ERK1/2. This kinase is responsive to tyrosine kinases and also to G protein-coupled receptors

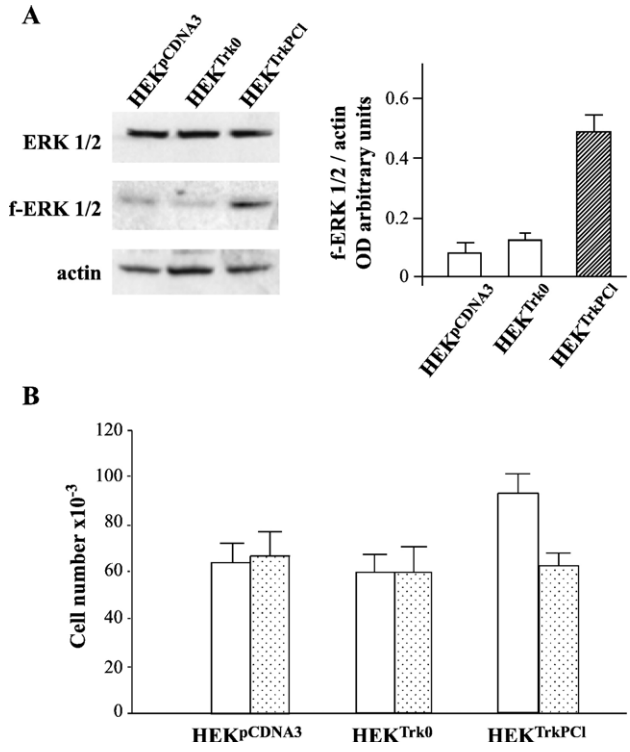

Fig. 8. TrkPC1 enhances the level of phospho-ERK1/2. (A) Whole cell lysates of HEK^{TrkPC1} and HEK^{Trk0} cells were analyzed by immunoblot for expression levels of ERK and phospho-ERK (f-ERK). Expression levels were normalized for the actin content. Histograms (mean ± SEM) show the normalized protein content in transfected clones in three different experiments. (B) HEK^{pCDNA3}, HEK^{Trk0}, and HEK^{TrkPC1} cells were grown in triplicate in the absence (white bars) or in the presence of the MEK1 inhibitor PD98059 (dotted bars). After 2 days, cells were collected and counted. Values (mean ± SEM) represent means from three different experiments.

through PKA, B-Raf, and MEK [48,49], but also through PKCα, Raf1, and MEK [50].

The TrkPC1-dependent increase in ERK1/2 activity found in our HEK^{TrkPC1} clones is in apparent contrast with previous data found in HEK293 cells transiently co-transfected with p44^{ERK1} and PC1 intracellular tail [22,51]; an increase in p44^{ERK1} activity was not observed, but co-transfection assays were performed in 16 h serum-starved cells.

We are able to rule out the possibility of a role for extracellular and transmembrane regions of Trk-A in the TrkPC1-dependent increase in cell proliferation, and PKCα and ERK1/2 activation because these effects are not found in HEK^{Trk0} control clones, which expressed the Trk0 chimeric receptor with the same TrkA extracellular and transmembrane sequences at the plasma membrane. Furthermore, the addition of NGF, the TrkA ligand, does not increase cell proliferation, but, on the contrary, reduces it in HEK^{pCDNA} and HEK^{Trk0} control clones and even more so in HEK^{TrkPC1} clones. As occurs in other epithelial cells [52], in HEK293 cells, NGF may induce a certain degree of cell differentiation by interacting with endogenously expressed TrkA receptor. In HEK^{TrkPC1} and HEK^{Trk0} cells such an effect takes place in presence of a transient antagonistic role on NGF-induced TrkA phosphorylation detected at the 100 ng/ml concentration, indicating that the interaction between the endogenous and the chimeric receptors do not markedly impair the NGF-signal. Nevertheless, since the NGF-induced reduction in cell proliferation was stronger (approximately 50%) in HEK^{TrkPC1} clones than the one found in control clones (approximately 25%), a ligand-dependent modification of TrkPC1 receptor could be hypothesized, making the PC1 domain unable to support the parallel serum-dependent increase in cell proliferation. We propose that, in absence of NGF, TrkPC1 is able to subtract functional partners to endogenous PC1, thus removing its growth-suppression activity [6,31], and that this interaction is reverted by the NGF binding to the chimera. This ligand-dependent effect is supported by the observation that, in NGF-treated HEK^{TrkPC1} clones, PKCα lost the ability to translocate from the cytoplasm to the plasma membrane (data not shown).

Since the PKCα-specific inhibitor blocks the TrkPC1-induced cell proliferation, it is presumable that Ca^{2+} is the upstream effector of the TrkPC1-induced activation of PKCα and cell proliferation. This suggestion is supported by (a) previous data reporting that intracellular Ca^{2+} concentrations and PKCα translocation in single cells are well correlated [46,47], (b) by our previous findings that in transiently transfected cells TrkPC1 induces a significant increase in ATP-evoked cytoplasmic Ca^{2+} levels [32], and c) by the TrkPC1-induced increase in serum-evoked cytoplasmic Ca^{2+} levels we have reported above. In this connection, we have demonstrated that the TrkPC1-dependent PKCα activation is serum-dependent through an increase in cytoplasmic Ca^{2+} levels. This leads us to the conclusion that Ca^{2+} and PKCα are the crucial downstream effectors for the PC1 tail effects on cell proliferation. This conclusion is supported by the observed association between serum-evoked intracellular Ca^{2+} and cell proliferation [53] mediated by a Ca^{2+}-dependent transcription of the c-*fos* oncogene [54], that indicates how short-lived intracellular signals cause alteration in gene expression leading to long-term cellular responses. Therefore, the TrkPC1-induced increase in serum-evoked calcium appears to be the first step in a sequence of events (phosphorylation, gene transcription, etc) that determine the observed increase in cell proliferation.

The role of intracellular Ca^{2+} levels in PKCα activation is of considerable interest, taking into consideration the ability of the PC1 tail to interact with PC2 [10,11], which is a Ca^{2+} permeable cation channel [13,14], capable of interacting, possibly, with other Ca^{2+} channels like TRPC1 [55]. In the present study, we are unable to predict the origin of the cytoplasmic Ca^{2+} increase. It is possible that undefined factors in 1% serum through their receptors cause a small increase in IP3 which, in turn, produces a Ca^{2+} release from internal stores that is higher in clones expressing TrkPC1, similarly to what we observed in

ATP-evoked cytoplasmic Ca^{2+} increase in TrkPC1 transiently transfected cells [32]. TrkPC1, by interacting with PC2 in a TrkPC1/PC2 complex, could trigger a comparable number of PC2 channels which, either as intracellular Ca^{2+} release channels [14] or as transient potential receptor channels on plasma membranes [56], produce an increase in cytoplasmic Ca^{2+} suitable to translocate PKCα from the cytoplasm to other cellular compartments, where it could act on substrates involved in ERK signalling pathways. In addition, TrkPC1 may interfere with the function of the endogenous full-length PC1, for instance by reducing its interaction with channel proteins or their regulators. This proposal is supported by a recent observation that in distal renal tubule cells the loss of PC1 function resulted in an increased cell Ca^{2+}, suggesting a role for PC1 in down-regulating agonist-stimulated intracellular Ca^{2+} increase [21]. Moreover, TrkPC1 may compete for the interaction with proteins involved in the JAK-STAT signal transduction pathway [57] (including PC2), resulting in down regulation of p21 expression, leading to an increase in cell proliferation. The growth suppressive effect of endogenous PC1 is also supported by a recent observation showing that the antisense oligonucleotide-mediated depletion of PKD1 expression increases cell proliferation rate [58].

The TrkPC1-induced increase in cell cycle progression and cell proliferation observed in our HEK293 clones is in agreement with a previous observation concerning the change in c-AMP-modulated growth properties of mouse cortical collecting duct cell lines stably expressing the mouse PC1 tail [29]. Unlikely control cell lines, the growth of PC1 overexpressing cells was stimulated by cAMP through PKA, and involved signaling pathways through the mitogen-activated MEK1 [59]. This observation was further supported by findings showing that cAMP activates ERK and increases cell proliferation in ADPKD epithelial cells, but not cells from normal human cortex [60].

The observation that clones overexpressing the C-terminal PC1 behave like human ADPKD cells provides evidence that a loss or gain of PC1 function is directly responsible for the abnormal cell growth phenotype in human ADPKD [29], and for the polycystic kidneys found in mice expressing the human PC1 transgene [30]. Conversely, it has been reported that the overexpression of the full-length PC1 in stably transfected MDCK cells leads to resistance to apoptosis, a decrease in proliferation, and promotion of tubulogenesis through the activation of the JAK-STAT signal transduction pathway and an increase in the p21 CKI [57]. It is, therefore, possible that different genomic or cDNA constructs in different cells have different cellular effects.

In conclusion, our data indicate that the intracellular C-terminal tail of PC1 selectively upregulates, in human HEK293 kidney epithelial cells, a serum factor-dependent cell proliferation signaling, mediated by the combined activation of the Ca^{2+}-dependent PKCα isoform and extracellular signal-regulated kinase ERK1/2. These data support a tentative model for different signaling pathways in which PKCα acts downstream the PC1–PC2 complex and upstream the activation of ERK and transcriptional activation of cyclin D1. Since the formation of cysts in ADPKD occurs by "turning on" specific signaling pathways involving cell growth, the pathways described above may be a promising target for therapeutic agents in the treatment of renal cystic diseases caused by an altered function of the PC1/PC2 complex.

Acknowledgments

We thank Prof. R. Gavioli (University of Ferrara) for providing us with anti-CDK and -CKI antibodies, and Dr. G. Szabadkai for the support in Fura-2 measurements. This work was supported by grants from Italian Telethon (E 1250, 1285, and GTF01011), Italian MIUR COFIN 2002, Cassa di Risparmio di Ferrara, University's Local Funds (ex-60%), Human Frontiers Science Program (Grant RGP0347) and the Italian Association for Cancer Research.

References

[1] P.A. Gabow, Autosomal dominant polycystic kidney disease: more than a renal disease, Am. J. Kidney Dis. 16 (1990) 403–413.
[2] J.J. Grantham, Mechanisms of progression in autosomal dominant polycystic kidney disease, Kidney Int., Suppl. 630 (1997) S93–S97.
[3] M. Trudel, L. Barisoni, J. Lanoix, V. D'Agati, Polycystic kidney disease in SBM transgenic mice: role of c-*myc* in disease induction and progression, Am. J. Pathol. 152 (1998) 219–229.
[4] K. Ramasubbu, N. Gretz, S. Bachmann, Increased epithelial cell proliferation and abnormal extracellular matrix in rat polycystic kidney disease, J. Am. Soc. Nephrol. 9 (1998) 937–945.
[5] P.C. Harris, Autosomal dominant polycystic kidney disease: clues to pathogenesis, Hum. Mol. Genet. 8 (1999) 1861–1862.
[6] M. Sutters, G.G. Germino, Autosomal dominant polycystic kidney disease: molecular genetics and pathophysiology, J. Lab. Clin. Med. 141 (2003) 91–101.
[7] J. Hughes, C.J. Ward, B. Peral, R. Aspinwall, K. Clark, J.L. San Millan, V. Gamble, P.C. Harris, The polycystic kidney disease 1 -(PKD1) gene encodes a novel protein with multiple cell recognition domains, Nat. Genet. 10 (1995) 151–160.
[8] C.P. Ponting, K. Hofmann, P. Bork, A latrophilin/CL-1-like GPS domain in polycystin-1, Curr. Biol. 9 (1999) 585–588.
[9] B.S. Weston, A.N. Malhas, R.G. Price, Structure-function relationships of the extracellular domain of the autosomal dominant polycystic kidney disease-associated protein, polycystin-1, FEBS Lett. 558 (2003) 8–13.
[10] L. Tsiokas, E. Kim, T. Arnould, V.P. Sukhatme, G. Walz, Homo- and heterodimeric interactions between the gene products of PKD1 and PKD2, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 6965–6970.
[11] F. Qian, F.J. Germino, Y. Cai, X. Zhang, S. Somlo, G.G. Germino, PKD1 interacts with PKD2 through a probable coiled-coil domain, Nat. Genet. 16 (1997) 179–183.
[12] T. Mochizuki, G. Wu, T. Hayashi, S.L. Xenophontos, B. Veldhuisen, J.J. Saris, D.M. Reynolds, Y. Cai, P.A. Gabow, A. Pierides, W.J. Kimberling, M.H. Breuning, C. Constantinou Deltas,

D.J.M. Peters, S. Somlo, PKD2, a gene for polycystic kidney disease that encodes an integral membrane protein, Science 272 (1996) 1339–1342.
[13] P.M. Vassilev, L. Guo, X.Z. Chen, Y. Segal, J.B. Peng, N. Basora, H. Babakhanlou, G. Cruger, M. Kanazirska, C. Ye, E.M. Brown, M.A. Hediger, J. Zhou, Polycystin-2 is a novel cation channel implicated in defective intracellular Ca(2+) homeostasis in polycystic kidney disease, Biochem. Biophys. Res. Commun. 282 (2001) 341–350.
[14] P. Koulen, Y. Cai, L. Geng, Y. Maeda, S. Nishimura, R. Witzgall, B.E. Ehrlich, S. Somlo, Polycystin-2 is an intracellular calcium release channel, Nat. Cell Biol. 4 (2002) 191–197.
[15] L.J. Newby, A.J. Streets, Y. Zhao, P.C. Harris, C.J. Ward, A.C. Ong, Identification, characterization, and localization of a novel kidney polycystin-1–polycystin-2 complex, J. Biol. Chem. 277 (2002) 20763–20773.
[16] M. Lakkis, J. Zhou, Molecular complexes formed with polycystins, Nephron Exp. Nephrol. 93 (2003) e3–e8.
[17] S. Somlo, B. Ehrlich, Human disease: calcium signalling in polycystic kidney disease, Curr. Biol. 11 (2001) R356–R360.
[18] G. Stayner, J. Zhou, Polycystin channels and kidney disease, Trends Pharmacol. Sci. 22 (2001) 543–546.
[19] K. Hanaoka, F. Qian, A. Boletta, A.K. Bhunia, K. Piontek, L. Tsiokas, V.P. Sukhatme, W.B. Guggino, G.G. Germino, Co-assembly of polycystin-1 and -2 produces unique cation-permeable currents, Nature 408 (2000) 990–994.
[20] G.M. Xu, S. Gonzalez-Perrett, M. Essafi, G.A. Timpanaro, N. Montalbetti, M.A. Arnaout, H.F. Cantiello, Polycystin-1 activates and stabilizes the polycystin-2 channel, J. Biol. Chem. 278 (2003) 1457–1462.
[21] S.M. Nauli, F.J. Alenghat, Y. Luo, E. Williams, P. Vassilev, X. Li, A.E. Elia, W. Lu, E.M. Brown, S.J. Quinn, D.E. Ingber, J. Zhou, Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells, Nat. Genet. 33 (2003) 129–137.
[22] T. Arnould, E. Kim, L. Tsiokas, F. Jochimsen, W. Gruning, J.D. Chang, G. Walz, The polycystic kidney disease 1 gene product mediates protein kinase C alpha-dependent and c-Jun N-terminal kinase-dependent activation of the transcription factor AP-1, J. Biol. Chem. 273 (1998) 6013–6018.
[23] E. Kim, T. Arnould, L.K. Sellin, T. Benzing, M.J. Fan, W. Gruning, S.Y. Sokol, I. Drummond, G. Walz, The polycystic kidney disease 1 gene product modulates Wnt signaling, J. Biol. Chem. 274 (1999) 4947–4953.
[24] P. Delmas, H. Nomura, X. Li, M. Lakkis, Y. Luo, Y. Segal, J.M. Fernandez-Fernandez, P. Harris, A.M. Frischauf, D.A. Brown, J. Zhou, Constitutive activation of G-proteins by polycystin-1 is antagonized by polycystin-2, J. Biol. Chem. 277 (2002) 11276–11283.
[25] S.C. Parnell, B.S. Magenheimer, R.L. Maser, C.A. Zien, A.M. Frischauf, J.P. Calvet, Polycystin-1 activation of c-Jun-N-terminal kinase and AP-1 is mediated by heterotrimeric G proteins, J. Biol. Chem. 277 (2002) 19566–19572.
[26] T. Arnould, L. Sellin, T. Benzing, L. Tsiokas, H.T. Cohen, E. Kim, G. Walz, Interaction between RGS7 and polycystin, Proc. Natl. Acad. Sci. U. S. A. 2596 (1999) 6371–6376.
[27] S.C. Parnell, B.S. Magenheimer, R.L. Maser, J.P. Calvet, Polycystin-1 activation of c-Jun N-terminal kinase and AP-1 is mediated by heterotrimeric G proteins, Biochem. Biophys. Res. Commun. 259 (1999) 539–543.
[28] P.D. Wilson, L. Geng, X. Li, C.R. Burrow, The PKD1 gene product, polycystin-1, is a tyrosine-phosphorylated protein that colocalizes with alpha2beta1-integrin in focal clusters in adherent renal epithelia, Lab. Invest. 79 (1999) 1311–1323.
[29] M. Sutters, T. Yamaguchi, R.L. Maser, B.S. Magenheimer, P.L. St. John, D.R. Abrahamson, J.J. Grantham, J.P. Calvet, Polycystin-1 transforms the cAMP growth-responsive phenotype of M-1 cells, Kidney Int. 60 (2001) 484–494.
[30] L. Pritchard, J.A. Sloane-Stanley, J.A. Sharpe, R. Aspinwall, W. Lu, V. Buckle, L. Strmecki, D. Walker, C.J. Ward, C.E. Alpers, J. Zhou, W.G. Wood, P.C. Harris, A human PKD1 transgene generates functional polycystin-1 in mice and is associated with a cystic phenotype, Hum. Mol. Genet. 9 (2000) 2617–2627.
[31] A. Boletta, F. Qian, L.F. Onuchic, B. Phakdeekitcharoen, K. Hanaoka, W. Guggino, L. Monaco, G.G. Germino, Polycystin-1, the gene product of PKD1, induces resistance to apoptosis and spontaneous tubulogenesis in MDCK cells, Mol. Cell 6 (2000) 1267–1273.
[32] G. Aguiari, M. Campanella, E. Manzati, P. Pinton, M. Banzi, S. Moretti, R. Piva, R. Rizzuto, L. del Senno, Expression of polycystin-1 C-terminal fragment enhances the ATP-induced Ca2+ release in human kidney cells, BBRC 301 (2003) 657–664.
[33] G. Aguiari, M. Banzi, S. Gessi, Y. Cai, E. Zeggio, E. Manzati, R. Piva, E. Lambertini, L. Ferrari, D.J. Peters, F. Lanza, P.C. Harris, P.A. Borea, S. Somlo, L. del Senno, Deficiency of polycystin-2 reduces Ca^{2+} channel activity and cell proliferation in ADPKD lymphoblastoid cells, FASEB J. 18 (2004) 884–886.
[34] L. Santella, The role of calcium in the cell cycle: facts and hypotheses, Biochem. Biophys. Res. Commun. 244 (1998) 317–324.
[35] M.J. Berridge, M.D. Bootman, P. Lipp, Calcium—A life and death signal, Nature 395 (1998) 645–648.
[36] M.S. Scheffers, H. Le, P. van der Bent, W. Leonhard, F. Prins, L. Spruit, M.H. Breuning, E. De Heer, D.J.M. Peters, Distinct subcellular expression of endogenous polycystin-2 in the plasma membrane and Golgi apparatus of MDCK-cells, Hum. Mol. Genet. 11 (2002) 59–67.
[37] G. Aguiari, R. Piva, E. Manzati, E. Mazzoni, G. Augello, E. Chiari, S. Moretti, L.M. Neri, L. del Senno, K562 erythroid and HL60 macrophage differentiation downregulates polycystin, a large membrane-associated protein, Exp. Cell Res. 244 (1998) 259–267.
[38] M. Brini, R. Marsault, C. Bastianutto, J. Alvarez, T. Pozzan, R. Rizzuto, Transfected aequorin in the measurement of cytosolic Ca2+ concentration ([Ca2+]c). A critical evaluation, J. Biol. Chem. 270 (1995) 9896–9903.
[39] G. Grynkiewicz, M. Poenie, R.Y. Tsien, A new generation of Ca2+ indicators with greatly improved fluorescence properties, J. Biol. Chem. 260 (1985) 3440–3445.
[40] J.W. Soh, I.B. Weinstein, Roles of specific isoforms of protein kinase C in the transcriptional control of cyclin D1 and related genes, J. Biol. Chem. 278 (2003) 34709–134706.
[41] O. Coqueret, Linking cyclins to transcriptional control, Gene 299 (2002) 35–55.
[42] C. Albanese, J. Johnson, G. Watanabe, N. Eklund, D. Vu, A. Arnold, R.G. Pestell, Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions, J. Biol. Chem. 270 (1995) 23589–23597.
[43] M. Shtutman, J. Zhurinsky, I. Simcha, C. Albanese, M. D'Amico, R. Pestell, A. Ben-Ze'ev, The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 5522–5527.
[44] E. Balciunaite, S. Jones, A. Toker, A. Kazlauskas, PDGF initiates two distinct phases of protein kinase C activity that make unequal contributions to the G0 to S transition, Curr. Biol. 10 (2000) 261–267.
[45] J.D. Black, Protein kinase C-mediated regulation of the cell cycle, Front. Biosci. 5 (2000) D406–D423.
[46] A. Tanimura, A. Nezu, T. Morita, N. Hashimoto, Y. Tojyo, Interplay between calcium, diacylglycerol, and phosphorylation in the spatial and temporal regulation of PKC-GFP*, J. Biol. Chem. 277 (2002) 29054–29062.
[47] P. Pinton, T. Tsuboi, E.K. Ainscow, T. Pozzan, R. Rizzuto, G.A. Rutter, Dynamics of glucose-induced membrane recruitment of protein kinase C beta II in living pancreatic islet beta-cells, J. Biol. Chem. 277 (2002) 37702–37710.
[48] W. Tian, Z. Zhang, D. Cohen, MAPK signalling and the kidney, Am. J. Physiol.: Renal., Physiol. 279 (2000) F593–F604.
[49] T. Yamaguchi, S. Nagao, D.P. Wallace, F.A. Belibi, B.D. Cowley Jr., J.C. Pelling, J.J. Grantham, Cyclic AMP activates B-Raf and ERK in

cyst epithelial cells from autosomal-dominant polycystic kidneys, Kidney Int. 63 (2003) 1983–1994.
[50] S. Nakashima, Protein kinase Cα (PKCα): regulation and biological function, J. Biochem. 132 (2002) 669–675.
[51] C. Nickel, T. Benzing, L. Sellin, P. Gerke, A. Karihaloo, Z.X. Liu, L.G. Cantley, G. Walz, The polycystin-1 C-terminal fragment triggers branching morphogenesis and migration of tubular kidney epithelial cells, J. Clin. Invest. 109 (2002) 481–489.
[52] Z.W. Zhu, H. Friess, L. Wang, F.F. Di Mola, A. Zimmermann, M.W. Buchler, Down-regulation of nerve growth factor in poorly differentiated and advanced human esophageal cancer, Anticancer Res. 20 (2000) 125–132.
[53] M. Wahl, Gruenstein, Intracellular free Ca^{2+} in the cell cycle in human fibroblasts: transition between G1 and G0 and progression into S phase, Mol. Biol. Cell 4 (1993) 293–329.
[54] A. Maturana, G. Van Haasteren, I. Piuz, C. Castelbou, N. Demaurex, W. Schlegel, Spontaneous calcium oscillations control c-*fos* transcription via the serum response element in neuroendocrine cells, J. Biol. Chem. 18 (2002) 39713–39721.
[55] L. Tsiokas, T. Arnould, C. Zhu, E. Kim, G. Walz, V.P. Sukhatme, Specific association of the gene product of PKD2 with the TRPC1 channel, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 3934–3939.
[56] Y. Luo, P.M. Vassilev, X. Li, Y. Kawanabe, J. Zhou, Native polycystin 2 functions as a plasma membrane Ca^{2+}-permeable cation channel in renal epithelia, Mol. Cell. Biol. 23 (2003) 2600–2607.
[57] A.K. Bhunia, K. Piontek, A. Boletta, L. Liu, F. Qian, P.N. Xu, F.J. Germino, G.G. Germino, PKD1 induces p21(waf1) and regulation of the cell cycle via direct activation of the JAK-STAT signaling pathway in a process requiring PKD2, Cell 109 (2002) 157–168.
[58] H. Kim, Y. Bae, W. Jeong, C. Ahn, S. Kang, Depletion of PKD1 by antisense oligodeoxynucleotide induces premature G1/S-phase transition, Eur. J. Hum. Genet. 12 (2004) 433–440.
[59] k.T. Yamaguchi, J.C. Pelling, N.T. Ramaswamy, J.W. Eppler, D.P. Wallace, S. Nagao, L.A. Rome, L.P. Sullivan, J.J. Grantham, Cyclic AMP activates B-Raf and ERK in cyst epithelial cells from autosomal-dominant polycystic kidneys, Kidney Int. 57 (2000) 1460–1471.
[60] K. Hanaoka, W.B. Guggino, cAMP regulates cell proliferation and cyst formation in autosomal polycystic kidney disease cells, J. Am. Soc. Nephrol. 11 (2000) 1179–1187.