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PROTEIN KINASE D1 UPREGULATES EXPRESSION AND ACTIVITY OF EXTRACELLULAR SIGNAL-REGULATED KINASE 1/2 AND EGFR IN HUMAN hTert KERATINOCYTES

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(*Submitted by Academician K. Kumanov on May 22, 2007*)

Abstract

Immortalized hTert keratinocytes have been obtained from normal human epidermal keratinocytes, which were infected with amphotropic retroviral vectors encoding hTert, the catalytic subunit of telomerase, followed by spontaneous loss of $p16^{INK4a}$ function. There are no any data in the literature concerning transduction pathways participating in regulation of proliferation and/or differentiation of this cell line, probably because it was previously supposed that immortalization of the cells does not disrupt other normal growth control mechanisms. In this study, we explored the effects of PKD1 inhibition in hTert keratinocytes, using siRNA approach, on expression and activation of ERK1/2 and EGFR which acts to promote proliferation and survival in normal human epidermal keratinocytes. Treatment of cultured hTert keratinocytes with siRNA for PKD1 effectively diminished protein levels of the kinase, ERK1/2 expression and activity, EGFR expression and activity and according to our previous results in K10 and involucrin expression. It is possible that PKD1, ERK1/2 and EGFR have adverse effects in immortalized human hTert keratinocytes compared to normal human epidermal keratinocytes in regulation of proliferation and differentiation. We showed for the first time that ERK1/2 MAPK, which are considered to be constitutively expressed kinases in mammalian cells, are inducible enzymes regulated by PKD1 in hTert keratinocytes.

Key words: hTert keratinocytes, PKD1, ERK1/2, EGFR, differentiation

Introduction. hTert keratinocytes have been obtained from normal human epidermal keratinocytes, which were infected with amphotropic retroviral vectors encoding hTert, the catalytic subunit of telomerase [1]. The hTert(+) cells have a normal karyotype and the cells have undergone more than 80 population doublings (PDs) after hTert retroviral transduction while control cells exhibit senescence-associated proliferation arrest after 8 PDs [2]. The mechanism that triggers $p16^{INK4a}$ accumulation appears to sense the senescence state of keratinocytes, but preventing telomere erosion does not

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avoid its activation. Loss of this mechanism, whether by p16^{INK4a} gene deletion, mutation, or altered regulation of expression, together with telomere stabilization effected by hTert expression is necessary to enable keratinocytes to become immortalized. It was supposed that immortalization of keratinocytes by forced expression of telomerase and subsequent spontaneous events leading to loss of this p16^{INK4a}–dependent mechanism generally does not disrupt either other normal growth control mechanisms or affects the ability of the cells to form a differentiated epithelium. hTert keratinocytes continued to express the differentiation-related markers involucrin and K10 in suprabasal cells of stratified colonies and form a differentiating epidermis in organotypic culture. They form more robust epithelium with a denser and more columnar basal cell layer and more layers of spinous cells than that formed by mid-life-span normal keratinocytes [3].

PKD (Protein Kinase D) is a new family of diacylglycerol (DAG)-stimulated serine/threonine protein kinases which consist of three isoforms – PKD1/ PKCμ (Protein Kinase C μ), PKD2 and PKD3/ PKCν. PKD1 is ubiquitously expressed and involved in diverse cellular functions like constitutive transport processes in epithelial cells, G protein-mediated regulation of Golgi organization, protection of apoptosis and promotion of proliferation, tumorogenesis, cell motility, osteoblastic and hTert keratinocyte differentiation. Despite the diverse roles of PKD1 relatively few in vivo substrates have been identified so far. One of them is the Ras effector RIN1 (RAS and RAB Interacting) which is phosphorylated by PKD1 at Ser 351, thereby causing dissociation from Ras and stimulating binding of RIN to 14-3-3 proteins. This in turn allows Raf to interact with Ras and to activate Ras/Raf/MEK1/ERK1/2 signal pathways [4–9].

ERK1/2 (Extracellular Signal-Regulated Kinase 1/2) are ubiquitously expressed in mammalian cells and serves to couple various cell surface stimuli to the alteration of cell function [10]. The activation of this signal pathway is able to promote different and some times opposite cellular processes. This wide variety of responses elicited by the activation of a single pathway has been shown to rely on the timing and strength of this activation. For instance, sustained high activation of ERK pathway induces cell cycle arrest that can be linked to senescence, apoptosis or differentiation, while transient activation followed by a sustained but lower level of ERK activity is a common feature of cell proliferation in many systems. The time and the strength of ERK1/2 activation can be regulated by specific ERKs phosphatases and by downregulation of upstream elements of the pathway like Ras or EGFR (Epidermal Growth Factor Receptor) [11–13]. Data in the literature suggest that ERK function to promote keratinocyte proliferation and survival, whereas p38 MAPK functions to promote differentiation and apoptosis [14].

ERK1/2 is one of the major downstream effectors of EGFR, member of an extended receptor tyrosine kinase family, named Human EGF Receptor (HER) family, and also known as HER1 or ErbB1. Activation of EGFR by several ligands, such as EGF (epidermal growth factor), HB-EGF (heparin binding-EGF), Amphiregulin and TGF-α (transforming growth factor α), leads to homo- and heterodimerization with other ErbB members, which promotes *trans* tyrosine phosphorylation of the intracellular C-terminal domain. These phosphorylated tyrosines function as docking sites for a variety of signal molecules and pathways including Ras/Raf/MEK/ERK pathway, that bring about cellular responses to EGFR ligands. Aberrant regulation of EGFR has been shown to promote tumorogenic processes by stimulating proliferation, angiogenesis and metastasis, which mean that its tyrosine phosphorylation must be strictly regulated in nontransformed cells [15]. It is known that one potential mechanism for its regulation is through PKC-dependent threonine phosphorylation of the receptor which in turn marks EGFR for degradation and decreases its intrinsic tyrosine kinase activity [12, 16].

In our previous research we have shown that inhibition of PKD1 expression in hTert keratinocytes, using siRNA approach, resulted in significant reduction of both the

level of mRNA of PKD1 and keratinocyte differentiation measured by decreased expression of early differentiation markers – involucrin and K10 [9]. So in the present study, using the same approach, we detected changes of PKD1 protein levels and effects of its inhibition on activity/phosphorylation and expression of ERK1/2 and EGFR, known to be connected with proliferation and migration of human epidermal keratinocyte [17].

Materials and methods. CELL CULTURE. hTert keratinocytes obtained by stable transfection of primary cell culture with human telomerase reverse transcriptase was kindly provided by Laboratory of Cellular Biochemistry and Biology, FUNDP, Namur, Belgium. Keratinocytes were grown in 75 cm^2 tissue-culture flasks with Epilife keratinocyte medium supplemented with growth factors, hormones and antibiotics (Cascade Biologics, UK). This medium was used for all subcultures of keratinocytes. The cultures were incubated at 37 °C in a humidified, 95% air, 5% CO_2 atmosphere. The cells were allowed to grow to 70%–80% confluence.

SMALL INTERFERENCE RNA (SIRNA) AND ITS TRANSFECTION. For transfection of siRNA for PKD1, hTert keratinocytes were seeded into 6-well plates with cell density 100 000/well one day before transfection. Transfection of siRNA for PKD1 (Santa Cruz Biotech. Inc.) was performed using Oligofectamine (Invitrogen Corp.) according to the manufacturer's protocol.

WESTERN BLOTTING. Whole cell extracts were made for different time intervals after transfection of siRNA for PKD1. The cells were lysed in sample buffer (0.125 M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.2 M DTT). Protein concentration was quantitated with Bio-Rad DC protein assay (Bio-Rad Laboratories, France) and equal amounts of protein were used for SDS-PAGE. The immunodetection was performed with rabbit polyclonal IgG anti-PKCμ antibody (Santa Cruz Biotech. Inc.), rabbit (polyclonal) anti-ERK1/2 and mouse monoclonal anti-phospho-ERK1/2 antibody (Upstate Biotechnology), rabbit (polyclonal) anti-EGFR [pY1173] phosphospecific antibody and mouse monoclonal anti-human EGFR Antibody Cocktail [R19/48] (Biosource). For control of protein loading, β-actin was detected with monoclonal anti-β-actin (Sigma, USA) antibody. Primary antibodies were revealed with horseradish peroxidase (HRP)-conjugated antibodies and Luminol reagent.

Results and discussion. In our previous research using Real Time PCR we have shown that siRNA for PKD1 inhibits expression of the kinase on the level of mRNA in hTert keratinocytes [9]. To detect changes of the kinase's protein levels we performed Western blotting of hTert keratinocytes transfected with siRNA for PKD1 for 6 hours and grown 48 hours after transfection. Nontransfected and oligofectamine-treated cells were used as controls. Figure 1*A* shows that expression of PKD1 was markedly decreased 48 hours after transfection. For determination of the total protein content in the samples, protein extracts were immunoblotted for β-actin (control of protein loading). Because there were some variations in the protein loading of the samples we decided to use densitometry to measure the effects of siRNA transfection. The obtained results show that siRNA for PKD1 indeed inhibit expression of PKD1 on protein level by more than 40% (Fig. 1*B*).

Little is known about the direct targets downstream of PKD1 and till now only few physiological substrates are identified. One of them is the RAS effector RIN1 which is phosphorylated by PKD1 at Ser 351. This in turn allows Raf to interact with RAS and to activate ERK1/2 signal pathways [4–7]. Because of that we next decided to detect changes of ERK1/2 activity/phosphorylation and total ERK1/2 after membrane stripping (Fig. 2*A*). Inhibition of PKD1 decreases activity of ERK1/2 48 hours after transfection by 40% compared to nontransfected sample (if we consider lower β-actin levels in treated cells) (Fig. 2*B*). Surprisingly ERK1/2 expression was inhibited either by 46% (Fig. 2*B*). According to these results decreased ERK1/2 activity/phosphorylation

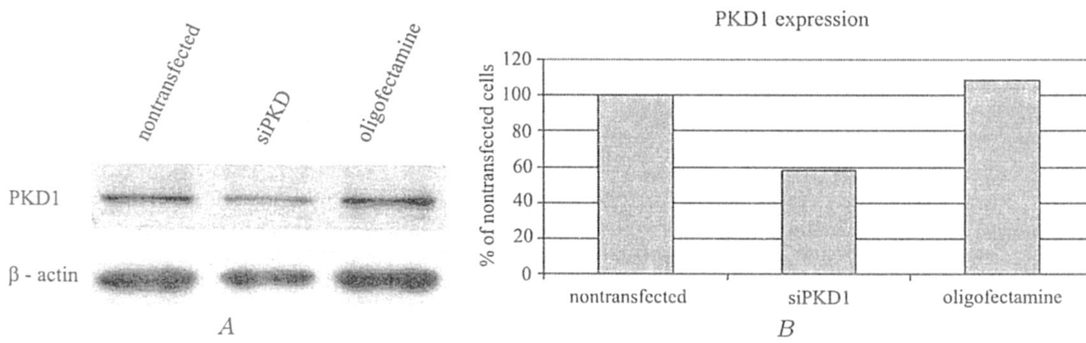

Fig. 1. Treatment of hTert keratinocytes with siRNA for PKD1 downregulates protein levels of the kinase. Keratinocytes were transfected with 100 nM siRNA for PKD1 for 6 h and then grown for 48 h. Nontransfected and oligofectamine-treated cells were used as controls. *A*) Whole cell extracts were immunoblotted for total PKD1 and for β-actin (control of protein loading). *B*) The intensity of each band was determined by densitometry and the ratio of the band intensities of PKD1: β-actin in the nontransfected cells 48 h after transfection was arbitrary set as 100%. The ratios obtained from the other samples were related to the value obtained for the control cells. Densitometry of the bands was performed with ImageJ software

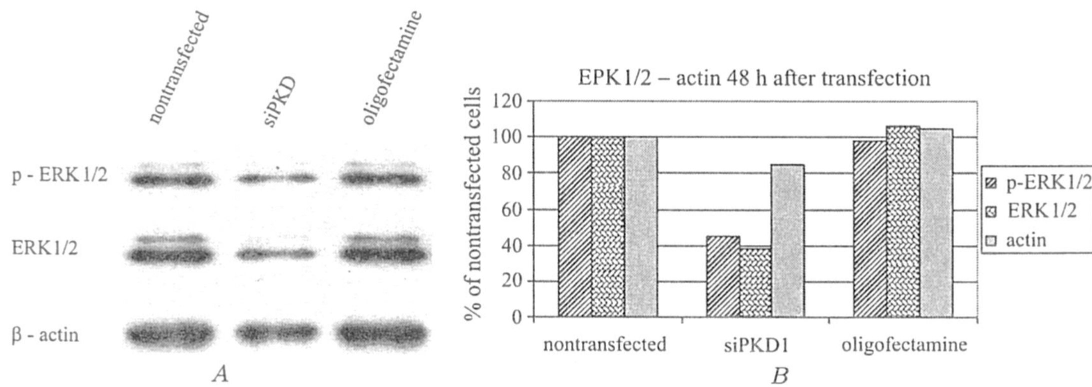

Fig. 2. Inhibition of PKD1 downregulates activity/phosphorylation and expression of ERK1/2. *A*) Immunoblots for detection of p-ERK1/2 and total ERK1/2 were performed with the same samples used for detection of changes of PKD1 levels. *B*) Densitometric analysis of changes of actin, p-ERK1/2 and total ERK1/2 48 hours after transfection. The intensity of the bands of each immunoblot depends on time-exposure of X-ray films so the intensity of nontransfected cells 48 hours after transfection of each blot were arbitrary set as 100%

48 h after transfection is a consequence of inhibition of the expression of ERK1/2 and was not a result of inhibition or lack of stimulation of Ras/Raf-1/ERK1/2 signalling cascade. This effect is surprising because ERK1/2 is thought to be an ubiquitously expressed constitutive enzyme and its expression is often used as a control of protein loading in Western blotting. The effect could not be a consequence of unsuccessful stripping because antibodies for detection of p-ERK1/2 and total ERK1/2 are from dif-

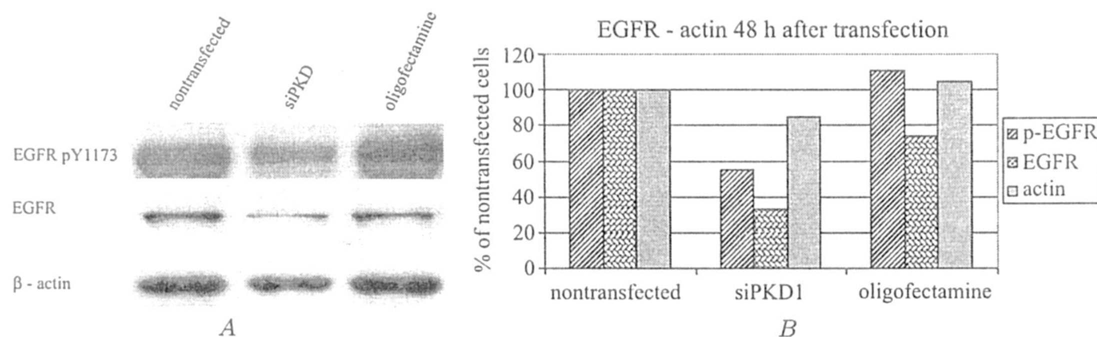

Fig. 3. Inhibition of PKD1 downregulates activity/phosphorylation and expression of EGFR. *A*) Immunoblots were performed with the same samples used for detection of changes of PKD1 levels. *B*) Densitometric analysis of changes of actin, p-EGFR and total EGFR 48 h after transfection. The intensity of nontransfected cells 48 h after transfection of each blot were arbitrary set as 100%

ferent species, mouse and rabbit respectively. These results clearly show that ERK1/2 is an inducible kinase in hTert keratinocytes and suggest that PKD1 participate in the transduction pathway that regulates its expression.

Next, we examined EGFR protein and its phosphorylation on Y-1173, *trans* tyrosine phosphorylation site which is a docking site for Shc-Grb2 complex, activating in this manner Shc/Grb2/SOS/Ras/Raf/MEK/ERK signalling pathway [18] (Fig. 3*A*). Transfection of hTert keratinocytes with siPKD1 reduced EGFR phosphorylation by 30% and its expression by 62%, 48 h after transfection (Fig. 3*B*).

In conclusion, it has been shown here that inhibition of PKD1 expression, using siRNA approach, effectively diminishes protein levels of the kinase, inhibits ERK1/2 expression and activity, as well as EGFR expression and activity 48 h after transfection, and, according to our previous results, inhibits K10 and involucrin expression 72 h after. Previously we have speculated that increased expression of PKD1 is connected with differentiation of human hTert keratinocytes and this statement was confirmed with the present results [9]. It is possible that PKD1, ERK1/2 and EGFR have adverse effects in immortalized human hTert keratinocytes compared to normal human epidermal keratinocytes on the cellular regulation of proliferation and differentiation. We show for the first time that ERK1/2, which are considered to be constitutively expressed kinases in mammalian cells, are inducible enzymes regulated by PKD1 in hTert keratinocytes.

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