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Research Article

Opposite roles of protein kinase C isoforms in proliferation, differentiation, apoptosis, and tumorigenicity of human HaCaT keratinocytes

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Abstract. We have previously shown that the protein kinase C (PKC) system plays a pivotal role in regulation of proliferation and differentiation of the human keratinocyte line HaCaT which is often used to assess processes of immortalization, transformation, and tumorigenesis in human skin. In this paper, using pharmacological and molecular biology approaches, we investigated the isoform-specific roles of certain PKC isoenzymes (conventional cPKCα and β; novel nPKCδ and ε) in the regulation of various keratinocyte functions. cPKCα and nPKCδ stimulated cellular differentiation and increased susceptibility of cells to actions of inducers of apoptosis, and they markedly inhibited cellular proliferation and tumor growth in immunodeficient mice. In marked contrast, cPKCβ and nPKCε increased both in vitro and in vivo growth of cells and inhibited differentiation and apoptosis. Our data present clear evidence for the specific, antagonistic roles of certain cPKC and nPKC isoforms in regulating the above processes in human HaCaT keratinocytes.

Key words. Human keratinocyte; HaCaT; protein kinase C; isoenzyme; recombinant overexpression; proliferation; differentiation; tumorigenesis.

Protein kinase C (PKC) comprises a family of serine/ threonine kinases that play crucial roles in the regulation of various cellular processes such as proliferation, differentiation, apoptosis, and tumorigenesis [1–4]. The members of the PKC family are the calcium- and phorbol ester-dependent 'conventional' isoforms (PKCα, βI, βII, and γ; cPKCs); the calcium-independent 'novel' isoforms (PKCδ, ε, η, and θ; nPKCs); and the calcium- and phorbol ester-independent 'atypical' (PKCζ and λ/ι; aPKCs) isoforms. These isoforms possess a characteristic expression pattern in a given cell type, and regulate in an isoenzyme-specific fashion various cellular processes including cell growth

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and death [4–6]. In addition, not only may some PKC isoforms be active whereas others not for a given response but different PKC isozymes may have antagonistic effects on the same cellular event [7–9], arguing for differential regulatory roles of specific PKC isoforms [4].

Emerging evidence suggests a pivotal role for PKC in the regulation of the proliferation and differentiation of normal human epidermal keratinocytes (NHEKs) as well as in the development of various skin tumors [10–13]. In addition, isoenzyme-specific roles of PKC in keratinocyte proliferation and differentiation have been described. For example, PKCα and δ were reported to be key components regulating the differentiation of NHEKs [10–15], and PKCε has been implicated in promoting skin tumor development in transgenic mice [12].

We have previously shown that immortalized HaCaT keratinocytes [16], which are very often used to model certain properties of NHEKs and to assess the processes of immortalization, transformation, and tumor progression in human skin [17, 18], possess a similar (although not identical) PKC system to that of NHEKs [19]. Namely, HaCaT cells express several PKC isoforms, the pattern of which alters with differentiation, and respond with cessation of proliferation and induction of differentiation upon the application of the PKC activator phorbol esters [19]. However, there are as yet little data available on the isoform-specific roles of the members of the PKC system in regulating HaCaT cells functions.

Exploiting the ability of HaCaT cells to be continuously passaged, in this study, in combination with the use of PKC inhibitors, we have constructed stable PKC transfectants of HaCaT cells and have investigated the possible roles of the PKC isozymes in regulating proliferation, differentiation, apoptosis, and the tumor induction properties of the cells. We report here that certain cPKC and nPKC isoforms play opposite roles in regulating the above processes, and that this antagonism could be seen even within members of the PKC sub-families.

Materials and methods

Antibodies

All primary antibodies against PKC isoenzymes were developed in rabbits and were shown to react specifically with the given PKC isoforms [19]. Anti-PKCα, β, and ε were from Sigma (St. Louis, Mo.), whereas anti-PKCδ was from Santa Cruz (Santa Cruz, Calif.). Specificities of anti-PKC antibodies were also tested by applying isoform-specific blocking peptides, which blocked the immunostaining in all cases (data not shown). Monoclonal mouse antibodies against involucrin (INV; Sigma), filaggrin (FIL; Biomedical Technologies, Stoughton, Mass.), and keratinocyte-specific transglutaminase-1 (TG; Biomedical Technologies) were used as markers of keratinocyte differentiation.

Generation of PKC constructs

PKC constructs were engineered as described previously [20, 21]. Briefly, the cDNA sequences of PKCα, β, δ, and ε were subcloned into a metallothionein promoter-driven eukaryotic expression vector (MTH) [22]. The vector sequence encodes a C-terminal PKCε-derived 12-amino-acid tag (εMTH) and attaches it to the end of the PKC proteins. As previously described [20], this epitope tag affects neither the localization nor the translocation of the given isoform.

Cell culture and transfection of cells

The human immortalized HaCaT keratinocyte cell line was a kind gift of Prof. N. E. Fusenig (Division of Differentiation and Carcinogenesis, German Cancer Research Center, Heidelberg, Germany). Cells were cultured in 25-cm^2 or 75-cm^2 tissue culture flasks (if not indicated otherwise) in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum (Sigma), 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 1.25 µg/ml fungizone (all from Biogal, Debrecen, Hungary) at 37°C in a 5% CO_2 atmosphere.

For transfection, HaCaT cells were seeded in 6-well tissue culture dishes and at 60–70% confluence were transfected by either the empty pεMTH vector (control cells) or by the vectors encoding the cDNA sequences of PKCα, β, δ, or ε [20, 21]. Transfections were performed using a lipofectamine anionic detergent (Invitrogen, Paisley, UK) in serum-free DMEM solution using 2–4 µg cDNA according to the protocol suggested by the manufacturer. Cells were selected in DMEM containing 750 µg/ml G418 (geneticin; Invitrogen) for 12–18 days. Then, single colonies were isolated. PKC-overexpressing cells were cultured in supplemented DMEM containing 500 µg/ml G418. Experiments were routinely carried out on pools of transfected cells, but the results were confirmed on at least three individual clones for each isoform. The efficacy of recombinant overexpression was monitored by Western blotting (see below and in fig. 2) using an anti-PKCε antibody (Sigma) which, beside recognizing the endogenously expressed PKCε (approximately 90 kDa), is able to detect the 'ε-tagged' transfected and overexpressed isoforms (approximately 80 kDa), resulting in double bands.

Determination of cellular proliferation

Proliferation was measured by a colorimetric bromodeoxyuridine (BrdU) assay kit (Boehringer Mannheim, Mannheim, Germany) and by analyzing standard growth curves. In those BrdU assays where the effects of PKC-acting agents were tested on cellular proliferation, cells were plated in 96-well multititer plates (5000 cells/well density) in quadruplicate, and 4 h later were treated with different concentrations of the agents and further incu-

bated for the time indicated. Cells were then incubated with 10 µM BrdU for 4 hours, and the cellular incorporation of BrdU (as the indicator of cellular proliferation) was determined colorimetrically according to the manufacturer's protocol. When BrdU assays were employed to investigate growth properties of PKC transfectants, cells were seeded at a density of 1000 cells/well and the BrdU incorporation was determined after the indicated days of culture, as described above.

To assess doubling times and maximal cell numbers of PKC overexpressers, 10^4 cells/well were plated in 12-well plates in triplicate in complete DMEM. Fresh medium was added every other day, and the cells in triplicate were harvested by trypsinization as indicated (usually on a daily basis) and counted using a hemocytometer. In determining the average doubling time, the 24-h time point was used as the starting point to avoid artifacts due to the initial lag period after plating [21]. The following equation was used to calculate doubling time: $\tau = D/\log_2(N/N_0)$ where τ is the doubling time, D is the number of days of culturing, N and N_0 are the number of cells at the end and the beginning of the experiments, respectively. To determine the maximal cell density, cells were grown in 12-well plates to confluence and kept post-confluent for 3 additional days with daily medium changes, and then counted as described above.

Western blot analysis

Cells were washed with ice-cold phosphate-buffered saline (PBS), harvested in homogenization buffer [20 mM TRIS-Cl, 5 mM EGTA, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 20 µM leupeptin, pH 7.4; all from Sigma] and disrupted by sonication on ice [23]. The protein content of samples was measured by a modified BCA protein assay (Pierce, Rockford, Ill.). Total cell lysates were mixed with SDS-PAGE sample buffer and boiled for 10 min at 100 °C. The samples were subjected to SDS-PAGE according to Laemmli [24] (8% gels were loaded with 20–30 µg protein per lane) and transferred to nitrocellulose membranes (BioRad, Vienna, Austria). Membranes were then blocked with 5% dry milk in PBS and probed with the appropriate primary antibodies against the given PKC isoforms or differentiation markers. Peroxidase-conjugated goat anti-rabbit or anti-mouse IgG antibodies (BioRad) were used as secondary antibodies, and the immunoreactive bands were visualized by an ECL Western blotting detection kit (Amersham, Little Chalfont, U.K.) on light-sensitive films (AGFA, Brussels, Belgium). To assess equal loading, nitrocellulose membranes were stripped in 200 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 2% SDS and 0.1 β-mercaptoethanol at 65 °C for 1 h and were reprobed with a mouse anti-cytochrome c antibody (Santa Cruz) followed by a similar visualization procedure as described above. When applicable, immunoblots were subjected to densitometric analysis as described previously [19] and normalized densitometric values of the individual lanes of several independent experiments were then determined.

PKC activity (kinase) assay

The PKC activity of transfected HaCaT cells was determined as described before [14, 20, 21]. Briefly, cells were lysed in the above lysis buffer, and the kinase activities of cell lysates (40 µg per reaction) were examined using histone III (H-III) or myosin light-chain kinase 20 (MLC20) as substrates (both from Sigma). The assay mixture contained 20 mM Tris (pH 7.5), 20 mM $MgCl_2$, 1 mM $CaCl_2$, 25 µM ATP, and 0.2 mg/ml substrate (all from Sigma), with 0.1 µCi of [γ-^{32}P]ATP (Amersham Biosciences, Freiburg, Germany) per assay. Data represent triplicate determinations.

Determination of apoptosis

Stable transfectants of HaCaT cells overexpressing the different PKC isoforms or the control empty vector were treated with vehicle, 10 µM 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] or 50 nM tumor necrosis factor α (TNFα) for 2 days. Cells were then collected by trypsinization and were incubated with 1 µl fluorescein isothiocyanate (FITC)-conjugated annexin V (Coulter-Immunotech, Hialeah, Fla.) for 10 min in the dark. Cells were then measured by a flow cytometer (Coulter EPICS XL-4) and the percentage of apoptotic cells compared to total cell number was determined.

Tumorigenicity in SCID mice

Cells overexpressing the various PKC isoforms (along with the control HaCaT cells) were grown in mass cultures, trypsinized and washed twice with PBS, and were then resuspended in culture medium at a density of $1-2 \times 10^6$ viable cells/200 µl. Severe combined immunodeficiency (SCID) mice were injected intradermally and observed over a period of 30 days (during this period, none of the animals showed lethal progression of tumor). Animals were finally euthanized and the averaged three-dimensional size and histological characteristics of the developed tumors (three to four animals for each group) were analyzed.

Histology and immunohistochemistry

The histological parameters were determined on formalin-fixed, paraffin-embedded, and hematoxylin-eosin (HE)-stained sections of the developed tumors. The averaged number of cell divisions was measured by counting the number of nuclei showing clear signs of mitosis in ten individual visual fields at high magnification using a light microscope, and results obtained in each tumor of the same group were then averaged and the mean values calculated.

In addition, to assess the number of proliferating cells, formalin-fixed, paraffin-embedded sections were immunostained against the nuclear marker Ki67 [25] using a streptavidine-biotin-complex (SABC) three-step immunohistochemical technique (DAKO, Hamburg, Germany). First, the inhibition of endogenous peroxidase activity was performed using 3% H_2O_2 in 100% methanol (both from Sigma). Then, non-specific binding was blocked by 1% bovine serum albumin (Sigma) in PBS buffer (pH 7.5). After testing various concentrations of the anti-Ki67 monoclonal mouse primary antibody (DAKO), an optimal 1:50 dilution was employed. The sections were then incubated in a humid chamber using a biotin-coupled anti-mouse secondary antibody (1:100; DAKO) followed by streptavidine conjugated with horseradish peroxidase (1:600; DAKO). To reveal the peroxidase activity, VIP SK-4600 (Vector, Burlingame, Calif.) was employed as a chromogen. The tissue samples were finally slightly counterstained with methyl green (DAKO) and mounted with Aquatex (Merck, Vienna, Austria). The averaged number of proliferating (Ki67-positive) cells was measured by counting the total number of Ki67-positive cells in ten individual visual fields at high magnification using a light microscope and the values were then normalized to the total number of cells measured in the fields.

Results

Effect of inhibition of PKC isoform activities on cellular proliferation and differentiation of HaCaT cells

Confirming our previous findings [19], we showed that the inhibition of endogenous PKC activity in HaCaT keratinocytes by a general PKC inhibitor GF109203X (inhibitor of the cPKC and nPKC isoenzymes) [26] suppressed the expression of the late differentiation markers INV, FIL, and TG, suggesting pivotal roles for the PKC

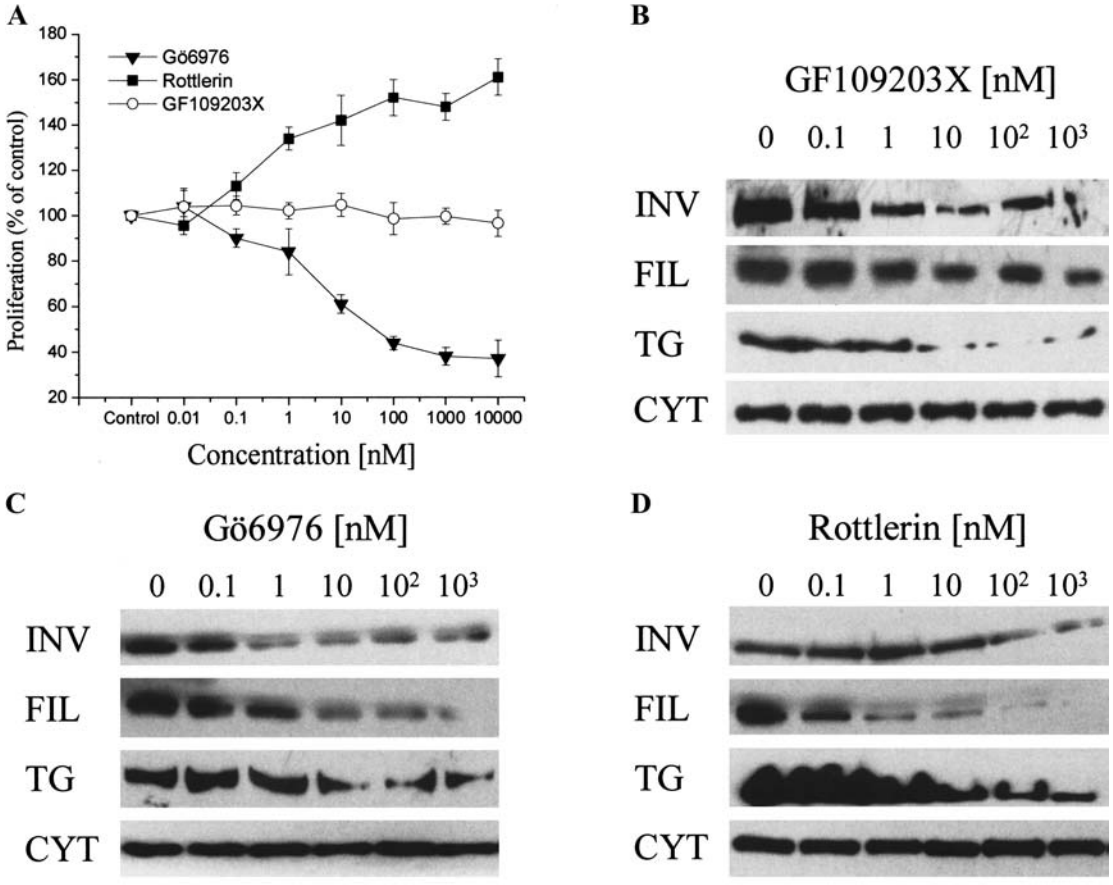

Figure 1. The inhibition of various PKC isoforms differentially modifies cellular proliferation and expression of keratinocyte differentiation markers in HaCaT cells. (*A*) HaCaT cells were seeded at densities of 5000 cells/well in 96-well microtiter plates, treated with various concentrations of PKC inhibitors for 2 days, and then BrdU assays were performed. Points represent the mean ± SE of quadruplicate determinations in one representative experiment. Two to three other experiments yielded similar results. (*B–D*) HaCaT cells were treated with different concentrations of GF109203X (*B*), Gö6976 (*C*), or rottlerin (*D*) for 3 days. Cells were then harvested, similar amounts of proteins were subjected to SDS-PAGE, and Western immunoblotting was performed using mouse antibodies against the differentiation markers INV, FIL, and keratinocyte-specific TG as described in Materials and methods. To assess equal loading, nitrocellulose membranes were stripped and reprobed with a mouse anti-cytochrome antibody (CYT). The figure is representative of two to three experiments yielding similar results.

system in regulating differentiation (fig. 1B). We also found that GF109203X (fig. 1A), in contrast to its dramatic effect on differentiation, did not modify the proliferation of these cells [19]. This latter result can be interpreted in (at least) two ways. First, these findings may suggest that the endogenous PKC activity does not contribute to the regulation of HaCaT cell proliferation. Alternatively, the inhibition of all of the existing cPKC and nPKC isoforms (we have previously shown that HaCaT keratinocytes express cPKCα and β, and nPKCδ, ε, η, and θ) [19] equally inhibited growth-promoting and growth-inhibiting PKC isoform activities, resulting in no net effect.

To test this latter hypothesis, we started to investigate the effects of other PKC inhibitors on HaCaT cell proliferation and differentiation. First, we employed the compound Gö6976, an inhibitor of the cPKC isoforms [27], i.e., cPKCα and β in HaCaT cells. As seen in figure 1A, in contrast to the effect of GF109203X, the inhibitor of the cPKC isoforms markedly inhibited the proliferation of the cells in a dose-dependent manner. Gö6976, however, induced a very similar pattern of changes in the expression of differentiation markers when compared to the effect of GF109203X (fig. 1B, C). Namely, the inhibitor decreased the expression of INV, FIL, and TG in a dose-dependent fashion as measured on Western blots. These results strongly argue for the positive roles of endogenous cPKC isoforms in the processes of proliferation and differentiation of HaCaT keratinocytes.

We then investigated the possible roles of the nPKC isoforms in these processes. Since commercially only the nPKCδ inhibitor rottlerin [28] was available, we measured the effects of this molecule on cellular functions of HaCaT keratinocytes. As seen in figure 1A, D, the inhibition of nPKCδ dose-dependently stimulated cellular proliferation whereas it inhibited the expression of the differentiation markers. Although confidence in the interpretation is limited because of possible effects of rottlerin on systems other than PKC, these findings at least suggested that endogenous nPKCδ activity is a positive regulator of differentiation whereas it functions as a negative modulator of proliferation.

Overexpression of certain cPKC and nPKC isoforms

The results with the inhibitors showed that certain isoforms might play important roles in the regulation of proliferation and differentiation of HaCaT keratinocytes. However, although GF109203X is widely used as a general PKC inhibitor, Gö6076 as an inhibitor of the classic PKCs, and rottlerin as a PKCδ inhibitor, none of these inhibitors is as specific and/or selective as was initially thought. For example, in some systems, GF109203X also blocks mitogen-activated protein kinase-activated protein kinase-1β and p70 S6 kinase [29], whereas rottlerin has been reported not to be selective for PKCδ [28]. Furthermore, the data could not differentiate, for example, between the putative isoform-specific roles of the cPKCα and β isoforms, and did not describe other nPKC isoform-specific functions. Therefore, using the previously introduced MTH vectors [20–23], we have stably transfected HaCaT keratinocytes with cPKCα and β and nPKCδ and ε. This latter isoform was chosen among the nPKC isoenzymes since its potential role in regulating cellular proliferation and differentiation has been extensively documented in several cell types including keratinocytes [5–7, 9, 12, 19, 30].

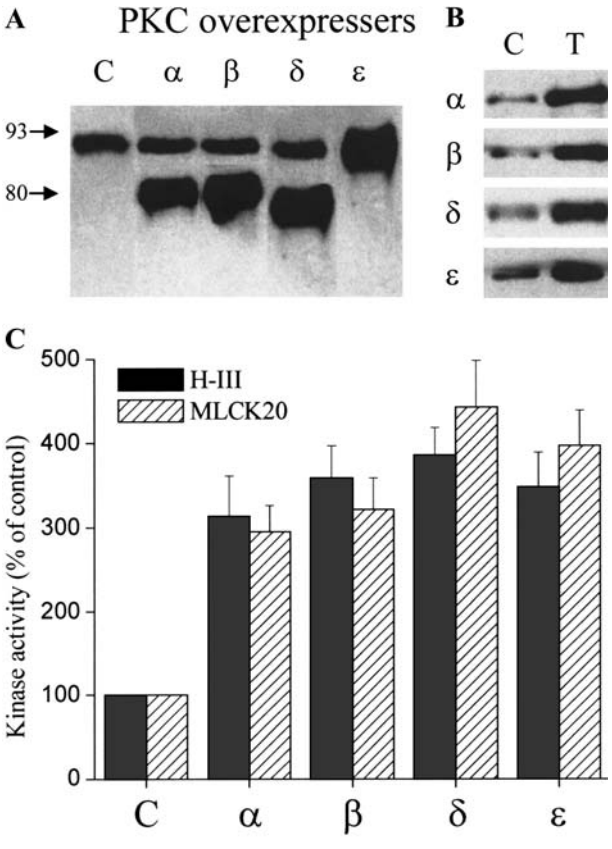

Figure 2. Overexpression of certain PKC isoforms in HaCaT cells. (*A*) Stable transfectants of HaCaT cells overexpressing the different PKC isoforms (Greek letters) or the empty vector (control, C) were harvested, similar amounts of proteins were subjected to SDS-PAGE, and Western immunoblotting was performed as described in Materials and methods. The membranes were then probed with an anti-PKCε antibody that recognizes both the endogenous nPKCε (approximately 90-kDa bands) and the ε-tag sequence of the recombinant, expressed PKC isoforms (approximately 80-kDa bands in the cases of the recombinant PKCα, β, and δ, and an approximately 90-kDa band in the case of the recombinant PKCε). (*B*) To detect the degree of overexpression, Western blot analysis was also performed on control (C) and PKC transfectant (T) HaCaT cells using isoform-specific antibodies that corresponded to the overexpressed recombinant PKC isoenzymes. (*C*) Cell lysates of overexpressors and control (empty vector-transfected, C) HaCaT cells were analyzed for kinase activity by measuring ^{32}P incorporation into H-III or myosin light-chain kinase 20 (MLCK20) substrates in triplicate determinations. The values are expressed as percent of control (mean ± SE). The figures are representative of three experiments for each isoenzyme yielding similar results.

We first examined the efficacy of recombinant overexpression. Cell lysates of pooled cultures (fig. 2) and several transfected clones (data not shown) were subjected to Western blotting. Using an anti-PKCε antibody, which recognizes both the endogenously present nPKCε (approximately 90-kDa bands) and the ε-tag sequence of the recombinant, expressed PKC isoforms (therefore, resulting in approximately 80-kDa bands in the cases of recombinant PKCα, β, and δ, and an approximately 90-kDa band in the case of recombinant PKCε), we were able to specifically detect the transfected isoenzymes (fig. 2A). Furthermore, using isoform-specific antibodies that corresponded to the overexpressed recombinant PKC isoenzymes, we found that the levels of the overexpressed PKCs (fig. 2B) were three- to seven-fold higher than those of the respective endogenous PKCs (data not shown). Finally, to establish that the overexpressed PKC isoforms were functionally active, we also measured kinase (PKC) activity on cell lysates. As seen in figure 2C, the cells expressing the recombinant PKC isoforms showed higher kinase activity, as assessed with both kinase substrates, compared with the control (empty vector-transfected) HaCaT cells.

Effects of overexpression of PKC isoforms on morphology of HaCaT cells

The overexpression of the PKC isoforms differentially affected the morphology of HaCaT keratinocytes. As seen in figure 3, the usual, characteristic cobblestone morphology of control (empty vector-transfected) HaCaT cells was dramatically changed by the overexpression of nPKCε; these cells exhibited a fibroblast-like, spindle-shaped phenotype. In addition, cells overexpressing cPKCβ also possessed elongated cell bodies (yet without processes) and formed denser islets during culture. The overexpression of PKCδ and α did not result in dramatic morphological changes; possibly, the nPKCδ-overexpressing HaCaT cells displayed a more cobblestone appearance.

Effects of overexpression of PKC isoforms on the proliferation and differentiation of HaCaT cells

We then investigated the effect of overexpression of the PKC isoforms on the proliferation of HaCaT keratinocytes. As revealed by BrdU assays (fig. 4A) and standard growth curve analyses (table 1), overexpression of the PKC isoforms markedly altered cell growth. Overexpression of cPKCα and nPKCδ decreased the proliferation of HaCaT cells. Conversely, keratinocytes overexpressing cPKCβ and nPKCε exhibited higher proliferation rates compared to the control (empty vector-transfected) cells.

The differences in proliferation were paralleled by the average doubling times and saturation densities of the cultures. Consistent with findings in the BrdU assays, cPKCα- and nPKCδ-overexpressing cells possessed prolonged doubling times and decreased saturation densities, while cells that overexpressed cPKCβ and nPKCε were characterized by markedly increased saturation densities and shortened doubling times (table 1).

To follow differentiation, we measured the expression of certain keratinocyte differentiation markers in the PKC-overexpressing cells. Since nPKCδ-overexpressing cells very often did not reach complete confluence and ceased

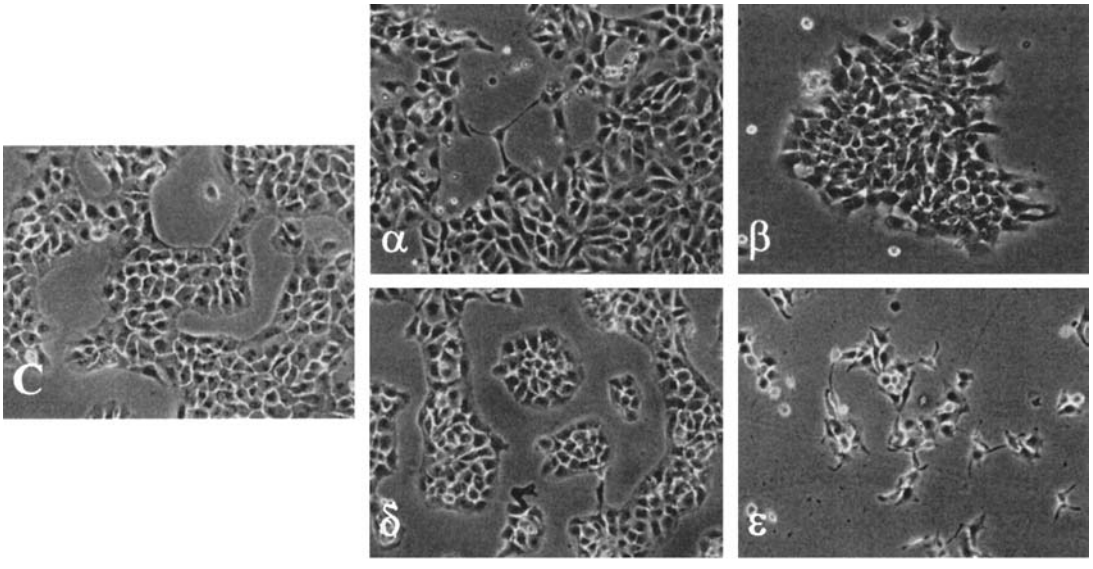

Figure 3. Overexpression of certain PKC isoforms alters morphology of HaCaT cells. Photomicrographs were taken using a phase contrast light microscope (magnification, × 200) on HaCaT cells stably overexpressing the PKC isoforms or the control empty vector (C). The figure is representative of three additional experiments with similar results.

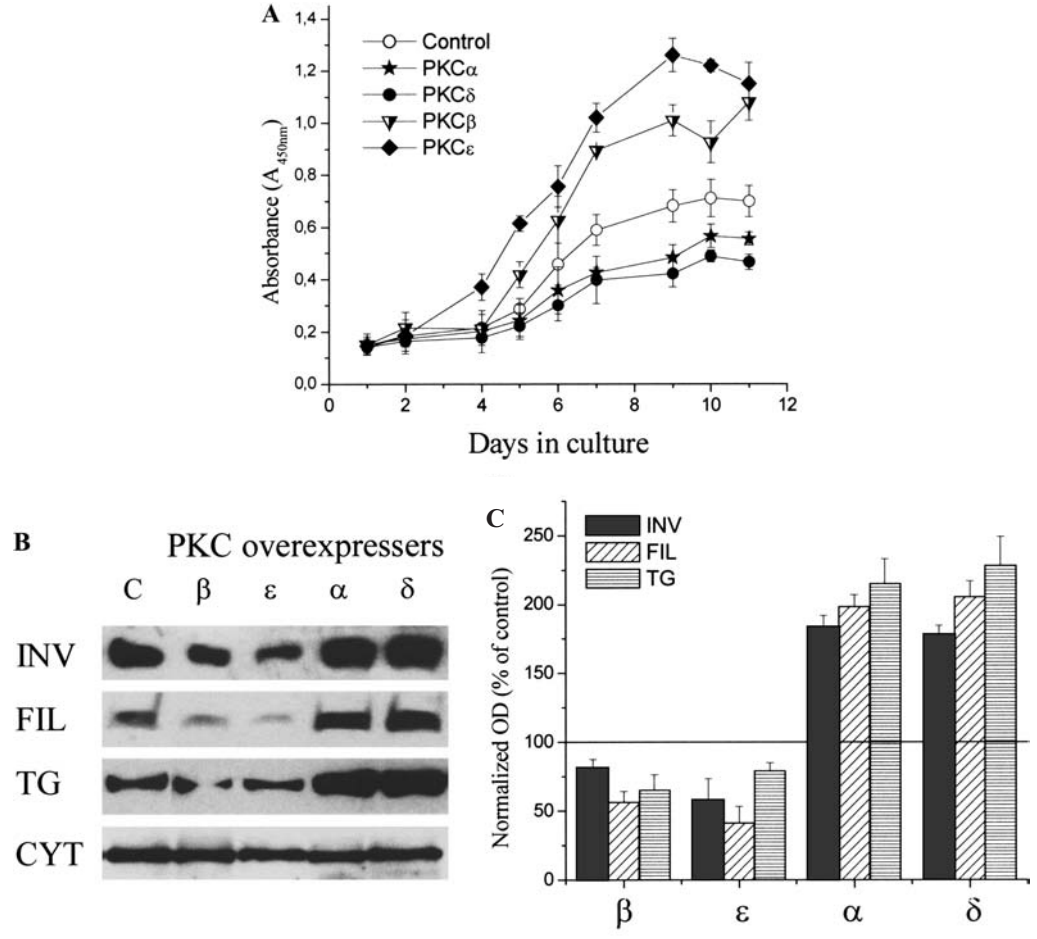

Figure 4. Overexpression of various PKC isoforms differentially alters cellular proliferation and expression of keratinocyte differentiation markers in HaCaT cells. (*A*) Control and PKC overexpresser HaCaT cells were seeded at densities of 1000 cells/well in 96-well microtiter plates and cell proliferation was determined after the indicated days of culture using BrdU assays as described in Materials and methods. Points represent the mean ± SE of quadruplicate determinations in one representative experiment for each isoform. At least three additional experiments for each PKC isoenzyme yielded similar results. (*B*) Stable transfectants of HaCaT cells overexpressing the different PKC isoforms or the control empty vector (C) were harvested, similar amounts of proteins were subjected to SDS-PAGE, and the Western immunoblotting was performed using mouse antibodies against the differentiation markers INV, FIL, and keratinocyte-specific TG as described in Materials and methods. To assess equal loading, nitrocellulose membranes were stripped and reprobed with a mouse anti-cytochrome antibody (CYT). The figure is representative of three experiments yielding similar results. (*C*) The amounts of the differentiation markers expressed in the PKC-overexpressing cells were quantitated by densitometry (optical density, OD), and expressed as the percentage of the OD value of immunoreactive bands of control cells (normalized OD). Bars represent the mean ± SE of three independent experiments.

Table 1. In vitro and in vivo growth analysis of HaCaT cells overexpressing various PKC isoforms.

Isoform	In vitro growth analysis		In vivo tumor growth analysis		
	doubling time (h)	saturation density (10^5 cells/cm^2)	averaged tumor size (mm)	number of cell division	percentage of Ki67-positive cells
Control	23.2 ± 2.5	1.4 ± 0.2	7 × 8 × 4.3	6.2 ± 1.0	25.2 ± 2.1
cPKCα	27.4 ± 3.2	1.0 ± 0.2	4.3 × 4 × 2.3	3.6 ± 0.5	13.2 ± 2.3
nPKCδ	30.5 ± 4.3	0.8 ± 0.1	4.6 × 4.3 × 2	4 ± 0.3	11.4 ± 3.1
cPKCβ	16.5 ± 3.3	2.1 ± 0.1	11 × 8.3 × 4.3	16 ± 1.2	34.9 ± 4.5
nPKCε	13.2 ± 3.9	2.8 ± 0.2	16 × 12 × 5.3	19.5 ± 2.8	41.4 ± 3.9

Various parameters were analyzed as described in Materials and methods. Data are expressed as the mean ± SE, except for averaged tumor size, where the three dimensional sizes of three to four tumors per group were averaged and the mean values are shown.

proliferating at about 80–90% of confluence (data not shown), reflecting a suppressed growth capacity, to obtain comparable data, all cell cultures were harvested at about 80–85% confluence, equal amounts of protein were subjected to SDS-PAGE, and the expression of various markers was investigated by Western blotting. As seen in figure 4B, C, in cells overexpressing cPKCα and nPKCδ isoenzymes, levels of the late-terminal differentiation markers increased, whereas in keratinocytes overexpressing the cPKCβ and nPKCε isoforms, the levels of the differentiation markers decreased compared to those of the control HaCaT cells.

Effects of overexpression of PKC isoforms on the apoptosis of HaCaT cells

Various PKC isoforms have been suggested to play pivotal roles in mediating apoptosis induced by various agents [4]. We therefore measured the effect of the known apoptosis inducers 1α,25(OH)$_2$D$_3$ and TNFα [31] on apoptosis in HaCaT cells overexpressing the various PKC isoenzymes. As determined by annexin V-based flow cytometry analysis (fig. 5), there were no major differences in the level of basal apoptosis for control and cPKCβ- and nPKCε-overexpressing HaCaT cells. In contrast, the basal apoptotic rates in the nPKCδ transfectants and, although to lesser extent, the cPKCα overexpressers were higher than in the control cells. In addition, the overexpression of PKCα and δ increased the susceptibility of cells to apoptosis induced by 10 µM 1α,25(OH)$_2$D$_3$ or 50 nM TNFα, whereas the PKCβ and ε overexpressers showed less sensitivity to the action of these agents than did the control cells.

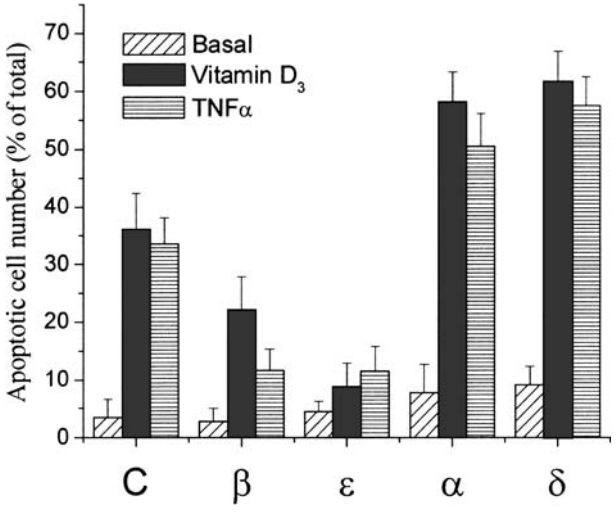

Figure 5. Overexpression of various PKC isoforms differentially alters apoptosis in HaCaT cells. Stable transfectants of HaCaT cells overexpressing the different PKC isoforms or the control empty vector (C) were treated with vehicle (Basal), 10 µM 1α,25 (OH)$_2$ D$_3$ (Vitamin D$_3$), or 50 nM TNFα for 2 days and then apoptosis was determined using an annexin V-based flow cytometry assay as described in Materials and methods. Data are expressed as the percentage of apoptotic cells compared to total cell number and represent the mean ± SE of three independent experiments.

Effects of overexpression of PKC isoforms on tumorigenicity in SCID mice

Finally, we investigated the behavior of PKC overexpressing cells in assays for tumor formation and in vivo growth. SCID mice (three to four in each group) were injected with a cell suspensions of HaCaT cells ($1-2 \times 10^6$ viable cells/200 µl) overexpressing different PKC isoforms and, after 30 days, tumors which had developed were characterized. As revealed on HE-stained sections, control HaCaT cells formed basal cell-enriched tumors with expansive growth properties at the periphery and with intense maturation and differentiation (formation of keratin islets, dyskeratotic cells, sometimes cyst formation) at the center of the tumor (fig. 6). The relative sizes of the proliferating and differentiating fields were approximately the same. The injection of HaCaT cells overexpressing the various PKC isoforms generally did not change the major histological characteristics of the tumors. In other words, all tumors maintained the expansive (i.e., non-infiltrative, benign) growth characteristics and histological features of peripheral proliferation and central differentiation. However, of greatest importance, we found marked differences in the average size of the tumors, the number of dividing cells, and the relative ratio of the proliferating and differentiating parts on the histological sections. Overexpression of nPKCε and cPKCβ resulted in increased tumor growth, as reflected by the markedly increased tumor sizes and number of dividing cells (table 1). Moreover, the relative ratio of the proliferating to the differentiating parts also increased in these tumors (more than 70% of the histological section was dominated by the proliferating part in the nPKCε- and cPKCβ-overexpressing HaCaT cell-induced tumors). In contrast, tumors initiated by cPKCα and nPKCδ overexpressers possessed suppressed averaged tumor sizes, decreased numbers of mitoses, and an increased dominance of the differentiating part (more than 70% of the tumor histological picture) (fig. 6 and table 1).

These differential features of PKC-overexpressing cells in tumorigenesis were also proven by analyzing the number of Ki67-positive cells in the tumors. As seen in table 1, in tumors induced by nPKCε- and cPKCβ-overexpressing cells, the number of Ki67-positive (proliferating) cells increased whereas in tumors induced by cPKCα and nPKCδ transfectants, the number decreased compared to that of the control cells.

Discussion

Previous experimental findings from our laboratory [19], in good accordance with a wide array of data in the liter-

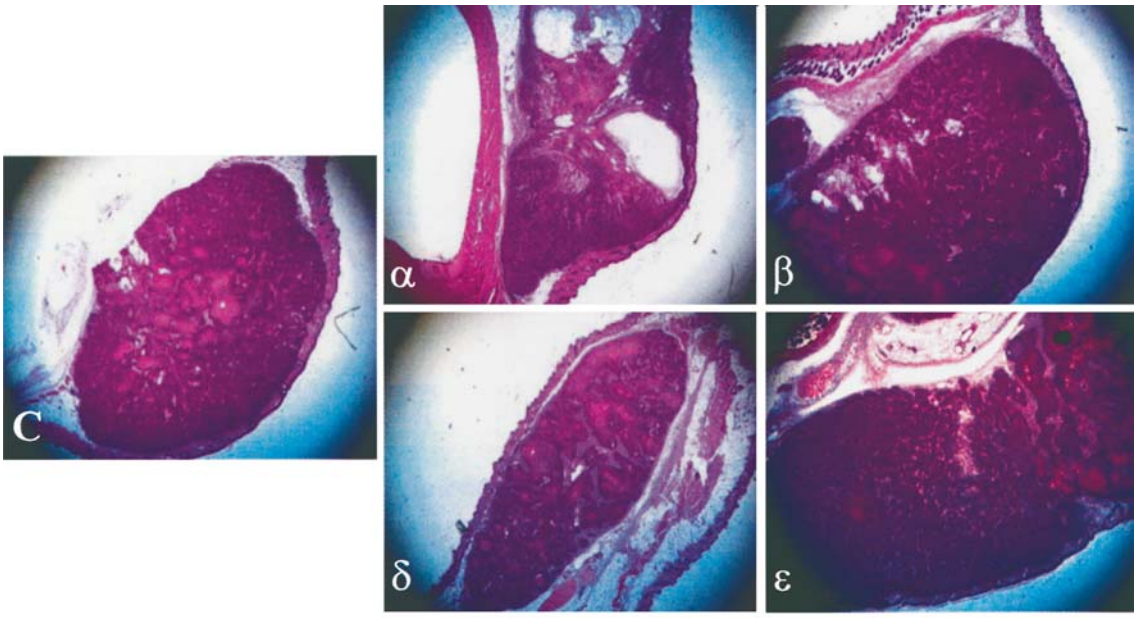

Figure 6. Cells overexpressing certain PKC isoforms induce different tumors in SCID mice. Stable transfectants of HaCaT cells overexpressing the different PKC isoforms or the control empty vector (C) at $1-2 \times 10^6$ viable cells/200 µl density were injected intradermally into SCID mice. After 30 days, animals were euthanized, the developed tumors were excised and HE staining was performed on formalin-fixed paraffin-embedded sections. Original magnification, × 20.

ature [10–15], unambiguously argued for the central role of the PKC system in regulation of numerous keratinocyte-specific cellular processes. Moreover, in the case of NHEKs, isoform-specific functions of certain isozymes (e.g., cPKCα and nPKCδ and η) were also described. With respect to the HaCaT cells, however, we possessed very little information about the exact role of the PKC system and, more importantly, of the individual isoforms in the cellular mechanisms. In the present paper, using combined pharmacological and molecular biological approaches, we report that among the several PKC isoenzymes expressed in HaCaT cells [19], certain members of the cPKC and nPKC sub-families possess opposite roles in regulating proliferation, differentiation, apoptosis, and tumorigenesis.

A key issue in the interpretation of our findings was the relevance of our data obtained with HaCaT keratinocytes for complete NHEKs. The cPKCα isoform, one of the most studied PKC isoforms in keratinocyte biology, was shown to play a central role in the calcium- and high cell density-induced terminal differentiation program in mouse and human keratinocytes [10–13, 15, 30]. This isoform was also shown to be rapidly and markedly down-regulated by phorbol esters both in NHEKs [10] and HaCaT cells [19], which was regarded as a key signal in mediating the action of the above agents to induce terminal differentiation. In our current experiments, we furthermore showed that the overexpression of cPKCα promoted differentiation and apoptosis but inhibited proliferation and tumor growth (figs 4–6). These findings strongly argue that, similar to its behavior in NHEKs, cPKCα may play a central role in the positive regulation of differentiation and negative regulation of growth of HaCaT keratinocytes.

Comparison of data obtained with cells overexpressing cPKCα (i.e., stimulation of apoptosis and differentiation, inhibition of growth) with those obtained using Gö6976, the inhibitor of the cPKC isoforms (i.e., inhibition of both proliferation and differentiation; fig. 1) suggested that cPKCβ (another cPKC isoform in HaCaT cells that was presumably also inhibited by Gö6976) may oppositely regulate the above processes. Indeed, the proliferation and tumorigenic activity of cells overexpressing cPKCβ was markedly increased whereas their differentiation and apoptotic tendencies were suppressed when compared to the control HaCaT cells (figs 4–6). These findings were in good accord with previous data stating that the expression of cPKCβ is altered in psoriasis where the sophisticated balance of keratinocyte proliferation and differentiation is impaired [32].

Data obtained using the compound Gö6976 highlight another important feature of the antagonistic roles of isoforms belonging to the conventional group of the PKC family. If cPKCα stimulates differentiation and inhibits growth and, conversely, cPKCβ stimulates proliferation and inhibits differentiation, the inhibitory effect of Gö6976 (the inhibitor of both cPKCα and β) on both proliferation and differentiation (fig. 1) could only be explained by the stimulatory actions of the two isoenzymes (i.e., cPKCα on differentiation and apoptosis, cPKCβ on

proliferation and tumorigenicity) being more effective than their inhibitory actions on the other responses. Nevertheless, our data clearly present the opposite roles of cPKC isoforms possessing similar activation mechanisms in regulating various cellular processes in HaCaT keratinocytes.

During the investigation of the novel PKC isoenzymes, a strikingly similar phenomenon was observed. The nPKCε has been extensively documented as a key positive regulator of cellular proliferation in various cell types [4, 5, 7, 9]. Overexpression of nPKCε was also shown to lead to pathological proliferation (hyperproliferative transformation) and down-regulation or overexpression of the dominant negative mutant of the enzyme results in inhibition of proliferation and induction of differentiation [5, 7, 9]. In addition, very recently, the promoting role of nPKCε in skin tumor formation in transgenic mice was also reported [12]. Since we have previously shown that, as in NHEK cells [30], nPKCε is almost exclusively expressed in the proliferating but not in the differentiating HaCaT cells [19], our current results that the growth rate and tumorigenicity of cells overexpressing nPKCε dramatically increased whereas the differentiation and apoptotic capacities decreased (figs 4–6) unambiguously argue for the positive regulatory role of this isoform in in vitro and in vivo growth of HaCaT keratinocytes as well.

Opposite findings were obtained regarding nPKCδ (figs 1, 4–6); i. e., this isoform functioned as a positive regulator of differentiation and apoptosis with a parallel inhibitory action on cellular and tumor growth. We have previously shown that the expression level of nPKCδ was remarkably elevated in differentiating HaCaT keratinocytes [19]. In addition, several groups have reported that the activation or overexpression of nPKCδ in keratinocytes inhibited proliferation, initiated the differentiation program [14], and mediated the apoptotic effect of several inducers both in NHEK [14, 33, 34] and HaCaT [35] cells. In addition, malignant transformation of HaCaT keratinocytes by Ha-ras overexpression was shown to result in the disappearance of nPKCδ from the cells [36] and treatment of control HaCaT cells by rottlerin induced an altered (proliferating) phenotype [37]. Consistent with these literature data, our findings further argue for the positive and central role of nPKCδ in the initiation and development of differentiation and apoptosis.

Finally, we should note that, to the best of our knowledge, this is the first demonstration of the tumor-inducing properties of HaCaT cells in SCID mice. In contrast to data obtained in nude mice, where control HaCaT cells formed characteristic cystic granules (which regressed after a few weeks) [16, 18], HaCaT keratinocytes induced expansively growing benign tumors in SCID animals which were histologically very similar to those developed by injecting benign ras-transfected HaCaT cells into nude mice [17, 18]. These data argue that, similar to the growth properties of other tumorigenic cell types [38–40], the tumor-inducing capabilities of HaCaT cells are more profound in SCID mice than in nude mice. Nevertheless, since the overexpression of none of the PKC isoforms (not even of cPKCβ or nPKCε) resulted in malignant transformation (in contrast to malignant ras transfection) [17, 18], although certain PKC isoforms stimulate in vitro and in vivo growth of HaCaT keratinocytes, their constitutive presence alone is apparently not enough for malignant transformation of the cells.

In summary, we can conclude that the isoform-specific roles of certain cPKC and nPKC isoforms enrolled in this study in the regulation of in vitro and in vivo growth, differentiation, and apoptosis of human HaCaT keratinocytes are very similar to those previously described in NHEKs, supporting the relevance of our findings for complete normal human epidermal cells.

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1 Nishizuka Y. (1988) The molecular heterogeneity of protein kinase C and its implication for cellular regulation. Nature **334:** 661–665
2 Ohno S., Akita Y., Hata A., Osada S., Kubo K., Kohno Y. et al. (1991) Structural and functional diversities of a family of signal transducing protein kinases, protein kinase C family; two distinct classes of PKC, conventional cPKC and novel nPKC. Adv. Enzyme Regul. **31:** 287–303
3 Nishizuka Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science **258:** 607–614
4 Gutcher I., Webb P. R. and Anderson N. G. (2003) The isoform-specific regulation of apoptosis by protein kinase C. Cell. Mol. Life Sci. **60:** 1061–1070
5 Goodnight J. A., Mischak H. and Mushinski J. F. (1994) Selective involvement of protein kinase C isozymes in differentiation and neoplastic transformation. Adv. Cancer Res. **64:** 159–209
6 Goodnight J. A., Mischak H., Kolch W. and Mushinski J. F. (1995) Immunocytochemical localization of eight protein kinase C isozymes overexpressed in NIN 3T3 fibroblasts: isoform-specific association with microfilaments, Golgi, endoplasmic reticulum, and nuclear and cell membranes. J. Biol. Chem. **270:** 9991–10001
7 Mischak H., Goodnight J. A., Kolch W., Martiny-Baron G. M., Schaechtle C., Kazanietz M. G. et al. (1993) Overexpression of protein kinase C-δ and -ε in NIH 3T3 cells induces opposite effects on growth, morphology, anchorage dependence, and tumorigenicity. J. Biol. Chem. **268:** 6090–6096
8 Murray N. R., Baumgardner G. P., Burns D. J. and Fields A. P. (1993) Protein kinase C isotypes in human erythroleukemia (K562) cell proliferation and differentiation: evidence that beta II protein kinase C is required for proliferation. J. Biol. Chem. **268:** 15847–15853
9 Brodie C., Kuperstein I., Ács P. and Blumberg P. M. (1998) Differential role of specific PKC isoforms in the proliferation of glial cells and the expression of the astrocytic markers GFAP and glutamine synthetase. Mol. Brain Res. **56:** 108–117
10 Lee Y. S., Yuspa S. H. and Dlugosz A. A. (1998) Differentiation of cultured human epidermal keratinocytes at high cell densi-

ties is mediated by endogenous activation of the protein kinase C pathway. J. Invest. Dermatol. **111:** 762–766
11 Bikle D. D., Ng D., Tu C. L., Oda Y. and Xie Z. (2001) Calcium- and vitamin D-regulated keratinocyte differentiation. Mol. Cell. Endocrinol. **177:** 161–171
12 Jansen A. P., Dreckschmidt N. E., Verwiebe E. D., Wheeler D. L., Oberley T. D. and Verma A. K. (2001) Regulation of the induction of epidermal ornithine decarboxylase and hyperplasia to the different skin tumor-promotion susceptibilities of protein kinase C alpha, -delta, and -epsilon transgenic mice. Int. J. Cancer. **93:** 635–643
13 Neill G. W., Ghali L. R., Green J. L., Ikram M. S., Philpott M. P. and Quinn A. G. (2003) Loss of protein kinase C alpha expression may enhance the tumorigenic potential of Gli1 basal cell carcinoma. Cancer Res. **63:** 4692–4697
14 Li L., Lorenzo P. S., Bogi K., Blumberg P. M. and Yuspa S. H. (1999) Protein kinase Cδ targets mitochondria, alters mitochondrial membrane potential, and induces apoptosis in normal and neoplastic keratinocytes when overexpressed by an adenoviral vector. Mol. Cell. Biol. **19:** 8547–8558
15 Lee Y. S., Dlugosz A. A., McKay R., Dean N. M. and Yuspa S. H. (1997) Definition by specific antisense oligonucleotides of a role for protein kinase Cα in expression of differentiation markers in normal and neoplastic mouse epidermal keratinocytes. Mol. Carcinog. **18:** 44–53
16 Boukamp P., Petrussevska R. T., Breitkreutz D., Hornung J., Markham A. and Fusenig N. E. (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J. Cell Biol. **106:** 761–771
17 Breitkreutz D., Boukamp P., Ryle C. M., Stark H.-J., Roop D. R. and Fusenig N. E. (1991) Epidermal morphogenesis and keratin expression in c-Ha-ras-tranfected tumorgenic clones of the human HaCaT cell line. Cancer Res. **51:** 4402–4409
18 Fusenig N. E. and Boukamp P. (1998) Multiple stages and genetic alterations in immortalization, malignant transformation, and tumor progression of human skin keratinocytes. Mol. Carcinog. **23:** 144–158
19 Papp H., Czifra G., Lázár J., Boczán J., Gönczi M., Csernoch L. et al. (2003) Protein kinase C isozymes regulate proliferation and high cell density-mediated differentiation of HaCaT keratinocytes. Exp. Dermatol. **12:** 811–824
20 Ács P., Bögi K., Marquez A. M., Lorenzo P. S., Bíró T., Szállási Z. et al. (1997) The catalytic domain of protein kinase C chimeras modulates the affinity and targeting of phorbol ester induced translocation. J. Biol. Chem. **272:** 22148–22153
21 Ács P., Wang Q. J., Bögi K., Marquez A. M., Lorenzo P. S., Bíró T. et al. (1997) Both the catalytic and regulatory domains of protein kinase C chimeras modulate the proliferation properties of NIH 3T3 cells. J. Biol. Chem. **272:** 28793–28799
22 Oláh Z., Lehel C., Jakab G. and Anderson W. B. (1994) A cloning and epsilon-epitope-tagging insert for the expression of polymerase chain reaction-generated cDNA fragments in *Escherichia coli* and mammalian cells. Anal. Biochem. **221:** 94–102
23 Lázár J., Szabó T., Kovács L., Blumberg P. M. and Bíró T. (2003) Distinct features of recombinant vanilloid receptor-1 expressed in various expression systems. Cell. Mol. Life Sci. **60:** 2228–2240
24 Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
25 Rose D. S. C., Maddox P. H. and Brown D. C (1994) Which proliferation markers for routine immunohistology? A comparison of five antibodies. J. Clin. Pathol. **47:** 1010–1014
26 Toullec D., Pianetti P., Coste H., Bellevergue P., Grand-Perret T., Ajakane M. et al. (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J. Biol. Chem. **266:** 15771–15781
27 Martiny-Baron G., Kazanietz M. G., Mischak H., Blumberg P. M., Kochs G., Hug H. et al. (1993) Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. J. Biol. Chem. **268:** 9194–9197
28 Gschwendt M., Müller H.-J., Kialbassa K., Zang R., Kittstein W, Rincke G. et al. (1994) Rottlerin, a novel protein kinase inhibitor. Biochem. Biophys. Res. Commun. **199:** 93–98
29 Alessi D. R. (1997) The protein kinase C inhibitors Ro 318220 and GF 109203X are equally potent inhibitors of MAPKAP kinase-1β and p70 S6 kinase. FEBS Lett. **402:** 121–123
30 Zang L. C., Ng D. C. and Bikle D. D. (2003) Role of protein kinase α in calcium induced keratinocyte differentiation: defective regulation in squamous cell carcinoma. J. Cell. Physiol. **195:** 249–259
31 Müller-Wieprecht V., Riebeling C., Stooss A., Orfanos C. E. and Geilen C. C. (2000) Bcl-2 transfected HaCaT keratinocytes resist apoptotic signals of ceramides, tumor necrosis factor α and 1α,25-dihydroxyvitamin D_3. Arch. Dermatol. Res. **292:** 455–462
32 Fisher G. J., Tavakkol A., Leach K., Burns D., Basta P., Loomis C. et al. (1993) Differential expression of protein kinase C isoenzymes in normal and psoriatic adult human skin: reduced expression of protein kinase C-betaII in psoriasis. J. Invest. Dermatol. **101:** 553–559
33 Denning M. F., Wang Y., Nickoloff B. J. and Wrone-Smith T. (1998) Protein kinase Cdelta is activated by caspase-dependent proteolysis during ultraviolet radiation-induced apoptosis of human keratinocytes. J. Biol. Chem. **273:** 29995–30002
34 Denning M. F., Wang Y., Tibudan S., Alkan S., Nickoloff B. J. and Qin J. Z. (2002) Caspase activation and disruption of mitochondrial membrane potential during UV radiation-induced apoptosis of human keratinocytes requires activation of protein kinase C. Cell Death Differ. **9:** 40–52
35 Fukunaga M., Oka M., Ichihashi M., Yamamoto T., Matsuzaki H. and Kikkawa U. (2001) UV-induced tyrosine phosphorylation of PKC delta and promotion of apoptosis in the HaCaT cell line. Biochem. Biophys. Res. Commun. **289:** 573–579
36 Geiges D., Marks F. and Gschwendt M. (1995) Loss of protein kinase Cδ from human HaCaT keratinocytes upon ras transfection is mediated by TGFα. Exp. Cell. Res. **219:** 299–303
37 Dietrich C., Gumpert N., Heit I., Borchert-Stuchltrager M., Oesch F. and Wieser R. (2001) Rottlerin induces a transformed phenotype in human keratinocytes. Biochem. Biophys. Res. Commun. **282:** 575–579
38 Xie X., Brunner N., Jensen G., Albrectsen J., Gotthardsen B. and Rygaard J. (1992) Comparative studies between nude and scid mice on the growth and metastatic behavior of xenografted human tumors. Clin. Exp. Metastasis **10:** 201–210
39 Taghian A., Budach W., Zietman A., Freeman J., Gioioso D. and Suit H. D. (1993) Quantitative comparison between the transplantability of human and murine tumors into the brain of NCr/Sed-nu/nu nude and severe combined immunodeficient mice. Cancer Res. **53:** 5018–5021
40 Kubota T., Yamaguchi H., Watanabe M., Yamamoto T., Takahara T., Takeuchi T. et al. (1993) Growth of human tumor xenografts in nude mice and mice with severe combined immunodeficiency (SCID). Surg. Today **23:** 375–377