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Differential localisation of nPKCδ during cell cycle progression

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Abstract

nPKCδ is a phospholipid-dependent and calcium-independent PKC isoform, whose over expression in BL6T murine melanoma cells, modifies their proliferative and metastatic potential in vivo. We focus here on the possible relationship between the subcellular localisation of nPKCδ and distinct phase of the cell cycle. Our findings show a dynamic localisation of nPKCδ in dependence of the phase of the cell cycle. Actually, this isoform is preferentially localised to the cytoplasm in serum-starved cells, shifting to the nucleus during the S-phase and becoming peri-nuclear, associated to the Golgi apparatus, in G2-M phase. Therefore, taken together our findings demonstrate that the subcellular localisation of nPKCδ changes dynamically during the cell cycle in dependence of the requirement of the enzyme at a particular place of the cell.

Keywords: nPKCδ; Melanoma cells; BL6 cells; Nucleus; Golgi apparatus

The term PKC stands for a whole family of serine/threonine kinases, comprising at least 11 mammalian isoforms, which show differences in structure and enzymatic proprieties [1]. Comparison of PKC activity in tumor cells and in the normal tissue counterpart reveals the specific involvement of individual PKC isoforms in tumor promotion and progression. For instance, a correlation between high PKC expression and malignant transformation and proliferation activity has been reported for various tumor-derived cell lines [2–4], whereas, in others, decreased PKC levels are associated with increased proliferation [5,6]. Thus, up-regulation, as well as down-regulation of PKC expression could be observed correlated with a malignant transformation and proliferative activity in different tumor cells.

Many studies, in which specific PKC isoforms have been over expressed, revealed that the effects of over expression strongly depend on the cellular system. In particular, in murine melanoma cells the over expression of nPKCδ, a phospholipid-dependent and calcium-independent isoform, modifies the melanin levels, the proliferative and metastatic ability in vivo [7]. nPKCδ is involved in keratinocyte differentiation [8], in *sis*-induced transformation of NIH3T3 via the activation of genes containing the TPA response element [9]. Moreover, over expression of such an isoform causes growth inhibition in CHO cells [10], in smooth muscle [11], in NIH3T3 cells [12], in human glioma cells [13], and in capillary endothelial cells [14], changing cell morphology, decreasing cell density, and suppressing G1 cyclin expression.

The different subcellular localisation of PKC isoforms is known to play an important role in determining the enzyme activity and specificity: PKCs have been found in the nucleus and in various subnuclear and subcellular compartments [15] in control condition and/or in response to different physiological stimuli and exogenous ligands [16]. Studies using conventional or confocal microscopy reveal a more complex and specific localisation of PKC isozymes. Most inactive isozymes are localised to subcellular structures and, upon activation, translocate to new distinct intracellular sites. For example, PKCδ is localised to the Golgi area in control NG108-15 neuroblastoma × glioma hybrid cells but, after activation by phorbol ester, it is found in the

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perinucleus and nucleus [17]. Correlation of nuclear localisation with differentiation and proliferation are indicative of a role of nuclear PKC in growth regulation [3,18–20].

To further address the role of nPKCδ during cell growth, in the present study we investigate the possible relationship between the intracellular localisation of the enzyme and cell cycle progression in BL6 murine melanoma cells over expressing nPKCδ (BL6T). Parental cell line (BL6 cells) does not express such an isoform [7].

Materials and methods

Reagents and antibodies. The polyclonal and monoclonal anti-nPKCδ antibodies were obtained from Santa Cruz Biotechnology (sc-213) and Transduction Laboratories, the competitive peptide was from Transduction Laboratories. The peroxidase-labelled secondary antibody for the Western blot was obtained from Roche (BM, Chemiluminescence Western blotting kit). The culture media and fetal bovine serum were from Life Technology. The vectastain universal elite ABC kit and the chromogene VIP for immunohistochemistry were from Vector. The FITC-conjugated anti-mouse and Texas-red conjugated anti-rabbit IgG were from Jackson Immunoresearch (West Grove, PA).

Cell culture and treatments. The BL6 and BL6T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1% non-essential amino acids, 2% MEM vitamin solution, 1% sodium pyruvate solution, 100 U penicillin, and 100 mg streptomycin at 37 °C in a humid atmosphere (5% CO_2, 95% air). The cells were detached by trypsinisation (trypsin 0.05% plus EDTA 0.02%). Over expressing nPKCδ cells were obtained and characterised as described previously [7].

Cells were made quiescent by serum starvation in DMEM plus 0.5% fetal calf serum (G0/G1) for 72 h, they were then given 20% fetal calf serum to induce the synchronous entry into the cell cycle and analysed up to 24 h after adding 20% fetal calf serum. To block the cells in early S-phase, the cells arrested in G0/G1 were incubated with complete medium plus 20% fetal calf serum and 3 mM hydroxyurea (Calbiochem) for 24 h. To obtain metaphase-arrested cells, an exponential growing culture was exposed to 50 ng/ml nocodazole for 14–15 h.

Cell cycle analysis. The cell cycle analysis was performed according to [7] with minor changes. Subconfluent cells were fixed in iced ethanol and kept at 4 °C until staining for the cytofluorimetric analysis. The fixed cells were treated with 1 mg/ml RNase (Sigma, St Louis, MO) for 1.3 h. Then, after brief washing in PBS, the cells were incubated with 50 μg/ml of propidium iodide (PI) for 20 min in ice to stain nucleic acids and immediately acquired from a FACSscanplus (Becton-Dickinson) equipped with an argon-ion laser (excitation wavelength 488 nm, laser power 200 mW). The sample flow rate during analysis did not exceed 500–600 cells/s. Typically 30,000 cells were analysed per sample. Only raw data have been used to prepare the experimental figures. DNA deconvolutions have been carried out using the software program CELLFIT (Becton-Dickinson). The experiments were repeated at least four times.

Immunostaining. The subcellular localisation of nPKCδ was determined by indirect immunostaining (1 μg/ml for anti-nPKCδ antibody). The cultures were plated onto sterile glass cover slips and fixed in 4% paraformaldehyde for 20 min or ice-cold methanol for 10 min. For immunohistochemical staining, immunoperoxidase system Vectastain ABC Elite kit was used according to the manufacturer's instructions. Briefly, endogenous peroxidase activity was blocked incubating the slides for 30 min in 0.3% H_2O_2. Then, after an incubation with normal blocking serum for 20 min, the slides were incubated with the primary antibody for 30 min, for other 30 min with biotinylated secondary antibody solution, and finally with VECTASTAIN Elite ABC reagent. Vector VIP was used as peroxidase substrate until an intense purple colour developed (2–10 min). The specificity of polyclonal nPKCδ signal was determined incubating the slides with antibody and competitive peptide (Santa Cruz) 1:2. Indirect immunofluorescence was performed as described [21]: after fixation the cells were permeabilised with 0.3% Triton X-100 in PBS containing 0.2% gelatin and incubated with the rabbit polyclonal or the mouse monoclonal antibody raised against the nPKCδ isoform. A polyclonal antibody against Giantin (a gift from Dr. M. Renz, see [22]) was used as a marker of the Golgi compartment. Fluorescein isothiocyanate (FITC)-conjugated or Texas-red conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG were used as secondary reagents. To label the nuclear compartment, after PKC staining, the cells were incubated with 1 mg/ml RNase for 30 min at room temperature, washed with PBS, and then re-incubated with 1 μg/ml propidium iodide (PrI) in PBS for 30 min.

The samples were examined with a Bio-Rad MRC-1024 confocal microscope.

Subcellular extracts and immunoprecipitation of nPKCδ. The soluble and nuclear extracts were obtained according to [23]. Purity of nuclear fractions was tested using cytoplasmic and plasma membrane markers [23], contamination being less than 2%. Eighty μg of proteins was immunoprecipitated with anti-nPKCδ (2 μg/sample) overnight at 4 °C. Immune complexes were precipitated with protein A–Sepharose beads, washed three times, and finally boiled with 2× sample buffer [24].

Western blot analysis. Immunoblot analysis was carried out according to [25]. After blocking non-specific binding sites with blocking solution provided by Roche, the sheet was incubated overnight with the primary antibody (anti-nPKCδ, 1 μg/ml) and then detected with peroxidase-labelled secondary antibody and the chemiluminescent substrate luminol according to the manufacturer's instructions. Peptides used to raise the antibody were used in competition studies to demonstrate the specificity of the antibody. In order to verify that similar amounts of samples were loaded, the same sheets were incubated with an anti-β-actin antibody (Sigma). The molecular weights were estimated using prestained markers. As negative control the anti-nPKCδ antibody (from Santa Cruz or from Transduction) was incubated with buffer instead of samples before incubation with Protein A–Sepharose. No significant signal occurs using buffer in comparison to samples (data not shown).

The results were analysed by densitometric analysis using ImageMaster software (Pharmacia Biotech).

Statistical analysis. Student's *t* test was used to determine statistical significance. A P value of less than 0.05 was considered to be statistically significant.

Results

Localisation of nPKCδ in BL6T cell line

A typical experiment of nPKCδ subcellular localisation is shown in Fig. 1: in the absence of stimuli, a cytosolic localisation of nPKCδ, often enriched in the perinuclear region (arrows), was observed in approximately 68% of the cells, while a nuclear staining was detected in the remaining 32%. Interestingly, the cytofluorimetric analysis revealed that 70% of normal growing BL6T exhibited a DNA content typical of G1 phase [7], thus suggesting the possibility that the nPKCδ distribution may change during the cell division cycle. In

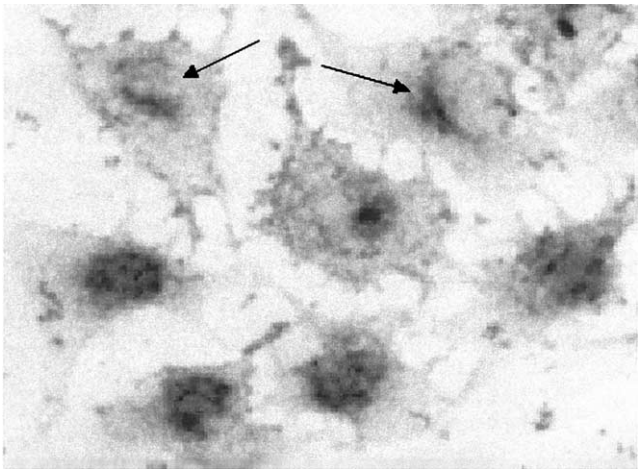

Fig. 1. Immunolocalisation of nPKCδ in BL6T cells. Subconfluent BL6T cells were fixed in 4% paraformaldehyde and incubated with anti-nPKCδ antibody for 30 min as described in the experimental procedures. The Vector VIP was used as peroxidase substrate until an intense purple colour developed (2–10 min). The arrows show the perinuclear localisation of nPKCδ. Magnitudine: 400×.

line with this idea, nPKCδ staining was always observed in the cytosol of cells undergoing cytokinesis (data not shown).

Differential localisation of nPKCδ during cell cycle progression

To understand how the distribution of nPKCδ could be regulated during the cell division cycle and to clarify the distribution of such an isoform in each phase of the cell cycle, we followed the localisation of nPKCδ in synchronous BL6T cells, arrested at different phases (early S-phase and G2-M phase of the cell cycle). Approximately 74% of BL6T cells serum starved for 72 h was arrested in G0 (as detected by FACS analysis, Fig. 2): a predominant nPKCδ cytoplasmic staining, often enriched in the perinuclear region, was observed by immunocytochemistry; no staining at the plasma membrane was detected (Fig. 3).

After addition of complete medium, cells re-enter into the cell cycle and 24 h later, approximately 56% exhibited a DNA content typical of the G2-M transition (Fig. 2). Under these conditions, nPKCδ labelling was detected in the perinuclear region of the cells, as shown in Fig. 3. The treatment with nocodazole (50 ng/ml) for 14–15 h caused a similar arrest in the G2-M transition (53% by FACS analysis, Fig. 2) and, in agreement with the above reported results, nPKCδ localised to the perinuclear region, in Golgilike structures, in approximately 60% of the cells (Fig. 3).

To study the cellular localisation of nPKCδ during the S-phase, transfected BL6T cells were exposed to 3 mM hydroxyurea for 24 h (see also experimental procedures). The results of FACS analysis indicated that approximately 85% of the cells was in S-phase (Fig. 2) and, as shown in Fig. 3, an evident nuclear staining of nPKCδ was detected. Interestingly, not only the nuclear envelope but also the nuclear matrix and nucleoli were labelled by the nPKCδ antibody. The nuclear localisation of nPKCδ in S-phase arrested BL6T cells was confirmed by means of double immunofluorescence experiments using propidium iodide to identify the nuclear compartment (Figs. 4a–c): nPKCδ co-localised with propidium iodide in the nucleus, as revealed by the bright staining in the merge. To identify the perinuclear compartment to which nPKCδ was found to accumulate during the G2-M transition, the cells were double stained with the anti-nPKCδ and anti-giantin (an integral protein of the Golgi compartment) [22] antibodies to visualise the Golgi network (Figs. 4d–f). Superimposition of the images showed co-localisation of nPKCδ and giantin in the perinuclear region (bright staining in the merge).

Using a biochemical approach, similar results were obtained: transfected cells, arrested at the indicated phases, were collected and the nuclear or the soluble fractions were separated as described in Materials and methods. Equal amounts of protein in each fraction were immunoprecipitated with the PKC antibody (anti-PKCδ antibody from Santa Cruz and from Transduction Laboratories), loaded onto a 10% SDS–polyacrylamide gel, and immunoblotted with anti-nPKCδ antibody. As showed in Fig. 5, nPKCδ was detected both in the nuclear and soluble fractions in control, untreated cells (C), and G0-arrested cells (G0). The level of such an isoform was found significantly higher in the soluble fraction than in the nuclear fraction in cells arrested in G2-M phase; in contrast, a predominant nuclear signal was detected in S-phase-arrested cells (Fig. 5).

Discussion

In this study we characterised the subcellular localisation of nPKCδ in connection with the phase of the cell cycle, in a high metastatic BL6 murine melanoma cell line (BL6T). Parental cell line did not express at significant level such an isoform [7]. We report for the first time that the subcellular localisation of nPKCδ depends on the phase of the cell cycle. Actually, while in BL6T cells an heterogeneous localisation of such an isoform occurs, in serum starved cells a predominant cytoplasmic staining has been detected and in the G2-M transition the enzyme appears preferentially perinuclear. This latter has been found either in cells re-entered into the cell cycle after 24 h and in nocodazole-treated cells arrested in G2-M phase, both showing the same percentage of cells in G2-M phase by FACS analysis. It is interesting to stress that, as demonstrated by double staining experiments, in G2-M phase, nPKCδ was detected in the perinuclear region of BL6T cells where it

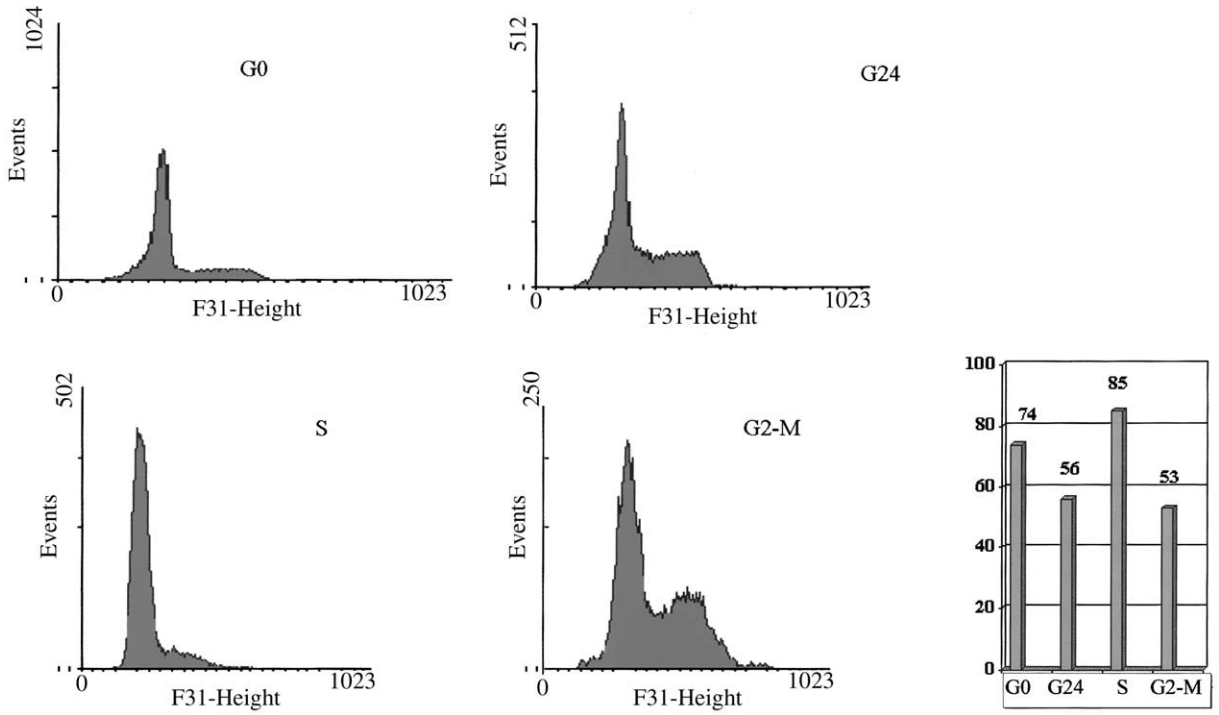

Fig. 2. Cytofluorimetric analysis of BL6T cells arrested at different phases of the cell cycle. Subconfluent BL6T cells were serum-starved for 72 h to induce a G0 arrest, incubated with new medium containing 20% serum for 24 h after serum-starvation (G24), S-phase blocked by exposition to 3 mM hydroxyurea (S), or metaphase arrested by treatment with 50 ng/ml nocodazole (G2-M), as described in the experimental procedures. After the treatments, the cells were fixed with ice-cold ethanol, treated with 1 mg/ml RNase (Sigma, St Louis, MO) for 1.3 h, and then incubated with 50 μg/ml of propidium iodide (PrI) for 20 min in ice to stain nucleic acids. The data were immediately acquired with a FACSscanplus (Becton-Dickinson) equipped with an argon-ion laser (excitation wavelength 488 nm, laser power 200 mW). Typically 30,000 cells were analysed per sample. The figure reported a typical result obtained.

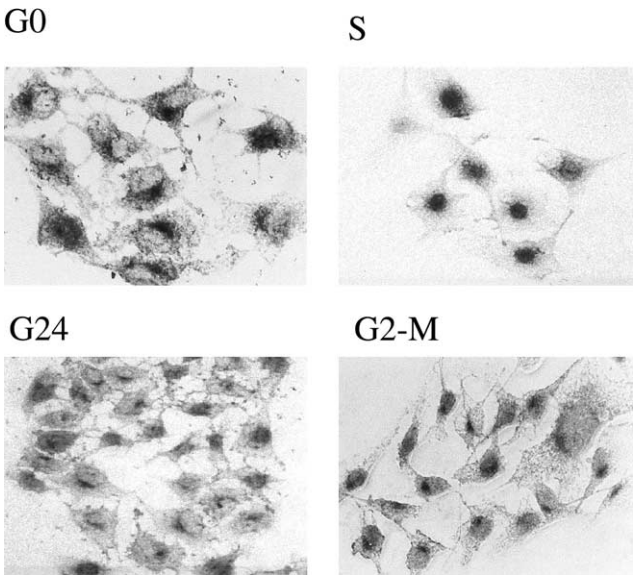

Fig. 3. nPKCδ subcellular localisation in subconfluent BL6T cells arrested at different phases of the cell cycle. Subconfluent BL6T cells, arrested in G0, G24, G2-M, and S-phase were fixed with 4% paraformaldehyde and incubated with anti-nPKCδ antibody for 30 min, as described in the experimental procedures. The Vector VIP was used as peroxidase substrate until an intense purple colour developed (2–10 min). Magnitude: 400×.

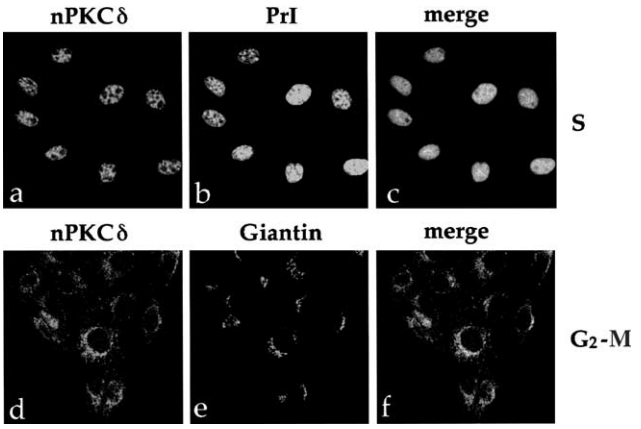

Fig. 4. Double immunofluorescence staining of subconfluent BL6T cells arrested in S-phase or G2-M phase. (a)–(c) To identify the nuclear compartment, subconfluent BL6T cells arrested in S-phase by hydroxyurea treatment were double stained with PrI and the anti-nPKCδ antibody. (d)–(f) The cells arrested in G2-M by nocodazole treatment were double stained with the anti-giantin antibody, a marker of the Golgi apparatus, and the anti-nPKCδ antibody. The merged confocal horizontal sections show co-localisation (bright staining) between nPKCδ and PrI (c) or giantin (f) in S-phase or G2-M phase arrested BL6T cells, respectively. Magnitude: 400×.

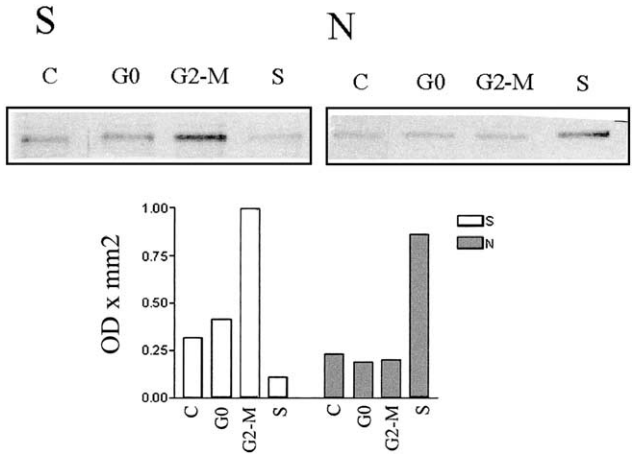

Fig. 5. Western blot analysis of soluble and nuclear nPKCδ in dependence of the cell cycle. The cells were blocked in G0, S-phase, and G2-M phase as described in the experimental procedures. The soluble (S) and nuclear (N) extracts were obtained, as described in the experimental procedures. Purity of nuclear fractions was tested using cytoplasmic and plasma membrane markers such as LDH and 5′NT, respectively. Contamination was found less than 2%. Eighty μg of proteins was immunoprecipitated with anti-nPKCδ and the immune complexes were precipitated with protein A–Sepharose beads. The samples were submitted to 10% SDS–PAGE and Western blotting with the polyclonal anti-nPKCδ antibody. Peptides used to raise the antibody were used in competition studies to demonstrate the antibody specificity. The molecular weights were estimated using prestained markers. The results were analysed by densitometric analysis using ImageMaster software (Pharmacia Biotech).

co-localised with a marker of the Golgi apparatus. During the M-phase, the Golgi apparatus disassemble: several studies have tried to identify whether specific kinases are required or sufficient for Golgi breakdown [26] and a set of mitotically active kinases and phosphatases has been characterised. Interestingly, a PKCα-dependent phosphorylation of Golgi proteins was demonstrated [27]. On the other hand, in BL6T cells arrested in early S-phase, a typical nuclear staining has been shown. nPKCδ is known to phosphorylate numerous nuclear proteins, in addition to cytoplasmic proteins, thus the presence of the nPKCδ in the nucleus may play a significant role in regulation of DNA duplication, transcription, and nuclear and nucleolar reorganisation.

In conclusion, nPKCδ shows a dynamic cell cycle-dependent subcellular localisation in dependence of the demands of the enzyme at a particular cell place.

Recently for CDK2 a relationship was demonstrated between cell proliferation and translocation of the enzyme from the cytoplasm to the nucleus, indicating that CDK2 like as nPKCδ, changes the subcellular localisation dynamically during the cell cycle [28,29]. Moreover, in glioma different chimeras of PKCs showed differential effects and distinct localisation in response to phorbol ester: the chimera PKCα/δ translocated to the plasma membrane, similar to translocation of cPKCδ, whereas the chimera PKCδ/α translocated to the nuclear and plasma membranes, similar to nPKCδ [30].

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