Proliferating and quiescent cells exhibit different subcellular distribution of protein kinase C activity

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The activity of calcium, phospholipid-dependent protein kinase (PKc), which is thought to play an important role in cell proliferation, has been measured in the particulate and soluble fractions of cultured cells, under different proliferative conditions. Our results indicate that proliferating cells display higher PKc activity than quiescent cells. Furthermore, in both normal and transformed cells, PKc is preferentially associated with the particulate fraction when the cells are proliferating, while in mitotically quiescent cells the majority of the enzyme activity is found in the soluble fraction. These data suggest that PKc activity and subcellular distribution undergo spontaneous changes according to the proliferative state of the cells.

Protein kinase C *Cell proliferation* *Phospholipid* *Diacylglycerol* *Phorbol ester*

1. INTRODUCTION

Calcium, phospholipid-dependent protein kinase (PKc) is thought to play an important role in the regulation of cell proliferation. This hypothesis is supported by the observations that: (i) the cascade of events leading to the activation of PKc can be induced by several growth factors [1,2]; (ii) direct activators of PKc (phorbol ester tumor promoters, diacylglycerols, etc.) have mitogenic activity [3–6]; (iii) important regulators of cellular proliferation are probable substrates of PKc-dependent phosphorylation [7,8]. PKc is found either in tight association with the plasma membrane or soluble in the cytoplasm and it is thought that the interaction with the phospholipid environment of the membrane is essential for the activity of the enzyme in the intact cell [5,9,10]. Recently it has been shown that an acute and transient translocation of PKc from the cytosolic to the membrane compartment can be induced by certain mitogenic stimuli [10–12]. We have therefore undertaken experiments to verify whether spontaneous changes in the proliferative activity of cultured cells were accompanied by changes in the activity and/or subcellular distribution of PKc.

2. EXPERIMENTAL

2.1. *Cell cultures*

Human fibroblasts were obtained and cultured according to standard procedures [13] in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and used at passage 3–7. Approx. 10^4 and 5×10^4 cells/cm^2 were plated to obtain, 36–48 h later, sparse proliferating and confluent quiescent cultures, respectively (as estimated by both phase-contrast microscopy and [^{3}H]thymidine incorporation).

BALB/c 3T3 cells, clone A31-1-1 [14,15], were cultured in MEM supplemented with 10% FBS and used at passage 10–16. BALB/c 3T3 cells were plated at 10^4 and 10^5 cells/cm^2 to obtain, 36–48 h later, sparse and confluent cultures, respectively.

3T3-DMBA cells (a cell line derived by cloning selection of 9,10-dimethyl-1,2-benzanthracene-treated BALB/c 3T3 A31-1-1 cells [15,16]) were grown in MEM supplemented with 10% FBS and

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seeded at either 10 or 200% confluency to obtain, 40–48 h later, sparse and dense cultures, respectively. At the time when protein kinase assays were conducted, [^{3}H]thymidine incorporation into sparse 3T3-DMBA cultures (approx. 50% confluency) was 135–150% that of dense cultures (based on cpm/mg protein).

Raji cells (a human lymphoblastoid cell line established from Burkitt's lymphoma) [17] were cultured in RPMI 1640 supplemented with 10% FBS. Raji cultures were seeded at either 3×10^5 or 10^6 cells/ml. The latter cultures were supplemented with 3 mM *n*-butyrate to arrest cell proliferation [18]. PKc assays were conducted approx. 48 h later, at which time the cell density of both groups was similar (10^6 cells/ml). Proliferation and viability of both groups of cultures was monitored daily by [^{3}H]thymidine incorporation and cell counting.

Culture media and sera were from Gibco, plasticware was from Nunc. Raji cells were a kind gift of Dr D.V. Ablashi, NCI, NIH.

2.2. *Cell homogenization and fractionation*

For enzyme assays, cultures were rinsed with Ca^{2+}, Mg^{2+}-free saline, scraped and collected by low-speed centrifugation. Cell homogenization and PKc assay methods were based on those described by Kikkawa et al. [9], modified as follows. Cell pellets were resuspended in 10 vols ice-cold buffer H (20 mM Tris-Cl, pH 7.4, 0.25 M sucrose, 50 mM β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 0.5% aprotinin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin), rapidly Dounce homogenized (40 strokes) and centrifuged at $100000 \times g$ for 1 h. The resulting pellet (particulate fraction) was extracted and solubilized for 30 min at 0°C with continuous stirring after being rehomogenized in buffer H supplemented with 2 mM EDTA, 10 mM EGTA, 0.5% Na deoxycholate. Before assaying the $100000 \times g$ supernatant (soluble fraction), EDTA (2 mM), EGTA (10 mM) and deoxycholate (0.5%) were added.

2.3. *Protein kinase assay*

PKc incubation mixture contained, in a final volume of 0.1 ml, 20 mM Tris-Cl, pH 7.4, 50 mM β-mercaptoethanol, 2 mM EDTA, 10 mM EGTA, 5 mM $MgCl_2$, 0.4 mg/ml histone type III-S (H1), 10 μM [γ-^{32}P]ATP (5 μCi/ml, NEN), 3–6 μg enzyme protein (measured as described by Bensadoun and Weinstein [19]) and, as appropriate, 2 mM $CaCl_2$, 40 μg/ml phosphatidylserine (PS), 4 μg/ml diolein (Diol), 10^{-7} M 12-*O*-tetradecanoyl phorbol 13-acetate (TPA). Assay reagents were from Sigma. The incubation (8 min at 30°C) was terminated by the addition of 3 ml/tube ice-cold 10% trichloroacetic acid, the precipitates washed 5 times with 5% trichloroacetic acid by centrifugation, solubilized with 2 N NaOH and counted by liquid scintillation spectrometry. The assay was linear with respect to incubation time and enzyme concentration over the range of conditions used. The activity measured in the presence of Ca^{2+} alone (no PS/Diol/TPA) was considered aspecific and subtracted from the activities obtained when PS + Diol or TPA were present in the incubation mixture.

3. RESULTS AND DISCUSSION

PKc activity was measured in the soluble and particulate fractions of human fibroblasts in early passage propagated cultures and of BALB/c 3T3. Both cell types actively proliferate under standard culture conditions until they reach confluency, when density-dependent inhibition of growth occurs. Basal and Diol- or TPA-stimulated PKc activities were measured in proliferating as well as contact-inhibited cells. In the experiments conducted on sparse proliferating human fibroblasts higher PKc specific activity was present in the particulate fraction compared to the soluble fraction (fig.1A). Conversely, when confluent quiescent human fibroblasts were assayed the specific activities of both fractions were lower than those found in proliferating fibroblasts and the specific activity of the particulate fraction was lower than that of the soluble fraction (fig.1B). This resulted in a reversed ratio of cytosolic to particulate PKc activity compared to proliferating cells. These results refer to both the basal activity, as measured in the presence of Ca^{2+} and PS, and the diolein (PS Diol) or TPA (PS TPA) stimulated activity. Similar results were obtained in experiments conducted on the immortalized cell line BALB/c 3T3. Sparse proliferating BALB/c 3T3 exhibited higher enzyme activity in the particulate fraction than in the soluble fraction (fig.1C). Conversely, in con-

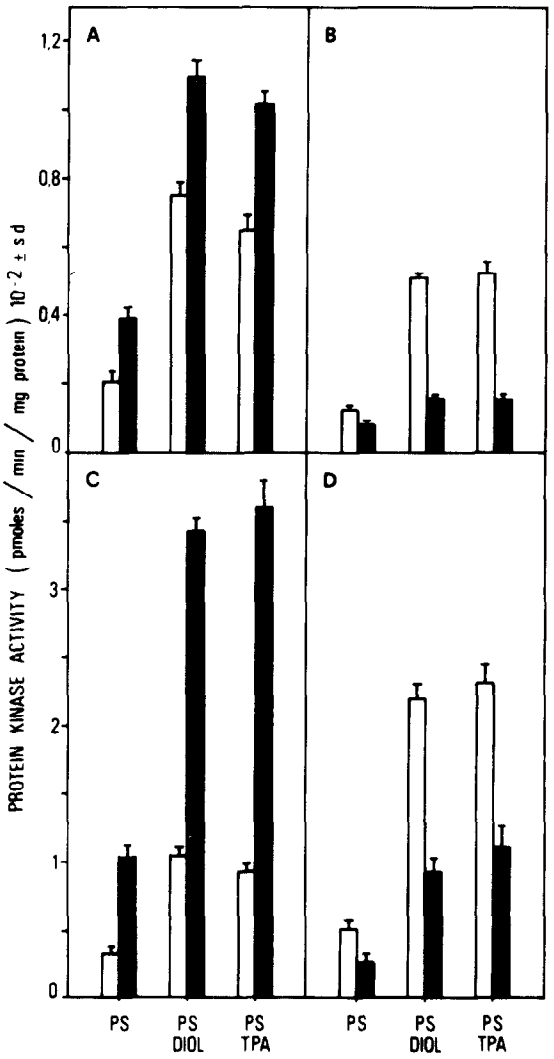

Fig.1. Protein kinase C activity of proliferating and quiescent cells. PKc activity was measured in the soluble (empty bars) and particulate (solid bars) fraction of the cells. Panels: (A) sparse, proliferating human fibroblasts; (B) confluent, quiescent human fibroblasts; (C) sparse, proliferating BALB/c 3T3; (D) confluent, quiescent BALB/c 3T3. Enzyme incubation mixtures contained Ca^{2+} and PS or Ca^{2+}, PS and either diolein (PS DIOL) or TPA (PS TPA).

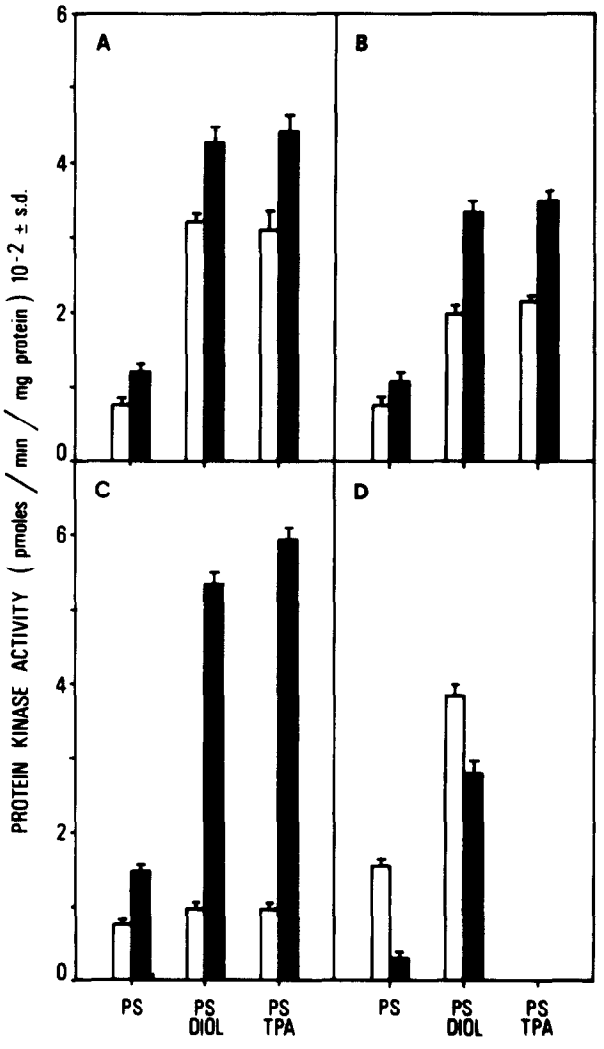

Fig.2. Protein kinase C activity of transformed cells. Panels: (A) sparse, actively proliferating 3T3-DMBA; (B) overconfluent, proliferating 3T3-DMBA; (C) actively proliferating Raji cells; (D) Raji cells inhibited to proliferate by *n*-butyrate treatment. Addition of 3 mM *n*-butyrate to the PKc reaction mixture of previously untreated Raji did not have any inhibitory effect on the enzyme activity. For symbols, see legend to fig.1.

fluent quiescent BALB/c 3T3 cultures, higher PKc activity was present in the soluble fraction than in the particulate fraction (fig.1D). This pattern resulted from both an increase of the soluble activity and a decrease of the particulate activity in quiescent cells compared to proliferating cells.

Table 1 shows that in both human fibroblasts and BALB/c 3T3 the total amount of PKc activity is higher in proliferating than in quiescent cells and that the enzyme is preferentially associated with the particulate fraction in proliferating cells, while it is mainly associated with the soluble fraction in

quiescent cells. It is possible that differences in the rate of synthesis and/or degradation of the enzyme occur between proliferating and quiescent cells.

To verify whether the described change in the distribution of PKc activity correlates with differences in cell density or proliferative state of cultured cells, protein kinase C was measured in a transformed tumorigenic cell line, 3T3-DMBA [14–16], at 2 different cell densities. 3T3-DMBA cells do not stop proliferating when they reach confluency, but continue dividing (although at a lower rate than sparse cultures) giving rise to multilayered areas. As shown in fig.2A,B, both sparse, at approx. 50% confluency, and overconfluent 3T3-DMBA exhibited higher PKc activity in the particulate fraction compared to the soluble fraction, a pattern of distribution similar to that of sparse normal cells. Also, the subcellular distribution of total soluble and particulate PKc activities was similar in sparse and confluent 3T3-DMBA (table 1). These results indicate that the activity of PKc (reflected by both its subcellular distribution and its specific activity) does not correlate with cell density and suggest that the activity of the enzyme is directly related to the proliferative state of the cells.

To determine whether the described phenomenon was limited to cells of fibroblastic phenotype or might have had broader relevance, Raji cells were used. These cells exhibit anchorage-independent growth and depend very little on exogenous growth factors for their proliferation [17]. Proliferation of Raji cells is arrested in the presence of 3 mM *n*-butyrate, a treatment that does not affect the viability of the cells [18]. The data presented in fig.2C,D indicate that in untreated actively proliferating Raji cells a higher PKc activity was found in the particulate than in the soluble fraction (fig.2C). Conversely, in *n*-butyrate-treated quiescent Raji cells (fig.2D) the specific activity of the enzyme was higher in the soluble than in the particulate fraction due to both an increase in the soluble activity and a decrease in the particulate activity (compared to proliferating Raji). It is interesting to note that the protein kinase activity found in the soluble fraction of proliferating Raji cells responded very poorly to either Diol or TPA. The distribution of total PKc activity

Table 1

Distribution of PKc activity between soluble and particulate fraction (pmol/min per ml homogenate) (± SD)

	Ca^{2+} PS			Ca^{2+} PS Diol		
	Sol.	Part.	% part.	Sol.	Part.	% part.
Human fibroblasts						
Sparse, proliferating	6.7 ± 1.0	18.7 ± 1.4	74	24.0 ± 1.1	52.5 ± 2.2	70
Confluent, quiescent	6.0 ± 0.5	3.9 ± 0.6	40	24.6 ± 0.8	7.7 ± 0.7	24
BALB/c 3T3						
Sparse, proliferating	7.6 ± 1.2	25.6 ± 2.9	77	24.2 ± 1.1	85.0 ± 2.0	78
Confluent, quiescent	11.7 ± 1.4	6.1 ± 1.2	34	50.5 ± 1.6	21.6 ± 1.5	30
3T3-DMBA						
Sparse	15.9 ± 1.5	28.4 ± 2.1	64	69.8 ± 2.5	100.4 ± 3.0	59
Confluent	17.8 ± 2.0	23.3 ± 2.3	57	47.5 ± 2.6	73.2 ± 3.6	61
Raji						
Control, proliferating	13.9 ± 0.8	24.7 ± 0.6	64	17.4 ± 1.4	89.0 ± 2.4	84
n-Butyrate, quiescent	29.9 ± 0.7	5.6 ± 0.8	16	74.8 ± 1.1	55.3 ± 0.8	42

Sol., soluble fraction; Part., particulate fraction; % part., percent of total (soluble + particulate) activity in particulate fraction

between the soluble and particulate fractions followed a pattern similar to that of the enzyme specific activity (table 1).

It is noteworthy that the transformed cells we used (3T3-DMBA and Raji) display a higher PKc activity compared to untransformed cells (human fibroblasts and BALB/c 3T3). Preliminary data from our laboratory (Adamo et al., unpublished) indicate that in both chicken myogenic and rat Sertoli cells progression of the cells along their differentiative program coincides with the reduction of PKc activity, especially in the particulate fraction.

It appears, therefore, that in normal or transformed cultured cells of different origin PKc exists in 2 physical states, either membrane-associated or cytosolic. On the basis of the particular lipid requirement for PKc activity, it is thought that the membrane-associated enzyme is physiologically functional in the intact cell, while the cytosolic enzyme is not [5,9,10]. Although conclusive evidence as to the identity between the cytosolic and the membrane-associated PKc is not yet available, membrane-associated brain PKc is indistinguishable from the cytosolic enzyme in physical, kinetic and catalytic properties [9], suggesting that the same protein constitutes both enzymes. Our data indicate that the subcellular distribution of protein kinase C may be related to the proliferative state of the cells: in proliferating cells most of the PKc activity is located in the particulate fraction, while the majority of the enzymatic activity of non-replicating cells can be recovered from the soluble fraction.

It has been shown that transient translocation of PKc activity from the cytosolic to the membrane compartment may be induced by appropriate stimuli [10–12]. Our data suggest that spontaneous changes of the localization and activity of PKc may occur under physiological growth conditions. It is possible to speculate that the different localization of PKc in growing and quiescent cells may be important for the ability of the cells to respond to physiological growth stimulatory signals, and that the onset of density-dependent inhibition of growth may coincide with dissociation of PKc from the membrane and its inactivation. Nevertheless, it remains to be demonstrated whether changes of PKc activity are the cause or consequence of changes in the proliferative state of the cells.

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