Protein expression of the α, γ, δ and ε subspecies of protein kinase C changes as C6 glioma cells become contact inhibited and quiescent in the presence of serum

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Abstract Total protein kinase C (PKC) activity and protein expression of the α and δ subspecies of PKC increases markedly as C6 glioma cells grow from low cell density to the contact-inhibited quiescent state (also known as G_o) in the presence of serum. At the same time protein expression of PKC subspecies γ and ε decreases while the β_I, β_{II}, ι and ζ subspecies did not change. Serum deprivation of growing C6 glioma cells does not induce the same changes in PKC subspecies protein expression. The findings support the growing view that there are significant differences between the G_0 states brought about by contact inhibition or serum deprivation.

Key words: Contact inhibition; C6 glioma cell; Protein kinase C subspecies expression changes; Quiescence; G_0

1. Introduction

The mitogenic effect of growth factors and phorbol esters is mediated through the protein kinase C (PKC) family of phospholipid-dependent serine/threonine kinases [1,2] which can activate proliferation-associated genes via Raf and the MAP kinase pathway [3,4]. The twelve subspecies of PKC identified to date fit into three main groups, the conventional (c) PKC subspecies α, β_I, β_{II} and γ requiring calcium and diacylglycerol (DAG), the novel (n) calcium-independent PKC subspecies δ, ε, η, μ and θ activated by DAG and the atypical (a) subspecies ζ, ι and λ being both calcium and DAG independent [5]. The c and n PKC subspecies are activated by phorbol esters [1,2]. Some PKC subspecies such as α, δ and ζ are widely expressed whereas the γ, η and θ subspecies have a restricted distribution [1,2,5]. The role of individual PKC subspecies in growth regulation is not well defined though PKC a activates Raf-1 by direct phosphorylation [4]. Over-expression of c and n group PKC subspecies in cells causes contrasting effects on growth rate and cell morphology (summarised in [6,7]) while the downregulation-sensitive PKC subspecies (α, β, δ, ε) regulate cyclin-dependent kinases in endothelial cells [8].

Serum deprivation or contact inhibition at high cell density causes cells to enter the quiescent stage of the cell cycle, also called G_0, characterised by a smaller cell size, decreased protein synthesis and monosomal ribosomes [9]. Quiescence due to contact-inhibition at high cell density has been ascribed to a lack of growth factors rather than to specific cell–cell interactions [10]. However, membrane tyrosine phosphatase activity is increased in density-dependent quiescent 3T3 cells but not in serum-deprived cells [11] while the serum deprivation response (*sdr*) gene is induced in serum starved but not contact-inhibited cells [10]. G_0 induced by high cell density may thus be different from the serum starvation condition. Here we show that PKC activity and PKC subspecies protein expression changes as C6 glioma cells become contact inhibited at high cell density. Such changes do not occur on serum starvation.

2. Materials and methods

2.1. Materials

C6 glioma cells were from the European Collection of Animal Cell Cultures (Porton Down, England, UK). Cell culture plastic was from Nunc (Life Technologies, Paisley, Scotland, UK) or Corning (Bibby-Sterilin, Stone, Staffs, UK). DMEM was from Flow Laboratories (Thame, Oxon, UK) and foetal bovine serum was from Advanced Protein Products. [^{3}H]Thymidine and the ECL detection system were from Amersham Int. (Little Chalfont, Bucks, UK). [^{33}P]ATP (1000–3000 Ci/mmol, NEG-302H) was from NEN/DuPont (UK) Ltd. (Stevenage, Herts, UK). Cellulose nitrate blotting membrane (0.2 μm) was from Schleicher and Schuell (Anderman, London, UK). BCA and Coomassie protein detection systems were from Pierce (Life Science Labs., Luton, England, UK). P-81 paper was from Whatman Int. Ltd. (Maidstone, England, UK). Leupeptin, aprotinin, PMSF, bovine serum albumin and peroxidase-conjugated second antibodies were from Sigma. Dried milk powder, Marvel, was from KwikSave (Hull Road, York, UK)

2.2. Cell culture and [^{3}H]thymidine incorporation

C6 cells were grown in DMEM/10% FBS in 25 or 75 cm^2 cell culture flasks. Medium was changed every two days. At confluency cells were split 1:4 and passaged into similar flasks or into six-well plates for thymidine incorporation and cell number assays. Cells were harvested at different stages of growth. 100% confluent cells were allowed to grow on further, with or without changes of medium, before being harvested. Cells were photographed under phase contrast optics onto T-max film.

C6 cells at different stages of growth in six-well plates were incubated at 37°C for 60 min with 1 ml DMEM containing 1 μCi [^{3}H]thymidine. Cells were then rinsed with cold Tris-saline, trypsinised from wells, counted using a haemocytometer and then recovered by low speed

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Abbreviations: ATP, adenosine-5-triphosphate; BCA, biocinchoninic acid; CB, column buffer; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; FBS, foetal bovine serum; HB, homogenisation buffer; MBP, myelin basic protein; PBS, phosphate-buffered saline; PKC, protein kinase C; PMSF, phenylmethyl sulphonyl fluoride; SDS, sodium dodecylsulphate; TBS, Tris-buffered saline; TBST, Tris-buffered saline/0.2% Tween 20.

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centrifugation. The cell pellet was rinsed with Tris-saline and homogenised in 1 ml water. Aliquots were taken for TCA precipitation. TCA-precipitated material was collected on GF/A filters which were rinsed and dried, prior to scintillation counting. Corresponding wells of C6 cells were scrapped into 1 ml distilled water and homogenised for protein estimation by the BCA method.

2.3. Protein kinase C assay

PKC activity was assayed by measuring the transfer of γ-phosphate from [^{33}P]ATP to a synthetic peptide substrate (MBP_{4-14}). It is known that PKC subspecies α, β, γ, δ and ε phosphorylate this substrate [12] although it is reported to be a poor substrate for PKC ζ [13]. Our procedure has been described in full previously [14] and is based on the previous method of Kikkawa et al [15]. C6 cells were rinsed twice with cold Tris-saline and scraped into homogenisation buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 2 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 5 mM PMSF) containing 1% Triton X-100. Extracts were homogenised, mixed on ice for 30 min and centrifuged at 100,000 × g for 15 min. The supernatants were fractionated on DE-52 columns and total PKC activity eluted with column buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.1% β-mercaptoethanol) containing 200 mM NaCl. PKC activity was assayed using MBP_{4-14} peptide (50 μmol) in the presence or absence of an inhibitor peptide (50 μmol) based on the pseudosubstrate site of PKC a. Additionally assays contained buffer (20 mM Tris-HCl, pH 7.5), Mg^{2+} (1.25 μmol), diolein (0.2 μg) and phosphatidylserine (10 μg). Assays (250 μl total volume) of calcium independent activity were performed in the presence of EGTA (125 nmol) and no calcium whereas total PKC was performed with calcium (125 nmol). Reactions were initiated by the addition of 2.5 nmol ATP containing 0.05 μCi [^{33}P]ATP (NEN) and were incubated for 15 min at 30°C. Reactions were terminated by spotting aliquots of reaction incubations onto P-81 phosphocellulose paper (in quadruplicate) which was then washed 4 times in phosphoric acid (80 mM). The P-81 paper strips were rinsed in distilled water, finally in acetone and then air-dried for scintillation counting. All results are expressed as specific activity (pmol ^{33}P-transferred/15 min/μg protein or as dpm/μg protein and are ± the standard deviation or are typical results from at least three repeats.

2.4. Immunodetection of PKC subspecies

Polyclonal antisera to the C-terminal sequences of rat PKC subspecies α, βI, βII, γ, δ, ε, ζ and η were raised and characterised as described [14,16]. C6 cells were rinsed twice in flasks with cold Tris-saline and were then scraped into homogenisation buffer (HB) without Triton X-100. Cells were homogenised, protein assayed using the Pierce BCA method and aliquots solubilised at a ratio of 2:1:1 (v/v) with 10% SDS and sample buffer followed by boiling for 5 min. Samples equivalent to 25 μg protein plus rat brain controls and suitable markers were resolved on 10% SDS-polyacrylamide gels at a constant 120 V and were then transferred to nitrocellulose membrane in 25 mM Tris base, 180 mM glycine containing 10% methanol (v/v) for 75 min with a constant current of 250 mA. Subsequent Western blotting with ECL detection was under standard conditions as we have described [14]. Primary antibodies were used at 1:2000. Detection of second antibody was by the ECL technique with X-O-graph blue film (X-O-graph, Malmsbury, Wilts., UK). Developed films were analysed using an enhanced laser densitometer (LKB).

3. Results

3.1. Cells growth

Phase contrast micrographs of C6 glioma cells at approximately 50%, 75% and 100% confluency, and quiescence, are shown in Fig. 1. Percent confluency was judged by the area of substratum in a field of view covered by growing cells. At 100% confluency no substratum was visible. Cells were cultured for up to seven more days (100/2, 4, etc.) with or without medium change depending on the experiment. At low confluency C6 cells were elongated in shape, about 15 μm in length. At 100% confluency the cells still retained this elongated shape but at quiescence they had rounded up and had a diameter of about 10 μm. Cell number per well increased steadily after the point where cells first completely covered the substratum (Fig. 2A). and doubled in the two days after cells first reached 100% confluency. After this point there was no further increase in cell numbers and incorporation of [^{3}H]thymidine decreased dramatically (Fig. 2B). After 4–7 days at full confluency, with or without changes of medium, C6 cells show little [^{3}H]thymidine incorporation indicative of the quiescent G_0 state.

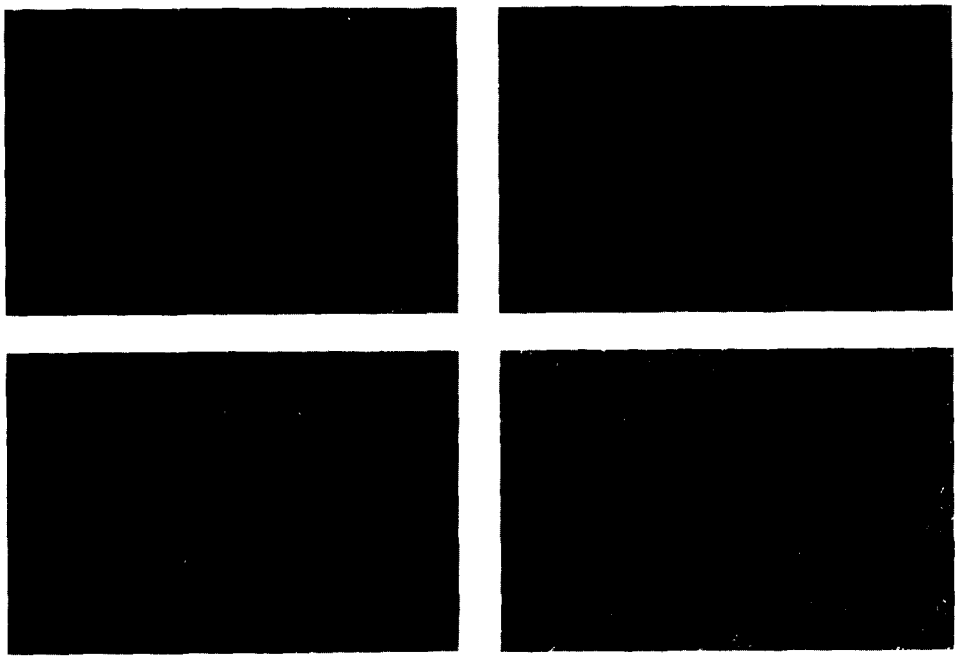

Fig. 1. Phase contrast micrographs of C6 glioma cells at different states of confluency in DMEM/10% FBS medium. (A) 50%; (B) 70–80%; (C) 100%; (D) quiescent. Magnification: ×330.

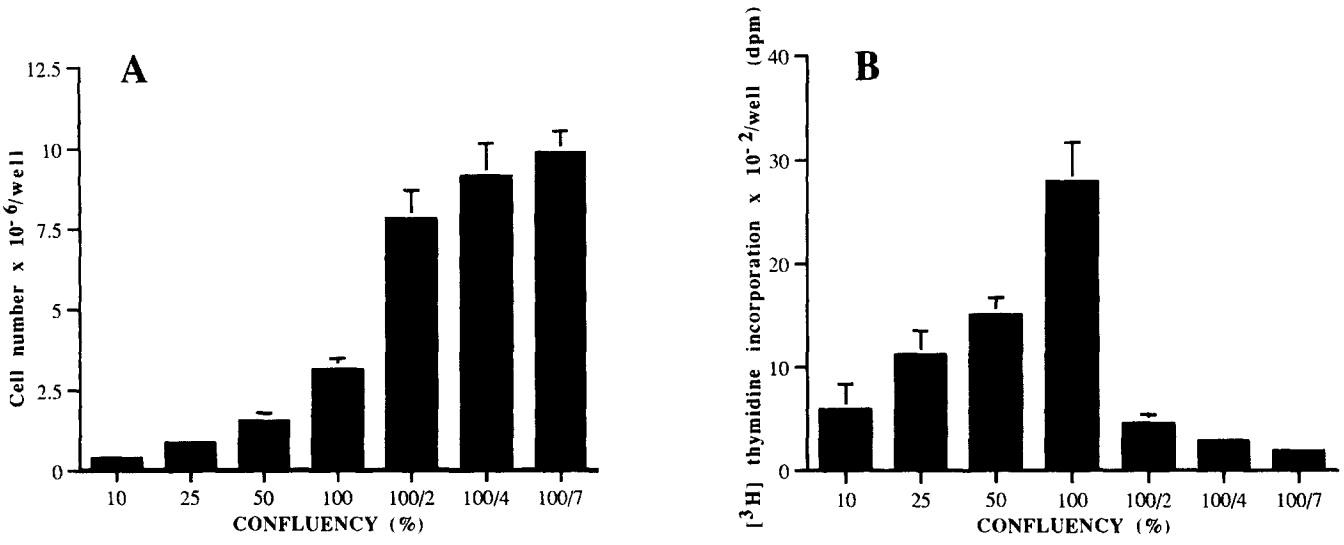

Fig. 2. Changes in (A) C6 cell number and (B) incorporation of [^{3}H]thymidine into C6 glioma cells growing in DMEM/10% FBS at different states of confluency. 100/2, 100/4 and 100/7 are 2, 4 and 7 days after cells become 100% confluent. Cell numbers were obtained by trypsinising cells from wells and counting with a haemocytometer. [^{3}H]Thymidine incorporation was as described in section 2.

3.2. Protein kinase C activity changes during C6 cell growth

As C6 cells grow to confluency the specific activity of both calcium-independent PKC subspecies and total PKC activity increases (Fig. 3), specific activity being highest in cells just reaching full confluency. PKC specific activity in quiescent C6 cells was decreased compared with the 100% confluency figure but values were still approximately double the levels in cells growing at 50% confluency with few cell–cell contacts. Serum deprivation for 24 h did not increase PKC specific activity compared with cells growing in the presence of serum (data not shown).

3.3. Protein kinase C subspecies protein expression during C6 cell growth

PKC subspecies protein expression was compared by loading equal amounts of C6 cell protein (25 μg) from cells at different growth stages onto gels which were then run, blotted and developed under standard conditions [14]. Samples of rat brain were always included as positive controls and peptide controls were also included for band identification. Blots were repeated at least twice with fresh samples of C6 cells at approximately similar stages of growth. The experiments confirmed [16] that C6 cells express PKC subspecies α, β_I, β_{II}, γ, δ, ε and ζ at the expected molecular weights with the exception of the β_I and β_{II} PKC subspecies which were always detected as bands of 40 kDa. We assume that these are proteolytic breakdown products since these were competed out with the appropriate peptide and appear in 6-week-old rat brain samples together with bands at the correct 80 kDa position. PKC subspecies η, μ amd θ were not detected in C6 cells but were detected in 6-week-old rat brain (η and μ) and rat muscle (θ) controls. PKC ι is expressed in C6 cells as a band at 90 kDa.

Protein expression of PKC subspecies α and δ increases markedly as cells grow to confluency and become quiescent by contact inhibition in the presence of serum (Fig. 4A,C). Laser scanning of ECL bands showed that in quiescent C6 cells PKC α subspecies expression increased by 2–3-fold and PKC δ by over 4-fold compared with cells at 50% confluency. For the γ and ε subspecies of PKC the opposite trend was detected: protein expression decreased as cells became confluent and these subspecies were barely detected in quiescent C6 cells (Fig. 4B,D). Protein expression of the ε subspecies decreased by as

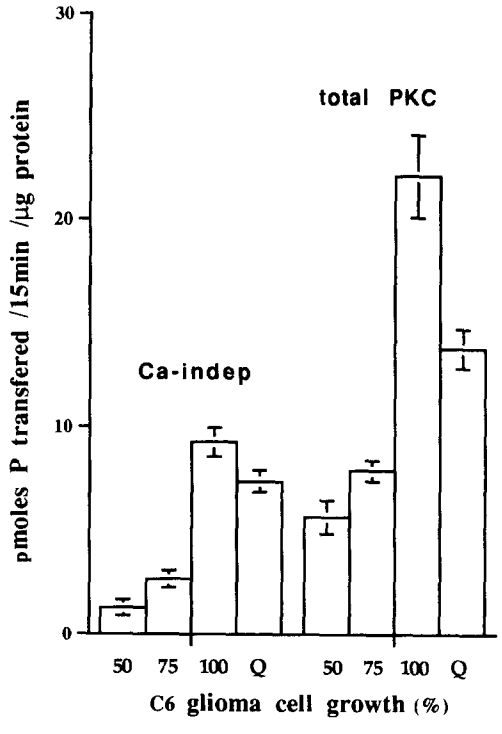

Fig. 3. Protein kinase C activity associated with C6 glioma cells at different stages of density-dependent growth arrest. Calcium-independent (nPKC) and total PKC activity were assayed as described in section 2. Results are means of specific activity from six replicates and are ±S.D. Quiescent cells (Q) were 100% confluent cells grown for a further four days in the presence of serum.

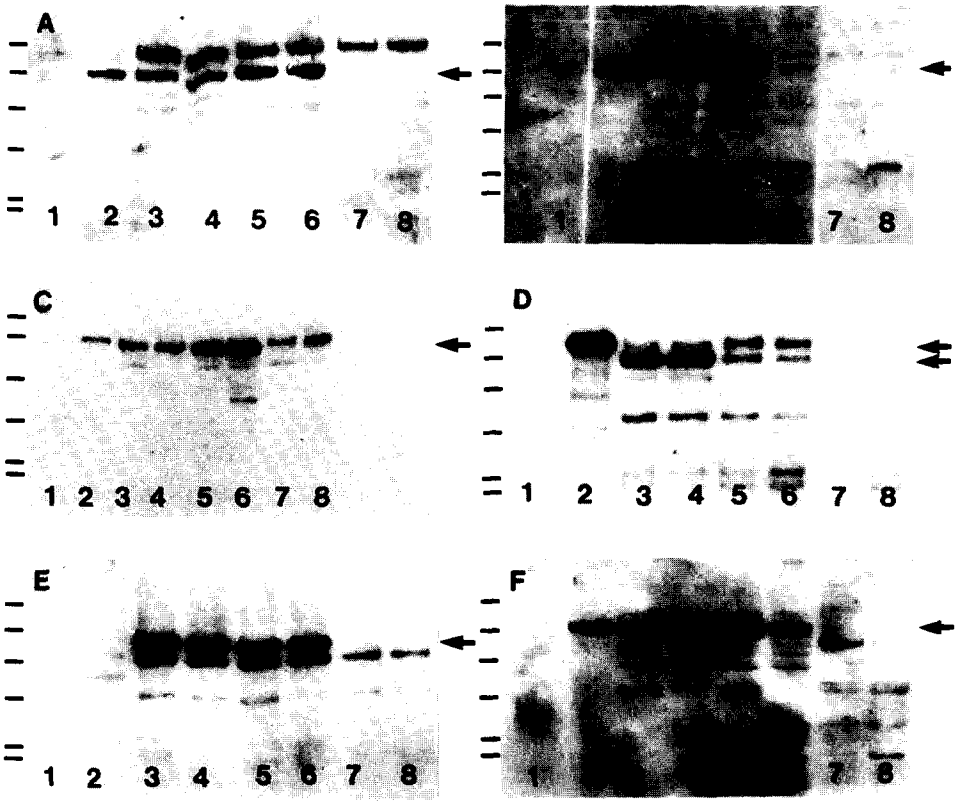

Fig. 4. Western blot analyses of PKC α (A), PKC γ (B), PKC δ (C), PKC ε (D), PKC ζ (E) and MARCKS (F) protein expression (arrows) in C6 glioma cells growing in DMEM/10% FBS at 50% (lane 3), 75% (lane 4), 100% confluency (lane 5) and quiescence − 100% + 4 days (lane 6). For all blots lane 1 is 6 week rat brain control + specific C-terminal peptide to block antibody, lane 2 is 6-week rat brain control, lanes 7 and 8 are 50% and quiescent C6 cells respectively + C-terminal peptide to block antibody. Bars indicate marker positions, from top 123, 89, 67, 50, 37.5 and 34 kDa.

much as 90% as cells became confluent and quiescent. Our anti-PKC ε antibody always detected two bands for the ε subspecies in C6 cells with molecular sizes of 95 kDa and 87 kDa (Fig. 4D). These both were competed out by the specific peptide. The 87 kDa molecular weight form was expressed strongly in rapidly growing C6 cells, expression decreasing as cells became contact inhibited at confluency and quiescence when the higher molecular weight form became more apparent. Protein expression of β_I, β_{II}, ι and ζ PKC subspecies did not change as C6 cells grew to confluency and quiescence. For these subspecies the blot for PKC ζ is shown as an example (Fig. 4E). The protein expression changes in PKC subspecies are summarised in Table 1. MARCKS protein expression increased as cells grew to confluency (Fig. 4F) but was decreased in contact inhibited quiescent C6 cells.

Removal of serum from growing C6 cells for 48 h did not result in the same changes in PKC subspecies protein expression as contact inhibition after 100% confluency (Fig. 5). There was no increase in the α and δ subspecies as found for cells becoming quiescent by contact inhibition (Fig. 5A,C), and there was no decrease in the γ and ε subspecies (Fig. 5B,D).

4. Discussion

Quiescent cells in an 'out of cycle' G_0 state have a decreased size and generally lowered rates of macromolecule synthesis and of enzymatic and membrane transport processes [9]. Such quiescent growth-arrested cells are still active, however, since synthesis of some specific RNA and proteins characteristic of

Table 1
Relative PKC subspecies protein expression as C6 cells reach quiescence

PKC isoform	Relative PKC expression		
	50% C6	100% C6	Quiescent C6
α	+	++	+++
β_I	+	+	+
β_{II}	+	+	+
γ	+	+/−−	+/−−−
δ	+	+++	++++
ε^{95}	+	>+	++
ε^{87}	+	+/−−	+/−−−
η	−	−	−
μ	−	−	−
θ	−	−	−
ζ	+	+	+
ι	+	+	+

'+' signifies 'normal' expression in rapidly dividing cells (50% confluent C6); and '−' signifies no protein expression of a PKC isoform. The increases and decreases are arbitary units derived from laser densitometric scanning of ECLs. ε^{95} is PKC ε of 95 kDa and ε^{87} is PKC ε of 87 kDa.

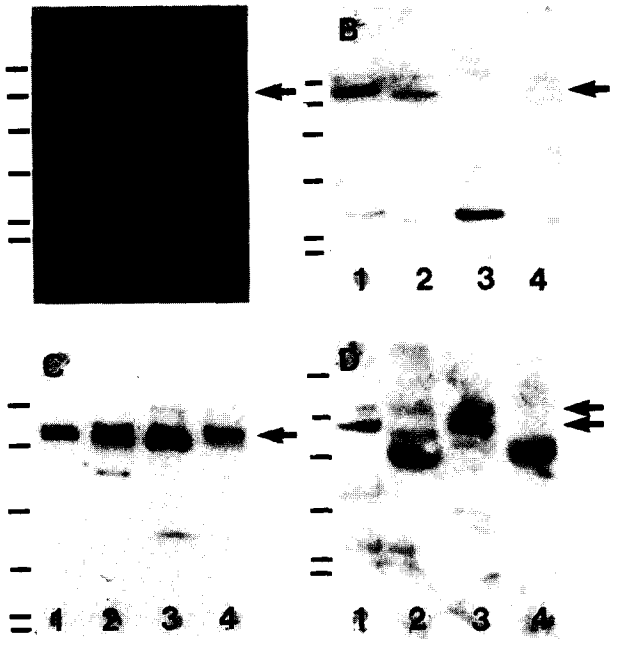

Fig. 5. Western blot analysis of protein expression changes in PKC α (A), PKC γ (B), PKC δ (C) and PKC ε (D) as C6 glioma cells reach 50% confluency (lane 1) or quiescence (lane 3) in DMEM/10% FBS compared with 50% confluent cells in DMEM/0.1% FBS for 48 h (lane 2). Lane 4 is as lane 2 but blots were incubated with the appropriate C-terminal peptide to block antibody. Bars indicate marker positions, from top 123, 89, 67, 50, 37.5 and 34 kDa.

the G_0 state occurs [10,17] and several *gas* (growth arrest-specific) genes, induced by both serum starvation or contact inhibition, have been defined (summarised in [10]). Differences in the G_0 state induced by serum starvation or density-dependent contact inhibition have been identified. For example, a serum deprivation response (*sdr*) gene is activated when NIH3T3 cells are made quiescent by serum removal but not by contact inhibition [10] while contact inhibited, but not serum-deprived, Swiss 3T3 cells in G_0 show elevated membrane tyrosine phosphatase activity [11]. MARCKS protein expression is increased in serum-starved cells in G_0 [18] but we find here that, though contact inhibition also increases protein expression of MARCKS protein as cells grow to confluency, in quiescence expression is subsequently decreased.

Involvement of PKC in the regulation of cell growth is well established [19,20] and in C6 cells PKC activity changes with different stages of the cell cycle being highest in G_0 [21]. However, the role of individual PKC subspecies in the regulation of cell growth is poorly understood [6–8] though PKC α is a RAF-1 kinase [4] which provides one route to activation of certain early response genes. Here we show that PKC activity and protein expression of the α and δ subspecies of PKC increases as C6 cells become growth-arrested by contact inhibition in the presence of serum while protein expression of the γ and ε subspecies decreases, these subspecies being barely detected in contact-inhibited quiescent C6 cells. The rise in PKC specific activity as C6 cells grow to 100% confluency can be ascribed to the increased protein expression of the α and δ subspecies since we have studied all PKC subspecies available and only the α and δ subspecies show an increase in protein expression. These changes in PKC subspecies expression do not occur when growing C6 cells are deprived of serum for 24–48 h (Fig. 5A,C). Thus we have found another difference between density-dependent contact inhibition entry into Go and the serum-deprivation mechanism.

The detection of two forms of PKC ε is particularly interesting and preliminary studies show that the low molecular weight form is exclusively associated with the cytosol while the high form, which is expressed as cells become quiescent, is principally membrane associated. Similarly, immunocytochemical staining shows that in growing C6 cells PKC ε is diffusely distributed throughout the cell but at quiescence it is largely plasma membrane associated and is detected as a rim of fluorescence at the cell periphery. Our current hypothesis is that the 95 kDa band is a more phosphorylated form.

The PKC subspecies changes we have observed occur in addition to any role for PKC subspecies in normal cell cycle control [8,21] since the cells go through several complete cell cycles of division before reaching quiescence. How the changes in PKC subspecies protein expression are triggered is not yet clear. Increasing surface contacts between cells can influence expression since addition of plasma membrane or surface membrane protein preparations to growing cells reduces cell growth rate (summarised in [22]). A purified plasma membrane glycoprotein, contactinhibin, inhibits growth of sparsely-seeded fibroblasts [22]. Further, growth of proliferating fibroblasts is inhibited by an antibody to N-CAM at the cell surface [23]. Integrin binding to the extracellular cell matrix causes changes in gene expression through the focal adhesion kinase $pp125^{FAK}$ [24]. Thus pathways to the nucleus from integrin, cell adhesion and other molecules involved in cell–cell contacts exist. The increased PKC subspecies expression may also be due to a reduction in signals from growth factors in the medium as cells are of much reduced size at quiescence [10]. This is less likely in our view since the major changes in PKC activity and subspecies expression are triggered while cells are growing to 100% confluency and are of approximately similar size. Why do C6 cells growing to quiescence increase expression of PKC α and δ and decrease expression of PKC γ and ε? Such changes may be regulating entry to G_0, slowing down the cell cycle. The negative cell cycle regulator protein p53 is a substrate for PKC [25]. It is also perhaps relevant that overexpression of PKC α in 3T3 cells decreases the cell surface density of epidermal growth factor receptors [26]. Thus the increase in PKC α expression we detect as C6 cells grow to quiescence may decrease cell growth by causing a reduction in positive signals from growth factors in the medium. Additionally, or alternatively, the change in PKC subspecies may be a preparation for subsequent re-entry to G_1. Activity of a membrane tyrosine kinase is also increased in contact-inhibited fibroblasts [8]. We find that similar PKC changes occur when L929 fibroblasts and astrocytes in primary culture grow to confluency (Williams, Moreton and Rumsby, in preparation). The results indicate that in studies on PKC the degree of cell confluency is clearly of importance when making activity or expression measurements.

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