Changes in protein kinase C ϵ phosphorylation status and intracellular localization as 3T3 and 3T6 fibroblasts grow to confluency and quiescence: a role for phosphorylation at Ser-729?

Karen ENGLAND*¹ and Martin G. RUMSBY*

*Department of Biology, University of York, P.O. Box 373, York YO10 5YW, U.K.

Protein kinase C (PKC) ϵ in 3T3 and 3T6 fibroblasts and in C6 glioma cells migrated on SDS/PAGE predominantly as a doublet with molecular masses of 87 and 95 kDa (PKC ϵ^{87} and PKC ϵ^{95} respectively). PKC ϵ^{95} predominates when cells reach confluency but PKC ϵ^{87} was the main form detected within 15 min when confluent cells were passaged at low cell density into fresh medium containing serum and allowed to adhere. Matrix-assisted laser-desorption ionization–time-of-flight MS analysis and experiments with phosphospecific antibodies revealed that PKC ϵ^{87} is phosphorylated at Thr-566 and Ser-703, and PKC ϵ^{95} is additionally phosphorylated at Ser-729. Cell fractionation studies revealed that PKC ϵ^{95} is associated with the nuclear fraction,

whereas PKC ϵ^{87} was found in the 100 000 *g* cytosol fraction. Immunofluorescence studies confirmed these findings and showed that PKC ϵ^{95} had a perinuclear, probably Golgi, localization and PKC ϵ^{87} was distributed in the cytosol. It is proposed that phosphorylation at Ser-729 may be important for determining the intracellular localization of PKC ϵ , and that a specific Ser-729 phosphatase may be activated on cell passage to convert PKC ϵ^{95} to PKC ϵ^{87} .

Key words: fibroblasts, Golgi, MALDI–TOF–MS, phosphorylation, protein kinase C.

INTRODUCTION

The protein kinase C (PKC) family of phospholipid-dependent serine/threonine kinases, at the present time, consists of 11 isoforms [1]. The conventional PKC isoforms, α , β_1 , β_2 and γ require calcium and diacylglycerol (DAG) for activation. The novel PKC isoforms δ , ϵ , η and θ are activated by DAG but are calcium-independent, whereas the atypical isoforms ζ , λ and ι are active in the absence of both calcium and DAG [1]. PKC isoforms control a diverse range of cellular functions, including proliferation, differentiation and morphology [1–3]. Evidence is accumulating that individual PKC isoforms may regulate specific functions in cells [2,4]. PKC isoforms are probably all synthesized in an inactive form and are then phosphorylated by phospholipid-dependent kinase-1 at a single threonine residue in the catalytic site (Thr-500 in PKC β_{11}) [5,6]. The protein can then undergo at least two further phosphorylations, probably by autophosphorylation at two C-terminal serine residues, at positions 641 and 660 in PKC β_{11} [7–9]. Evidence so far points to other PKC isoforms being phosphorylated similarly, as these serine and threonine residues are conserved throughout the PKC family [9,10]. These phosphorylations seem to be necessary for the activation of PKC by DAG. Activation of PKC may occur at discrete sites within a cell, possibly because of interaction with isotype-specific binding proteins [11–13]. Subsequent down-regulation of a PKC isoform may occur at the proteasome and/or via calpain-mediated proteolysis [14–17].

PKC ϵ is the only isoform which exhibits full oncogenic potential [18,19]. This may occur by the enhancement of Raf

kinase activation by PKC ϵ [20], perhaps by inducing production of autocrine growth factors [21]. PKC ϵ is also unique in having Golgi- [13,22,23] and actin-binding domains [24,25]. PKC ϵ translocates from the Golgi to the plasma membrane on phorbol ester activation [13,22,23]. PKC ϵ purified from rat brain migrates as two distinct bands with molecular masses of 93 kDa and 96 kDa; on phosphatase treatment these are reduced to a single 90 kDa species [26]. These results suggest that at least two phosphorylated forms of PKC ϵ exist. In other studies, PKC ϵ has often been detected as a doublet, but this has been rarely commented on [19,21,27]. In C6 glioma cells, PKC ϵ is clearly identified as a doublet with molecular masses of 87 and 95 kDa [28]. At low cell density the 87 kDa form, PKC ϵ^{87} , predominates but as the cells grow to confluency and become contact inhibited and quiescent the 95 kDa form, PKC ϵ^{95} , becomes the major species. It has been noted previously [29] that, when quiescent C6 glioma cells and 3T3 and 3T6 mouse fibroblasts are passaged, PKC ϵ^{87} rapidly (within 15 min) becomes the predominant form of the protein. This change in PKC- ϵ mobility as cells are passaged has also been observed in primary chondrocytes and primary astrocytes and thus may be a general feature of adherent cells upon passage.

In the present work, the phosphorylation status of PKC ϵ *in vivo* has been examined, and this has been linked to the intracellular localization of the protein. Using antibodies and matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) MS analysis, it was found that PKC ϵ^{95} was phosphorylated at Thr-566, Ser-703 and Ser-729, whereas PKC ϵ^{87} was phosphorylated at Ser-703 but not Ser-729. It was noted

Abbreviations used: AEBF, 4-(2-aminoethyl)benzenesulfonyl fluoride; BODIPY® TR ceramide, N-([4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diazas-indacen-3-yl]phenoxy)acetyl)sphingosine; DAG, diacylglycerol; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; MBP, myelin basic protein; PKC, protein kinase C.

¹ To whom correspondence should be addressed (e-mail ke5@york.ac.uk).

that the Ser-729-phosphorylated form of PKC ϵ , PKC ϵ^{95} , had a different intracellular localization from that of the 87 kDa form, which was not phosphorylated at Ser-729. PKC ϵ^{95} was detected in the perinuclear region, probably Golgi associated, whereas PKC ϵ^{87} had a cytoplasmic localization. It is suggested that phosphorylation at Ser-729 may be important for determining the intracellular localization of the protein and, thus, its cellular function. In addition, it is proposed that a Ser-729-specific protein phosphatase may be activated on cell passage to convert PKC ϵ^{95} to PKC ϵ^{87} .

EXPERIMENTAL

Materials

Cells were obtained from the European Collection of Animal Cultures (Porton Down, Wilts., U.K.) and the William Dunn Cell Bank (Oxford, Oxon, U.K.). Cell culture plasticware was from Nunc (Life Technologies, Paisley, Scotland, U.K.). All other cell culture reagents were from Gibco-BRL Life Technologies (Paisley, Scotland, U.K.) except for serum, which was from PAA Laboratories (Linz, Austria). All other chemicals were from Sigma (Poole, Dorset, U.K.) unless otherwise stated. Nitrocellulose membrane (Hybond C) and [γ - 32 P]ATP (AH9968) were from Amersham International (Little Chalfont, Bucks, U.K.). Dried milk powder was Marvel (Premier Beverages, Stafford, U.K.). Bicinchnonic acid protein assay reagents were from Pierce (Luton, Beds., U.K.). P81 phosphocellulose paper was from Whatman (Maidstone, Kent, U.K.).

Antibodies

The polyclonal PKC ϵ antibody to the C-terminal peptide sequence, used for Western blotting and immunoprecipitations, was generated by Professor N. Groome (Oxford Brookes University, Oxford, U.K.) as described previously [28]. For immunofluorescence studies, a polyclonal antibody to PKC ϵ (Sigma) was used. The anti-phospho Ser-729-PKC ϵ was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). The anti-phospho-Thr-566-PKC ϵ was a gift from Mr D. B. Parekh and Professor P. J. Parker (Imperial Cancer Research Fund, London, U.K.). Anti-phosphothreonine and anti-phosphoserine antibodies were from Zymed (San Francisco, CA, U.S.A.). The anti-phosphotyrosine antibody was from Sigma. Peroxidase- and FITC-conjugated secondary antibodies were from Sigma.

Tissue culture

Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal-calf serum in a humidified incubator at 5% CO₂, as described previously [29].

Western blotting

Cells were scraped from the flasks into lysis buffer [50 mM Tris/HCl (pH 7.5)/0.5 mM EDTA/2 mM EGTA, containing 0.5% (v/v) Nonidet P40, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 2 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), 50 mM sodium fluoride, 5 mM sodium pyrophosphate and 10 μ M sodium orthovanadate]. Protein concentration was determined using the bicinchnonic acid assay (Pierce). Laemmli loading buffer was added and equal amounts of protein (30 μ g) were loaded and separated by SDS/PAGE (7.5% gels); the

separated proteins were transferred to nitrocellulose membranes and probed for PKC ϵ , as described previously [29].

Cell fractionation

3T3 and 3T6 fibroblasts were fractionated as described by Ohmori and Arteaga [30]. Briefly, cells were harvested in homogenization buffer [20 mM Tris/HCl (pH 7.5)/5 mM EGTA containing 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 2 mM AEBSF, 50 mM NaF, 5 mM sodium pyrophosphate and 10 μ M sodium orthovanadate). Samples were homogenized with ten strokes in a hand-held homogenizer. The homogenate was passed through a 26 gauge needle several times and protein concentration was estimated using the bicinchnonic acid assay (Pierce). Samples containing equal amounts of total cell protein were then centrifuged to obtain the various cell fractions. The nuclear fraction was obtained as a pellet after centrifugation at 800 *g*; the supernatant of this centrifugation was designated the postnuclear fraction. The nuclear pellet was treated with 1% Triton X-100 for 30 min and centrifuged at 100 000 *g* for 1 h. The detergent-insoluble fraction is the nuclear scaffold and the detergent-soluble fraction contains the nuclear membrane. The postnuclear supernatant was centrifuged for 1 h at 100 000 *g*. The supernatant of this centrifugation procedure was designated the cytosolic fraction. The postnuclear pellet was treated with Triton X-100 and re-centrifuged as described above. The detergent-insoluble fraction represents the cytoskeleton and the soluble fraction is the postnuclear membrane. Pellets and supernatants were freeze-dried and rehydrated in 100 μ l of lysis buffer by sonication using an MSE 100 W ultrasonic probe disintegrator. Aliquots (20 μ l) of each fraction were used for protein separation by SDS/PAGE and the resolved proteins were analysed by Western blotting.

Immunofluorescence

Cells, grown on glass cover-slips as described previously, were rinsed three times with warm PBS, fixed for 10 min in 3% (w/v) paraformaldehyde in PBS and quenched in 50 mM NH₄Cl. Cells were permeabilized with 0.2% (v/v) Triton X-100 for 4 min, blocked by incubation with 2% (w/v) BSA, 5% (v/v) normal goat serum in PBS and labelled with an anti-PKC ϵ antibody for 1 h in 2% BSA. The coverslips were rinsed several times and then the cells were labelled with a FITC-conjugated secondary antibody in 2% BSA. After rinsing, the coverslips were inverted on to mounting media (Dako, Ely, Cambs, U.K.) and sealed with nail varnish. Cells were examined using a Nikon Labophot fluorescence microscope. To detect Golgi, cells were stained with 0.01 μ g/ml *N*-({[4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diazas-indacen-3-yl]phenoxy}acetyl)sphingosine (BODIPY[®] TR ceramide) (catalogue no. D-7540; Molecular Probes, Eugene, OR, U.S.A.) during incubation with the secondary antibody.

Immunoprecipitation

Cells were harvested in immunoprecipitation buffer [10 mM Tris/HCl, 150 mM sodium chloride, 1 mM EDTA, 0.5% SDS, 1% Triton X-100, 1% deoxycholic acid, 0.2 mM AEBSF, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 μ M sodium orthovanadate). Samples were precleared with Protein A-Sepharose at 4 °C for 1 h on a rotary stirrer. Equal amounts of cell protein were incubated with anti-PKC ϵ antibody (1:250) overnight and recovered by centrifugation with Protein A-Sepharose. After washing with immunoprecipitation buffer, the pellet was resuspended in 10% (w/v) SDS and Laemmli loading buffer.

PKC activity assay

Quiescent and newly passed cells were harvested in homogenization buffer and equal amounts of cell protein were used to prepare nuclear and cytoplasmic fractions as described above. PKC ϵ was immunoprecipitated from the fractions, as described above, except that the immunoprecipitate was washed three times with and resuspended in 20 mM Tris/HCl (pH 7.5). Half of this sample was analysed for PKC ϵ by Western blotting. The remainder was assayed for PKC activity using the pseudo-substrate inhibitor peptide method of Yasuda et al. [31]. Briefly, 200 μ l PKC activity assay buffer [20 mM Tris/HCl (pH 7.5), 25 mM magnesium acetate, 2.5 mM EDTA, 50 μ M myelin basic protein (MBP) peptide₄₋₁₄, 20 μ g phosphatidylserine, 0.4 μ g diolein] was added to each sample, with or without the PKC inhibitor peptide RFAEKGLRQKNV [31], and the reaction was initiated with 10 μ l of 2.5 nM ATP containing 1.86 kBq [γ -³³P]ATP. The assay temperature was 37 °C. The reaction was stopped after 30 min by spotting 20 μ l aliquots of the reaction mixture, in triplicate, on to P81 phosphocellulose paper squares. These were washed four times in 80 mM phosphoric acid, then in distilled water and finally in acetone. The dried P81 paper squares were added to Ultima Gold scintillant and radioactivity was estimated by scintillation spectroscopy (1900c Tri-Carb; Hewlett-Packard).

Alkaline phosphatase and λ phosphatase treatment

Total cell protein (100 μ g) in 100 μ l of 50 mM Tris/HCl (pH 9.0)/1 mM MgCl₂ containing 20 μ g/ml leupeptin and 2 μ g/ml aprotinin was triturated through a 26 gauge needle and 30 units of alkaline phosphatase were added with or without 10 mM sodium pyrophosphate as a control. Samples were incubated at 37 °C for 2 h and then dissolved in Laemmli loading buffer and analysed for PKC ϵ by Western blotting. For λ phosphatase treatment, cells (100 μ g protein) were harvested into 100 μ l 50 mM Tris/HCl (pH 7.8)/5 mM dithiothreitol/2 mM MnCl₂ containing 100 μ g/ml BSA and incubated with 200 units λ phosphatase (Calbiochem) with or without 200 mM EGTA and 50 mM NaF at 37 °C for 30 min.

MALDI-TOF-MS analysis

PKC ϵ was immunoprecipitated, as described above, from quiescent, newly passaged 3T3 and 3T6 cells and from 1-day old rat brain and resolved by SDS/PAGE (7% gels). Proteins were revealed with Coomassie Blue staining and protein kinase C ϵ bands at 87 kDa and 95 kDa were excised with a razor. Protein was digested as described in [31a]. Briefly, gel pieces were macerated in a homogenizer and destained by washing several times in 50% acetonitrile in 25 mM NH₄HCO₃. After drying, the gel particles were resuspended in 25 mM NH₄HCO₃ with 0.1 μ g/ μ l trypsin or Glu C (Boehringer Mannheim, Lewes, East Sussex, U.K.) and digested overnight according to the manufacturer's instructions. Peptides were extracted with acetonitrile/trifluoroacetic acid (10:1, v/v). The samples were concentrated (Speed-Vac) and desalted using Zip Tips (Millipore, Bedford, MA, U.S.A.); 1 μ l of sample was mixed with 1 μ l of matrix (10 mg/ml α -cyano-4-hydroxycinnamic acid) and spotted on to a MALDI-TOF-MS sample plate. MS was performed in both linear and reflector modes. A multipoint MS calibration was used. Peptides were analysed using a Voyager-DE™ STR Biospectrometry Workstation and Voyager and Grams software (Perspective Biosystems).

All data shown are representative of at least three separate experiments.

Figure 1 Western blots of PKC ϵ in quiescent and newly passaged cells

(A) 3T3 cells were grown to confluency and total cell protein was extracted (q) or cells were passaged into fresh medium and flasks for 15 min before harvesting adherent cells (p). Immunoblotting was carried out for PKC ϵ as described in the Experimental section. Molecular-mass markers at 97 and 79 kDa are indicated by arrows on the left. PKC ϵ^{95} was the major band in quiescent cells and PKC ϵ^{87} was the major form in newly passaged cells. (B) 3T3 cells were grown to confluency (q) and total protein was extracted or cells were passaged into new medium and flasks for 15 min before harvesting adherent cells (p). Immunoblotting was carried out for PKC ϵ as described in the Experimental section, except that a 10% polyacrylamide gel was used to resolve PKC ϵ fragments (*). Molecular-mass markers at 97, 79, 57 and 38 kDa are indicated by arrows on the left. It can be seen that there was no change in the intensity of the bands representing PKC ϵ proteolytic fragments on passage. (C) Total cell protein from quiescent 3T3 cells was incubated with alkaline phosphatase (ap+) or λ phosphatase (λ p+) for 30 min with or without phosphatase inhibitors (ap-, λ p-), as described in the Experimental section, before analysis for PKC ϵ by Western blotting. Molecular-mass markers at 97 and 79 kDa are indicated by arrows on the left. Treatment with alkaline and λ phosphatase decreased the intensity of the PKC ϵ^{95} band.

RESULTS

Changes in PKC ϵ molecular mass with cell growth to confluency and on passage

When 3T3 and 3T6 cell lines were grown to confluency PKC ϵ was detected largely as a 95 kDa form, PKC ϵ^{95} . However, upon

Figure 2 Detection of phosphorylation sites in PKC ϵ using phosphospecific antibodies

(A) Immunoprecipitations from quiescent (q) and newly passed (p) 3T3 cells were immunoblotted for phosphoserine (PSer) and phosphothreonine (PThr), as described in the Experimental section. The positions of PKC ϵ^{87} and PKC ϵ^{95} bands are indicated on the right, molecular-mass markers at 97 and 79 kDa are indicated by arrows on the left. The gels indicate that both PKC ϵ^{87} and PKC ϵ^{95} are phosphorylated on serine and threonine residues. (B) Total cell protein from quiescent (q) and newly passed (p) 3T3 cells were immunoblotted for PKC ϵ with (i) a PKC ϵ specific antibody, (ii) an anti-phospho-Thr-566-PKC ϵ antibody or (iii) an anti-phospho-Ser-729-PKC ϵ specific antibody, as described in the Experimental section. The positions of PKC ϵ^{87} and PKC ϵ^{95} are indicated on the right, molecular-mass markers at 97 and 79 kDa are indicated by arrows on the left. The gels show that PKC ϵ^{87} and PKC ϵ^{95} are phosphorylated at Thr-566 but only PKC ϵ^{95} is phosphorylated at Ser-729.

passage of cells, the principal PKC ϵ form detected had a molecular mass of 87 kDa (Figure 1A). Formation of PKC ϵ^{87} upon passage was rapid and was detected within 15 min of cells being passaged into new flasks and adhering. PKC can be cleaved by calpain to yield the catalytic and regulatory domains [31b] and the C-terminal PKC ϵ cleavage fragments (approx. 50 kDa) are frequently detected by Western blotting, but, in the present study, there was no consistent change in the immunoreactivity of these PKC ϵ fragments as cells were grown to confluency and passaged (Figure 1B).

Treatment of 3T3 cell homogenates with alkaline phosphatase or λ phosphatase decreased the intensity of the 95 kDa band of PKC ϵ relative to controls (Figure 1C) and led to an increased intensity in the 87 kDa band. Similar results were obtained with 3T6 fibroblasts and C6 glioma cells (results not shown). This suggests that PKC ϵ^{95} is a phosphorylated form of the protein and would account for its decreased mobility on SDS/PAGE.

Phosphorylation of PKC ϵ^{87} and PKC ϵ^{95}

PKC ϵ was immunoprecipitated from quiescent and newly passed 3T3 and 3T6 fibroblasts to analyse the phosphorylation

status of the 95 and 87 kDa forms of this protein. Immuno-precipitates were resolved by SDS/PAGE and blots were probed with anti-phosphothreonine and anti-phosphoserine antibodies. As shown in Figure 2(A), both PKC ϵ^{95} and PKC ϵ^{87} from quiescent and newly passaged 3T3 cells reacted with these antibodies, indicating that both forms of PKC ϵ were phosphoproteins and contained phosphate groups on both serine and threonine residues. Neither band reacted with an anti-phosphotyrosine antibody indicating the absence of phosphotyrosine residues (results not shown). Detection of both PKC ϵ^{95} and PKC ϵ^{87} with an anti-phospho-Thr-566-PKC ϵ -specific antibody [32] showed that Thr-566 was phosphorylated in both forms of PKC ϵ (Figure 2B).

An anti-phospho-Ser-729-PKC ϵ antibody was used to gain further information on how the 95 and 87 kDa forms of PKC ϵ differed in their phosphorylation status. Figure 2(B) shows that PKC ϵ^{95} but not PKC ϵ^{87} in 3T3 cell lysates reacted with this antibody. Thus Ser-729 was not phosphorylated in the PKC ϵ^{87} found in newly passaged fibroblast cells but was phosphorylated in PKC ϵ^{95} . Similar results were obtained with 3T6 cells.

MALDI-TOF-MS analysis of the phosphorylation status of PKC ϵ

Further examination of phosphorylation of both PKC ϵ bands was undertaken using MALDI-TOF-MS. PKC ϵ^{95} and PKC ϵ^{87} were immunoprecipitated, extracted from Coomassie-Blue-stained gels and digested with trypsin. The peptide masses detected by MALDI-TOF-MS (Figure 3) were compared with those of predicted digests using peptide mass software from Expasy (www.expasy.ch). Phosphorylation increased the peptide mass by 80 Da; it follows therefore that the mass of a phosphorylated peptide will be 80 Da greater than predicted. Peptides of mass 1604 were detected in both PKC ϵ^{95} and PKC ϵ^{87} , which correspond to phosphorylation on peptides containing Ser-703. Peptides of mass 1358, matching peptides containing a phospho group at Ser-729, were detected only in PKC ϵ^{95} . A typical MALDI-TOF-MS trace, with these peaks labelled, is shown in Figure 3. The MALDI-TOF-MS data from PKC ϵ^{95} and PKC ϵ^{87} immunoprecipitated from 3T3, 3T6 and rat brain are summarized in Table 1 and show that both PKC ϵ^{95} and PKC ϵ^{87} are phosphorylated on Ser-703. Corresponding non-phosphorylated peptides were not detected, suggesting that both of these sites are fully phosphorylated in both forms of PKC ϵ from quiescent and newly passaged cells. It also confirmed that only PKC ϵ^{95} is additionally phosphorylated on Ser-729 (Table 1 and Figure 3) and, again, corresponding non-phosphorylated peptides were not detected. The phosphorylation status of Thr-566 could not be determined using this method, as the trypsin-cleaved peptide containing this site was large and carried a weak charge which made ionization and detection by MALDI-TOF-MS difficult.

Differential localization of PKC ϵ^{95} and PKC ϵ^{87} within the cell

Western-blot analysis of nuclear membrane, nuclear matrix, postnuclear membrane, cytoskeleton and cytoplasmic fractions from quiescent and newly passaged 3T3 fibroblasts revealed that PKC ϵ^{95} was principally found in the nuclear membrane fraction (Figure 4). This fraction may have included Golgi apparatus, since a Golgi-specific dye (BODIPY[®] TR ceramide) co-fractionated with the nuclear fraction. As seen in Figure 4, PKC ϵ^{87} was recovered only in the 100 000 g cytoplasmic fraction. Neither form of PKC ϵ was detectable in the cytoskeletal or postnuclear-membrane fractions (Figure 4).

These observations were confirmed by immunofluorescence microscopy using the anti-phospho-Ser-729-PKC ϵ antibody,

Figure 3 MALDI-TOF-MS analysis of phosphorylation of PKC ϵ^{87} and PKC ϵ^{95}

(A) PKC ϵ^{95} and (B) PKC ϵ^{87} were excised from gels and digested with trypsin prior to MALDI-TOF-MS analysis, as described in the Experimental section. Representative traces are shown with the two phosphopeptides indicated. These represent phosphorylation of peptides containing sites Ser-703, (m/z 1604), detected in both PKC ϵ^{95} and PKC ϵ^{87} , and Ser-729 (m/z 1358), detected only in PKC ϵ^{95} .

which, as shown in Figure 2(B), specifically detected PKC ϵ^{95} , and an anti-PKC ϵ antibody, which detected both forms. Fluorescent labelling with the anti-phospho-Ser-729-PKC- ϵ antibody in quiescent cells was exclusively localized in the perinuclear region (Figure 5), whereas when cells were stained with the PKC ϵ antibody a similar but slightly more diffuse localization around

Figure 4 Association of PKC ϵ^{87} and PKC ϵ^{95} with the cytosolic and nuclear fractions of 3T3 cells

Total cell protein from quiescent and passaged 3T3 cells was fractionated into nuclear (n), nuclear scaffold (ns), cytoskeleton (sk), post-nuclear membrane (m) and cytoplasm (cy). Western blotting was carried out for PKC ϵ as described in the Experimental section. Molecular-mass markers at 97 and 79 kDa are indicated by arrows on the left.

the perinuclear region was seen. This was consistent with the results of Western blotting, which showed that the predominant form of PKC ϵ in these cells was PKC ϵ^{95} . In small rounded cells, 15 min after passage, fluorescent labelling with the PKC ϵ antibody produced diffuse staining of the cytoplasm. At this time point, only faint labelling with the anti-phospho-Ser-729-PKC ϵ antibody was observed, which was consistent with the decrease in PKC ϵ^{95} protein levels at 15 min after passage detected by Western blotting. PKC ϵ co-localized with BIODIPY® TR ceramide, a Golgi specific stain, which also had a perinuclear localization (results not shown). Similar results were seen in 3T6 cells (results not shown).

Phosphorylation of MBP peptide by both PKC ϵ^{95} and PKC ϵ^{87}

To investigate whether phosphorylation at Ser-729 is critical for the catalytic activity of PKC- ϵ , both PKC ϵ^{95} and PKC ϵ^{87} were examined for their ability to phosphorylate a suitable substrate in the presence of appropriate cofactors. Immunoprecipitates of PKC ϵ from the nuclear fraction (which contained predominantly PKC ϵ^{95}) and the cytoplasmic fraction (which contained predominantly PKC ϵ^{87}) were prepared and were incubated with MBP peptide₄₋₁₄ in the presence of DAG and phosphatidylserine; PKC ϵ activity was measured as the difference between radioactivity incorporated into the substrate in the presence or absence of a PKC pseudosubstrate inhibitor peptide [31]. Incubation with the PKC inhibitor peptide decreased phosphorylation of MBP by approx. 50% in all samples. In Figure 6, the results, shown after subtraction of the values obtained with the PKC inhibitor peptide present, demonstrate that both forms of PKC ϵ can phosphorylate MBP peptide₄₋₁₄ in the presence of DAG and phosphatidylserine. These results indicate that, in the presence of the appropriate cofactors, PKC ϵ^{87} lacking a phosphate at Ser-729 is still catalytically active and can phosphorylate a suitable substrate.

Table 1 Summary of MALDI-TOF-MS analysis of PKC ϵ

PKC ϵ^{87} and PKC ϵ^{95} were excised from gels and digested with either Glu C or trypsin as described in the Experimental section. Peptide masses detected from MALDI-TOF-MS were compared with those of predicted digests. A difference in mass of 80 indicates the addition of a phosphate group. The data show that both PKC ϵ^{87} and PKC ϵ^{95} were phosphorylated at site Ser-703 and that PKC ϵ^{95} was additionally phosphorylated at Ser-729.

Phosphorylation site	Peptide	Enzyme	m/z	Detected in PKC ϵ^{87}	Detected in PKC ϵ^{95}
Ser-703	678–724	Glu C	5737.7	Yes	Yes
	693–704	Trypsin	1604.7	Yes	Yes
	693–717	Trypsin	3157.3	Yes	Yes
Ser-729	727–737	Trypsin	1358.6	No	Yes
	719–737	Trypsin	2361.7	No	Yes

Figure 5 Differential localization of PKC ϵ^{87} and PKC ϵ^{95} in 3T3 cells

PKC ϵ in quiescent and newly passaged 3T3 cells was revealed by immunofluorescence with anti-PKC ϵ antibody, which recognizes both PKC ϵ^{95} and PKC ϵ^{87} , and an anti-phospho-Ser-729-PKC ϵ antibody, which recognizes only PKC ϵ^{95} , as described in the Experimental section. (A) Perinuclear localization of PKC ϵ in quiescent 3T3 cells. (B) Perinuclear localization of PKC ϵ^{95} in quiescent 3T3 cells. (C) Diffuse cytoplasmic localization of PKC ϵ in newly-passaged cells. (D) Weak staining of PKC ϵ^{95} in newly-passaged 3T3 cells. The scale bar represents 10 μ m.

DISCUSSION

Recent work has highlighted the importance of phosphorylation in the regulation of PKC isotypes, notably PKC α and β_{II} [5–9,33–35]. It seems clear that these PKC isotypes are initially

phosphorylated by a PKC kinase, recently identified as phospholipid-dependent kinase-1, at a conserved threonine residue at the lip of the catalytic site [5,6,36,37]. This corresponds to Thr-566 in PKC ϵ [5,6]. Phosphorylation of this threonine residue, which may occur at a membrane site [32], allows the protein to

Figure 6 Phosphorylation of MBP peptide₄₋₁₄ by PKC ϵ^{87} and PKC ϵ^{95} in the presence of appropriate cofactors

PKC ϵ was immunoprecipitated from the nuclear and cytoplasmic fractions from quiescent and newly passaged 3T3 cells. The nuclear fraction contains predominantly PKC ϵ^{95} and the cytoplasmic fraction predominantly PKC ϵ^{87} . The ability of these immunoprecipitates to phosphorylate MBP peptide₄₋₁₄ was assessed in the presence of cofactors, with and without PKC inhibitor peptide [31]. Incorporation of [γ - 32 P]ATP into MBP peptide₄₋₁₄ was measured as described in the Experimental section. The results shown are after subtraction of values obtained in the presence of PKC inhibitor peptides and are means $\pm$ S.E.M.

become phosphorylated at two C-terminal serine residues [7–9], which correspond to Ser-703 and Ser-729 in PKC ϵ . It has been shown that Ser-703, or its equivalent, is an autophosphorylation site but it is possible that Ser-729 is autophosphorylated or phosphorylated by another kinase [32,38]. Once phosphorylated on all three residues the protein is primed for activation by other cofactors such as DAG.

In the present study, we have examined the phosphorylation status of the two major PKC ϵ forms, PKC ϵ^{95} and PKC ϵ^{87} , which are found in 3T3 and 3T6 fibroblasts. These changes were studied in untransfected cells, since this will accurately reflect the changes that occur normally in PKC ϵ as confluent and quiescent cells are passaged. Our results showed that both PKC ϵ^{95} and PKC ϵ^{87} are phosphoproteins. The lower molecular mass form, PKC ϵ^{87} , was phosphorylated at Ser-703 and Thr-566, whereas the form with higher molecular mass, PKC ϵ^{95} , was additionally phosphorylated at Ser-729.

We observed that treatment of cell lysates with alkaline or λ phosphatase reduced the intensity of the 95-kDa band on Western blots of PKC ϵ and increased the intensity of the 87-kDa band. With alkaline phosphatase treatment, the formation of an even lower molecular mass, faster migrating form of PKC ϵ was not detected, which might have been expected given that PKC ϵ^{87} is also a phosphoprotein. This is possibly because the phosphate groups on Thr-566 and Ser-703 were inaccessible to alkaline phosphatase; there is evidence that PKC cofactors such as DAG and calcium are required for the phosphate groups on PKC to become accessible to phosphatases [39]. In fact, when cell extracts were analysed a faster migrating form of PKC ϵ at 84 kDa was occasionally found (see Figure 1B). This may correspond to the 84 kDa form of PKC ϵ identified by Koide et al. [26], and may represent a mixture of non-phosphorylated and mono-phosphorylated PKC ϵ [7].

We have shown that PKC ϵ^{95} has a perinuclear, probably Golgi, localization, whereas PKC ϵ^{87} was located in the cytoplasm. This suggests that the presence or absence of a phosphate group at Ser-729 is associated with the change in localization of the protein. Additionally, we have shown that both forms of PKC ϵ can phosphorylate MBP peptide₄₋₁₄, and therefore both

forms have the potential to be active in the presence of appropriate cofactors. It is not yet clear whether one or both forms of PKC ϵ are catalytically active in the cell and if they have independent functions. The necessary cofactors may not be available to PKC ϵ^{87} in the cytoplasm but should be available to PKC ϵ^{95} in a perinuclear/Golgi site, where both phosphatidylserine and DAG have been identified [40,41]. This transfer of PKC ϵ from the cytosol to the perinuclear/Golgi region as cells grow to confluency may therefore represent an activation. It is possible that phosphorylation at Ser-729 may facilitate PKC ϵ binding to the Golgi via its Golgi-localization signal [22,23], perhaps by inducing a conformational change which makes the Golgi-localization signal accessible.

Given that PKC ϵ is the only isoform to have full oncogenic potential, it is possible that this change in the intracellular localization of PKC ϵ is important in regulating cell growth/survival or the adherence and mobility of cells. PKC ϵ has been reported to activate Raf, either directly or indirectly [20,22,42,43]. PKC ϵ has also been reported to be anti-apoptotic through its interactions with Bcl2 [44,45]. This suggests a role for PKC ϵ^{95} , which accumulates as cells become growth arrested. PKC ϵ is also reported to modulate Golgi function and to inhibit glycosaminoglycan sulphation, perhaps by its reported interaction with β' -COP, a selective binding protein ('RACK') for PKC ϵ [13]. There is, thus, a possibility that PKC ϵ^{95} accumulates in the perinuclear/Golgi region as cells grow to confluency, and binds to β' -COP with concomitant effects on vesicle trafficking.

One theory for the observed change in phosphorylation status in PKC ϵ as cells are passaged, is that this kinase has a role in quiescence (G_0), implying that PKC ϵ^{95} is the active form, possibly regulating Golgi activity [23] or suppressing apoptosis [44,45]. After passage, this activity is no longer required and the phosphate at Ser-729 is removed, releasing the enzyme from its peri-nuclear/Golgi localization into the cytosol. Alternatively, PKC ϵ^{95} may be an inactive form which is maintained in the perinuclear region of quiescent cells and is activated on cell passage when cofactors, such as DAG, are generated in response to signalling pathways. When active, PKC ϵ is more susceptible to phosphatase attack [39] and so, as it is activated by the presence of cofactors, the phosphate on Ser-729 is removed by a phosphatase which releases PKC ϵ^{87} into the cytosol. In each of these cases it is possible that PKC ϵ^{95} is first released into the cytosol where dephosphorylation of Ser-729 takes place and other factors control the localization of PKC ϵ . Our observations support both of the above hypotheses and further studies are underway to determine which is correct.

Our findings indicate that PKC ϵ^{95} in confluent cells is rapidly converted to PKC ϵ^{87} on passage. How this occurs is not resolved but one obvious possibility is that a phosphatase specific for Ser-729 is activated upon cell passage, as the phosphorylation at sites at Thr-566 and Ser-703 remain unattacked in PKC ϵ^{87} . We have been unable to detect rapid incorporation of [35 S]methionine into PKC ϵ in newly passaged fibroblasts, suggesting that the fast formation of PKC ϵ^{87} is not due to new protein synthesis (K. England, unpublished work). This supports the idea that a specific Ser-729 protein phosphatase may be involved in converting PKC ϵ^{95} into PKC ϵ^{87} . We are currently attempting to identify the nature of the phosphatase involved in this process, as it may represent yet another level of control of the PKC family of proteins. Previous reports indicate that C-terminal phosphorylation in PKC α and β_{II} regulates enzyme stability, sensitivity to phosphatase attack and localization [9,34,35,39]. The present work shows that the presence or absence of phosphorylation at Ser-729 is also important in the regulation of novel PKC ϵ .

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REFERENCES

- Nishizuka, Y. (1988) The molecular heterogeneity of protein kinase-C and its implications for cellular-regulation. *Nature (London)* **334**, 661–665
- Goodnight, J., Mischak, H. and Muschinski, J. F. (1994) Selective involvement of protein-kinase-C isozymes in differentiation and neoplastic transformation. *Adv. Cancer Res.* **64**, 159–209
- Mellor, H. and Parker, P. J. (1998) The extended protein kinase C superfamily. *Biochem. J.* **332**, 281–292
- Dekker, L. V. and Parker, P. J. (1994) Protein-kinase-C - a question of specificity. *Trends Biochem. Sci.* **19**, 73–77
- Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P. and Parker, P. J. (1998) Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* **281**, 2042–2045
- Chou, M. M., Hou, W., Johnson, J., Graham, L. K., Lee, M. L., Chen, C.-S., Newton, A. C., Schaffhausen, B. S. and Tokar, A. (1998) Regulation of protein kinase C zeta by PI 3-kinase and PDK-1. *Curr. Biol.* **8**, 1069–1077
- Newton, A. C. (1997) Regulation of protein kinase C. *Curr. Opin. Cell Biol.* **9**, 161–167
- Dutil, E. M., Keranen, L. M., DePaoli-Roach, A. A. and Newton, A. C. (1994) *In-vivo* regulation of protein kinase C by trans-phosphorylation followed by autophosphorylation. *J. Biol. Chem.* **269**, 29359–29362
- Keranen, L. M., Dutil, E. M. and Newton, A. C. (1995) Protein kinase C is regulated *in vivo* by three functionally distinct phosphorylations. *Curr. Biol.* **5**, 1394–1403
- Garcia-Paramio, P., Cabrero, Y., Bornancin, F. and Parker, P. J. (1998) The broad specificity of dominant inhibitory protein kinase C mutants infers a common step in phosphorylation. *Biochem. J.* **333**, 631–636
- Jaken, S. (1996) Protein kinase C isozymes and substrates. *Curr. Opin. Cell Biol.* **8**, 168–173
- Newton, A. C. (1996) Protein kinase C: Ports of anchor in the cell. *Curr. Biol.* **6**, 806–809
- Csukai, M., Chen, C.-H., De Matteis, M. A. and Mochly-Rosen, D. (1997) The coatomer protein beta-COP, a selective binding protein (RACK) for protein kinase C epsilon. *J. Biol. Chem.* **272**, 29200–29206
- Lee, H.-W., Smith, L., Pettit, G. R. and Bingham-Smith, J. (1997) Bryostatins 1 and phorbol ester down-modulate protein kinase C-alpha and -epsilon via the ubiquitin/proteasome pathway in human fibroblasts. *Mol. Pharmacol.* **51**, 439–447
- Lu, Z., Liu, D., Hornia, A., Devonish, W., Pagano, M. and Foster, D. A. (1998) Activation of protein kinase C triggers its ubiquitination and degradation. *Mol. Cell. Biol.* **18**, 839–845
- Dwyer, L. D., Miller, A. C. K., Parkes, A. L., Jaken, S. and Malkinson, A. M. (1994) Calpain-induced down-regulation of activated protein-kinase C-alpha affects lung epithelial cell morphology. *Am. J. Physiol.* **266**, L569–L576
- Eto, A., Akita, Y., Saido, T. C., Suzuki, K. and Kawahima, S. (1995) The role of the calpain-calpastatin system in thyrotropin-releasing hormone-induced selective down-regulation of a protein kinase-C isozyme, nPKC-epsilon, in rat pituitary gh(4)c(1) cells. *J. Biol. Chem.* **270**, 25115–25120
- Mischak, H., Goodnight, J., Kolch, W., Martiny-Baron, G., Schaehtle, C., Kazanietz, M. G., Blumberg, P. M., Pierce, J. H. and Muschinski, J. F. (1993) Overexpression of protein kinase C-delta and -epsilon in NIH 3T3 cells induces opposite effects on growth, morphology, anchorage dependence and tumorigenicity. *J. Biol. Chem.* **268**, 6090–6096
- Cacace, A. M., Guadagno, S. N., Krauss, R. S., Fabbro, D. and Weinstein, I. B. (1993) The epsilon isoform of protein kinase-C is an oncogene when overexpressed in rat fibroblasts. *Oncogene* **8**, 2095–2104
- Cacace, A. M., Ueffing, M., Philipp, A., Han, E. K.-H., Kolch, W. and Weinstein, I. B. (1996) PKC epsilon functions as an oncogene by enhancing activation of the Raf kinase. *Oncogene* **13**, 2517–2526
- Ueffing, M., Lovric, J., Philipp, A., Mischak, H. and Kolch, W. (1997) Protein kinase C-epsilon associates with the Raf-1 kinase and induces the production of growth factors that stimulate Raf-1 activity. *Oncogene* **15**, 2921–2927
- Lehel, C., Olah, Z., Petrovics, G., Jakob, G. and Anderson, W. B. (1996) Influence of various domains of protein kinase C epsilon on its PMA-induced translocation from the Golgi to the plasma membrane. *Biochem. Biophys. Res. Commun.* **223**, 98–103
- Lehel, C., Olah, Z., Jakon, G. and Anderson, W. B. (1995) Protein kinase C-epsilon is localized to the Golgi via its zinc-finger domain and modulates Golgi function. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1406–1410
- Prekeris, R., Mayhew, M. W., Cooper, J. B. and Terrain, D. M. (1996) Identification and localization of an actin-binding motif that is unique to the epsilon isoform of protein kinase C and participates in the regulation of synaptic function. *J. Cell Biol.* **132**, 77–90
- Prekeris, R., Hernandez, R. M., Mayhew, M. W., White, M. K. and Terrain, D. M. (1998) Molecular analysis of the interactions between protein kinase C-epsilon and filamentous actin. *J. Biol. Chem.* **273**, 26790–26798
- Koide, H., Ogita, K., Kikkawa, U. and Nishizuka, Y. (1992) Isolation and characterization of the epsilon subspecies of protein kinase-C from rat-brain. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1149–1153
- Saido, T. C., Mizuno, K., Konno, Y., Osada, S., Ohno, S. and Suzuki, K. (1992) Purification and characterization of protein kinase-C-epsilon from rabbit brain. *Biochemistry*, **31**, 482–490
- Moreton, K., Turner, R., Blake, N., Paton, A., Groome, N. and Rumsby, M. G. (1995) Protein expression of the alpha-subspecies, gamma-subspecies, delta-subspecies and epsilon-subspecies of protein-kinase-C changes as C6 glioma-cells become contact inhibited and quiescent in the presence of serum. *FEBS Lett.* **372**, 33–38
- Littlebury, P., Watson, J., Williams, T., Beale, G. and Rumsby, M. G. (1997) Protein expression of the epsilon subspecies of protein kinase C ceases as Swiss 3T6 fibroblasts increase in cell density even though message for the protein is still present. *FEBS Lett.* **400**, 304–308
- Ohmori, T. and Arteaga, C. L. (1998) Protein kinase C epsilon translocation and phosphorylation by cis-diaminedichloroplatinum(II) (CDDP): Potential role in CDDP-mediated cytotoxicity. *Cell Growth Diff.* **9**, 345–353
- Yasuda, I., Kishimoto, A., Teneka, S. I., Tominaga, M., Sakurai, A. and Nishizuka, Y. (1990) A synthetic peptide substrate for selective assay of PKC. *Biochem. Biophys. Res. Commun.* **166**, 1220–1227
- Rosenfeld, J., Capdevielle, J., Guillemot, J. C. and Ferrara, P. (1992) In-gel digestion of proteins for internal sequence analysis after 1-dimensional or 2-dimensional gel-electrophoresis. *Anal. Biochem.* **203**, 173–179
- Kishimoto, A., Kajikawa, N., Shiota, M. and Nishizuka, Y. (1983) Proteolytic cleavage of calcium-activated, phospholipid-dependent protein kinase by calcium-dependent neutral protease. *J. Biol. Chem.* **258**, 1156–1164
- Parekh, D. B., Ziegler, W. and Parker, P. J. (2000) Multiple pathways control protein kinase C phosphorylation. *EMBO J.* **19**, 496–503
- Zhang, J., Wang, L., Schwartz, J., Bond, R. W. and Bishop, W. R. (1994) Phosphorylation of Thr(642) is an early event in the processing of newly synthesized protein kinase C-beta(1) and is essential for its activation. *J. Biol. Chem.* **269**, 19578–19584
- Gysin, S. and Imber, R. (1997) Phorbol-ester-activated protein kinase C-alpha lacking phosphorylation at Ser657 is down-regulated by a mechanism involving dephosphorylation. *Eur. J. Biochem.* **249**, 156–160
- Bornancin, F. and Parker, P. J. (1996) Phosphorylation of protein kinase C-zeta on serine 657 controls the accumulation of active enzyme and contributes to its phosphatase-resistant state. *Curr. Biol.* **6**, 1114–1123
- Cazaubon, S., Bornancin, F. and Parker, P. J. (1994) Threonine-497 is a critical site for permissive activation of protein kinase C-alpha. *Biochem. J.* **301**, 443–448
- Orr, J. W. and Newton, A. C. (1994) Requirement for negative charge on activation loop of protein kinase-C. *J. Biol. Chem.* **269**, 27715–27718
- Parekh, D., Ziegler, W., Yonezawa, K., Hara, K. and Parker, P. J. (1999) Mammalian TOR controls one of two kinase pathways acting upon nPKC delta and nPKC epsilon. *J. Biol. Chem.* **274**, 34758–34764
- Sweatt, J. D., Atkins, C. M., Johnson, J., English, J. D., Roberson, E. D., Chen, S.-J., Newton, A. and Klann, E. (1998) Protected-site phosphorylation of protein kinase C in hippocampal long-term potentiation. *J. Neurochem.* **71**, 1075–1085
- Roth, M. G. (1999) Lipid regulators of membrane traffic through the Golgi complex. *Trends Cell Biol.* **9**, 174–179
- Divecha, N., Banfic, H. and Irvine, R. F. (1993) Inositides and the nucleus and inositides in the nucleus. *Cell* **74**, 405–407
- Cai, H., Smola, U., Wixler, V., Eisenmann-Tappe, I., Diaz-Meco, M. T., Moscat, J., Rapp, U. and Cooper, G. M. (1997) Role of diacylglycerol-regulated protein kinase C isotypes in growth factor activation of the Raf-1 protein kinase. *Mol. Cell Biol.* **17**, 732–741
- Perletti, G. P., Concari, P., Brusarferri, S., Marras, E., Piccinini, F. and Tashjian, A. H. (1998) Protein kinase C epsilon is oncogenic in colon epithelial cells by interaction with the ras signal transduction pathway. *Oncogene* **16**, 3345–3348
- Sawai, H., Okazaki, T., Takeda, Y., Tashima, M., Sawada, H., Okuma, M., Kishi, S., Umehara, H. and Domae, N. (1997) Ceramide-induced translocation of protein kinase C-delta and -epsilon to the cytosol - implications in apoptosis. *J. Biol. Chem.* **272**, 2452–2458
- Gubina, E., Rinaudo, M. S., Szallasi, Z., Plumberg, P. M. and Mufson, R. A. (1998) Overexpression of protein kinase C isoform epsilon but not delta in human interleukin-3-dependent cells suppresses apoptosis and induces bcl-2 expression. *Blood* **91**, 823–829