Protein kinase C isoforms play differential roles in the regulation of adipocyte differentiation

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In this study we first established, by immunoblotting with specific antibodies, the temporal changes in cellular levels of protein kinase C (PKC) isoforms during differentiation of 3T3-F442A pre-adipocytes. Both pre-adipocyte and adipocyte 3T3-F442A cells were found to express PKC-α, -γ, -δ, -ϵ, -ζ and -μ. However we were unable to detect PKC-β, -η or -θ. The same PKC isoform expression profile was found in rat adipocytes. The α, δ and γ isoforms displayed similar temporal patterns of expression during differentiation of 3T3-F442A cells; all increased rapidly, peaking at day 2 of differentiation. Subsequently, the expression of these isoforms decreased, resulting in lower levels in fully differentiated adipocytes than in pre-adipocytes. The expression of PKC-ϵ increased steadily during differentiation, resulting in markedly elevated levels in adipocytes. Although expression of PKC-μ increased during differentiation, this was attributable to prolonged confluence rather than to the differentiation process itself. No change was observed in PKC-ζ levels during adipocyte development. Anti-sense oligodeoxynucleotides (ODNs) were used to deplete selectively the individual PKC subtypes. Each of the ODNs used effectively depleted the specific isoforms to undetectable levels and did not affect expression of the other PKC subtypes. This approach indicated that pre-adipocyte differentiation is not dependent upon PKC-ζ but that PKC-α, -δ and -μ each exert an inhibitory influence upon differentiation. Use of anti-sense ODNs to deplete PKC-ϵ and -γ revealed that pre-adipocyte differentiation is dependent upon each of these isoforms. However, PKC-γ, but not PKC-ϵ, appeared to be necessary for the clonal expansion of differentiating cells, suggesting that PKC-ϵ is required at a later phase in the differentiation process, when its expression is elevated, for the attainment and maintenance of the adipocyte phenotype.

INTRODUCTION

The differentiation of pre-adipocytes is a complex process dependent upon the strict temporal regulation of multiple and interacting signalling events, ultimately leading to the modulation of expression of an array of genes necessary for the attainment of the adipocyte phenotype [1,2]. Several murine fibroblast cell lines (e.g. 3T3-L1 and 3T3-F442A) have the capacity to undergo differentiation into cells which exhibit most of the morphological and biochemical characteristics of normal adipocytes. Extensive studies using these pre-adipocyte lines indicate that they represent a faithful model of the process of adipocyte differentiation *in vivo*. As such, many of the key hormonal regulators of the process have now been identified. However, the intracellular molecular events that regulate the adipocyte differentiation process and which are required to maintain the adipocyte phenotype remain largely unknown.

The protein kinase C (PKC) family is a multigene family of phospholipid-dependent serine/threonine kinases consisting, so far, of eleven members divided into groups on the basis of their structural and biochemical properties. The conventional PKCs (α, βI/βII and γ) are regulated by Ca^{2+} ions and diacylglycerol or phorbol esters, and the novel PKCs (δ, ϵ, η and θ) are sensitive to diacylglycerol or phorbol esters but are insensitive to calcium [3,4]. There are also atypical PKCs (ζ and λ/ι) which, although structurally related to the other PKCs, are insensitive to Ca^{2+} ions, diacylglycerol and phorbol esters [3,5]. PKC-μ, which has regulatory properties similar to the novel PKCs, appears to be structurally quite distinct [6]. Increasing evidence suggests that different PKC isoforms may serve diverse functional roles, which is consistent with their displaying distinct regulatory properties and tissue distribution [3–11]. Although the precise roles played by different PKC isoforms have still to be elucidated, it is clear, from a wide range of studies, that PKCs function in the regulation of cell growth and differentiation [3,12–16]. Indeed, a number of lines of evidence implicate this family in adipocyte differentiation. First, PKCs are involved in a number of signalling pathways, including the mitogen-activated protein kinase [17], $p70^{s6k}$ [18] and phosphatidylinositol 3-kinase [19,20] pathways, all of which have been implicated in adipocyte differentiation [21–23]. Secondly, studies show that the phorbol ester, PMA, can modulate adipocyte differentiation [24,25]. However, such studies, done with phorbol esters, can be difficult to interpret, as the relatively chronic treatments involved will lead to the down-regulation of particular subsets of PKC isoforms [26]. More direct evidence for a role of PKC in adipocyte development has recently emerged from studies demonstrating differential changes in the expression of PKC isoforms during the differentiation of 3T3-L1 pre-adipocytes [27,28]. In the present study we have used 3T3-F442A pre-adipocytes to examine the temporal expression patterns of the conventional, novel and atypical PKCs and of the recently identified PKC-μ during adipocyte development. Furthermore,

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GPDH, glycerol-3-phosphate dehydrogenase; ODN, oligodeoxynucleotide; PKC, protein kinase C.

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by selective depletion of individual PKC subtypes with anti-sense oligodeoxynucleotides (ODNs), we show that individual PKC isoforms may assume distinct functional roles in the regulation of adipocyte differentiation.

MATERIALS AND METHODS

Materials

Culture media, calf serum, fetal calf serum and lipofectin were obtained from Life Technologies (Paisley, U.K.). Enhanced chemiluminescence solutions were obtained from Pierce (Chester, U.K.).

Antibodies

Monoclonal antibodies to PKC-α, -βI/II, -γ, -δ, -ϵ, -θ and -μ were purchased from Affiniti Research Products Limited (Nottingham, U.K.), and the monoclonal antibody to PKC-η was from Santa Cruz Biotechnology Inc. Rabbit polyclonal antiserum to PKC-βI/βII was a kind gift from Dr. P. Parker (I.C.R.F., London, U.K.) and polyclonal antisera to PKC-ζ and PKC-βI/βII were raised in-house as described previously [26].

Cell culture

3T3-F442A and 3T3-C2 cells (from Dr. H. Green, Harvard Medical School) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % calf serum (growth medium), as described previously [29]. Just-confluent cultures were induced to differentiate by replacing the growth medium with DMEM containing 2 mM glutamine, 10 % fetal calf serum and 1 μM insulin. This differentiation medium was replenished every 72 h. Under these conditions at least 80 % of the cells exhibited adipocyte morphology when examined after 7–10 days. As a control, parallel cultures were maintained at confluence in growth medium for 10 days. These cells, in which adipocyte conversion was less than 5 %, are referred to as 10 day confluent cells. NIH-3T3 cells transfected to stably over-express PKC-α, -β, -γ, -δ, -ϵ and -ζ (a gift from Dr. J. Goodnight, N.C.I., Bethesda, MD, U.S.A.) were grown as described previously [26] and cell lysates used as positive controls on immunoblots.

Treatment of cells with anti-sense ODNs

Phosphorothioate-modified ODNs were purchased from Genosys Biotechnologies (Cambridge, U.K.). The anti-sense sequences used were 5′-CGG-GTA-AAC-GTC-AGC-CAT-3′ for PKC-α [30], 5′-GAA-GGA-GAT-GCG-CTG-GAA-3′ for PKC-δ [30], 5′-GCC-ATT-GAA-CAC-TAC-CAT-3′ for PKC-ϵ [31], 5′-CGT-CCT-GCT-GGG-CAT-3′ for PKC-ζ [32], 5′-AGG-GCC-CAG-ACC-CGC-CAT-3′ for PKC-γ and 5′-GAC-CGG-AGG-GGC-GCT-CAT-3′ for PKC-μ. The anti-sense sequences for PKC-α, -γ, -ϵ and -ζ are based on the start codons (ATG) plus the 15 (or 12 for PKC-ζ) additional downstream bases in the appropriate murine PKC sequences [30–33], and the anti-sense sequence for PKC-μ is based on the start codon plus the 15 additional downstream bases in the human PKC-μ sequence [6]. The anti-sense sequence for PKC-δ is based on bases 10–27 of the murine coding sequence [30]. The appropriate isoform-specific sense sequences were used as controls. 3T3-F442A cells were treated with ODNs as described previously. Briefly, 3T3-F442A fibroblasts (typically 70–80 % confluent in 30 mm dishes) were incubated for 6 h at 37 °C with 10 μM ODN/20 μg/ml lipofectin. Cells were then washed prior to the addition of fresh medium containing 2 mM glutamine, 10 % heat-treated calf serum and 10 μM ODN but no lipofectin. After a further 42 h cells were either assessed for immunoreactive PKC levels or induced to differentiate by addition of medium containing 10 % heat-treated fetal calf serum, 1 μM insulin and 10 μM ODN. The differentiation medium was replaced at 72 h periods and cells were harvested at the appropriate times, indicated in legends, after the induction of differentiation. The appropriate ODN was present throughout the entire differentiation period.

Preparation of rat brain homogenate

Rat-brain homogenates, for use as a positive control on immunoblots, were prepared as described previously [26].

Western blotting

Cells were incubated in serum-free medium for 16–18 h prior to preparation of lysates. Cells were scraped into ice-cold lysis buffer [25 mM Hepes (pH 7.4)/5 mM EDTA/50 mM NaCl/50 mM NaF/30 mM sodium pyrophosphate/1 % (v/v) Triton X-100/10 % (v/v) glycerol/1 mM sodium orthovanadate/1 mM PMSF/2 μg/ml each of aprotinin, pepstatin A and leupeptin], clarified by centrifugation (14000 ***g*** for 10 min at 4 °C) and proteins denatured by adding 0.25 volumes of 5 × concentrated SDS sample buffer [312.5 mM Tris (pH 6.7)/6.25 % SDS/62.5 % glycerol/12.5 % β-mercaptoethanol] and boiling for 5 min. Lysate proteins were separated by SDS/PAGE on 9 % gels. Following transfer to nitrocellulose, blots were probed with antibodies to PKC isoforms. Immunoreactive bands were detected using the enhanced chemiluminescence (ECL) system (Pierce). Densitometric analysis of immunoreactive bands was performed using a Molecular Dynamics Personal Densitometer.

When carrying out direct comparisons of immunoreactive PKC levels in cells at different stages of differentiation, cellular lysates were loaded on to gels on the basis of equal protein. However, in accordance with others, we have found that the amount of protein per cell in fully differentiated adipocytes is approx. 2.3 times that in fibroblasts [34]. Hence when comparing immunoreactive PKC levels at different stages of the differentiation process it is necessary to normalize results on a per-cell basis. This was achieved by setting up parallel cultures for determination of the number of cells and total protein per plate. These values permitted the calculation of the amount of protein per cell, which was used to convert immunoreactive PKC levels, assessed by densitometry of gels, into PKC level per cell.

GPDH and DNA assays

Glycerol-3-phosphate dehydrogenase (GPDH) activity was measured spectrophotometrically [35]. All GPDH activities were expressed relative to DNA content rather than amount of cellular protein. The DNA content of cellular homogenates was determined fluorimetrically [36].

Oil Red O staining

Formalin-fixed cell cultures were stained with Oil Red O as described by Kuri-Harcuch and Marsch-Moreno [37].

Protein assay

The protein content of cell lysates was determined by the Bradford method [38] using the Bio-Rad Protein Assay Kit (Bio-Rad Labs. Ltd., Herts, U.K.) and BSA as a standard.

Microscopy

Cells were photographed under Phase Contrast Optics ($\times 24$ magnification) using an Olympus IMT3 inverted microscope.

Statistical analysis

Results are presented as means $\pm$ S.E.M. and, unless otherwise stated, statistical analysis was by Student's *t*-test of paired samples.

RESULTS

Characterization of PKC isoform expression in 3T3-F442A cells and rat adipocytes

We recently described the PKC isoform complement of 3T3-F442A pre-adipocytes and showed the presence of the α, γ, δ, ϵ and ζ isoforms [26]. Using the same antibodies we now demonstrate the presence of these PKC isoforms in 3T3-F442A adipocytes and also rat adipocytes (Figure 1). In addition, we detected PKC-μ in rat adipocytes as well as both differentiated and undifferentiated 3T3-F442A cells (Figure 1). In our hands, under the experimental conditions used, the polyclonal antiserum to PKC-ζ detected an immunoreactive band with an apparent molecular mass of 83 kDa (Figure 1). This is higher than the 67 kDa molecular mass predicted from the amino acid sequence for PKC-ζ [39]. However, this band was specifically competed out by the peptide immunogen and an identically migrating 83 kDa band was detected when a monoclonal antibody to PKC-ζ (Affiniti Research Products Limited) was used for immunoblotting the samples (results not shown). Thus we conclude that this 83 kDa band does indeed represent PKC-ζ and this is consistent with reports from others who have also observed this PKC isoform migrating at around this size [40,41].

We were unable to detect PKC-β in these cells, using either the commercially available monoclonal antibody from Affiniti (results not shown) or two different polyclonal antibodies, one which was raised in house (Figure 1) and another a gift from Dr. P. Parker (I.C.R.F., London, U.K.; results not shown). However, the polyclonal anti-PKC-β antibodies used detected two immunoreactive bands (83 kDa and 75 kDa), both in rat-brain homogenates and in lysates prepared from NIH-3T3 cells overexpressing PKC-βI/βII (Figure 1). Competition studies demonstrated that the peptide immunogen selectively abolished detection of the upper (83 kDa) band while having no effect upon appearance on immunoblots of the lower (75 kDa) band (results not shown). Furthermore, the monoclonal antibody to PKC-βI/βII detected only the upper band (83 kDa). We therefore conclude that it is this upper (83 kDa) band which represents PKC-βI/βII: we were unable to detect this band both in 3T3-F442A cells, either before or after differentiation, and in rat adipocytes. Using antibodies commercially available, we were also unable to detect expression of PKC-η or PKC-θ in both the 3T3-F442A cells and rat adipocytes (results not shown).

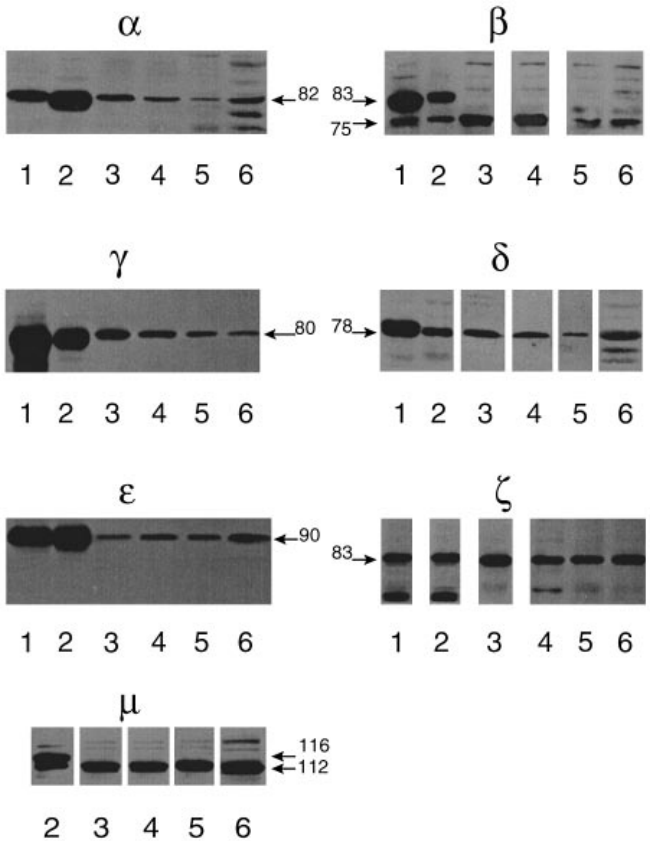

Figure 1 PKC subtypes present in 3T3-F442A cells and rat adipocytes

Cell lysates (75 μg of protein) prepared from just-confluent 3T3-F442A pre-adipocytes (lane 3), 12-day-confluent 3T3-F442A pre-adipocytes (lane 4) or fully differentiated 3T3-F442A adipocytes (lane 5) were run alongside NIH-3T3 cells transfected to overexpress the appropriate PKC isoform (lane 1, 50 μg of protein), a rat adipocyte homogenate (lane 6, 75 μg of protein) and a rat mid-brain homogenate (lane 2, 50 μg of protein). PKC subtype expression was assessed by immunoblotting as described in the Materials and methods section. Molecular masses (kDa) of the major immunoreactive bands are indicated by arrows. Immunoblots are representative of results obtained in at least four separate experiments.

Changes in expression of PKC subtypes during the differentiation of 3T3-F442A cells

Changes in PKC isoform expression were measured by immunoblot analysis at different stages during the differentiation of 3T3-F442A cells and compared with changes occurring in 3T3-C2 cells treated identically with differentiation-inducing agents (Figure 2). 3T3-C2 is a line that does not undergo terminal adipocyte differentiation [42]. All values for PKC-isoform expression levels presented in Figure 2 have been normalized on a per-cell basis, as described in the Materials and methods section. In 3T3-F442A cells the levels of PKC-γ and -δ declined significantly between 2 days prior to confluence (day -2) and the initiation of differentiation at confluence (day 0; Figure 2). Although a similar decline in the cellular levels of PKC-α was apparent prior to confluence, this was not significant (Figure 2). The expression levels of PKC-α, -γ and -δ all increased rapidly in 3T3-F442A cells with maxima at around day 2 following the induction of differentiation (Figure 2). Subsequently, the expression of these isoforms decreased and fell to below day 0 levels by the end of the treatment period (day 10), which coincided with maximal differentiation. The expression of PKC-α, -γ and -δ followed a similar pattern in identically treated 3T3-C2 cells; however, their expression did not subsequently fall to below day 0 levels by day 10 (Figure 2). The overall decreases in expression of PKC-α, -γ and -δ in 3T3-F442A cells did not occur in cells maintained in normal growth medium for 10 days (Table 1), indicating that they occurred as a result of adipocyte differentiation itself rather than as a result of prolonged confluency.

The expression of PKC-ϵ in 3T3-F442A cells increased steadily following the induction of differentiation, peaking between days 5 and 10 when cellular levels of PKC-ϵ were 2.86 ± 0.09-fold

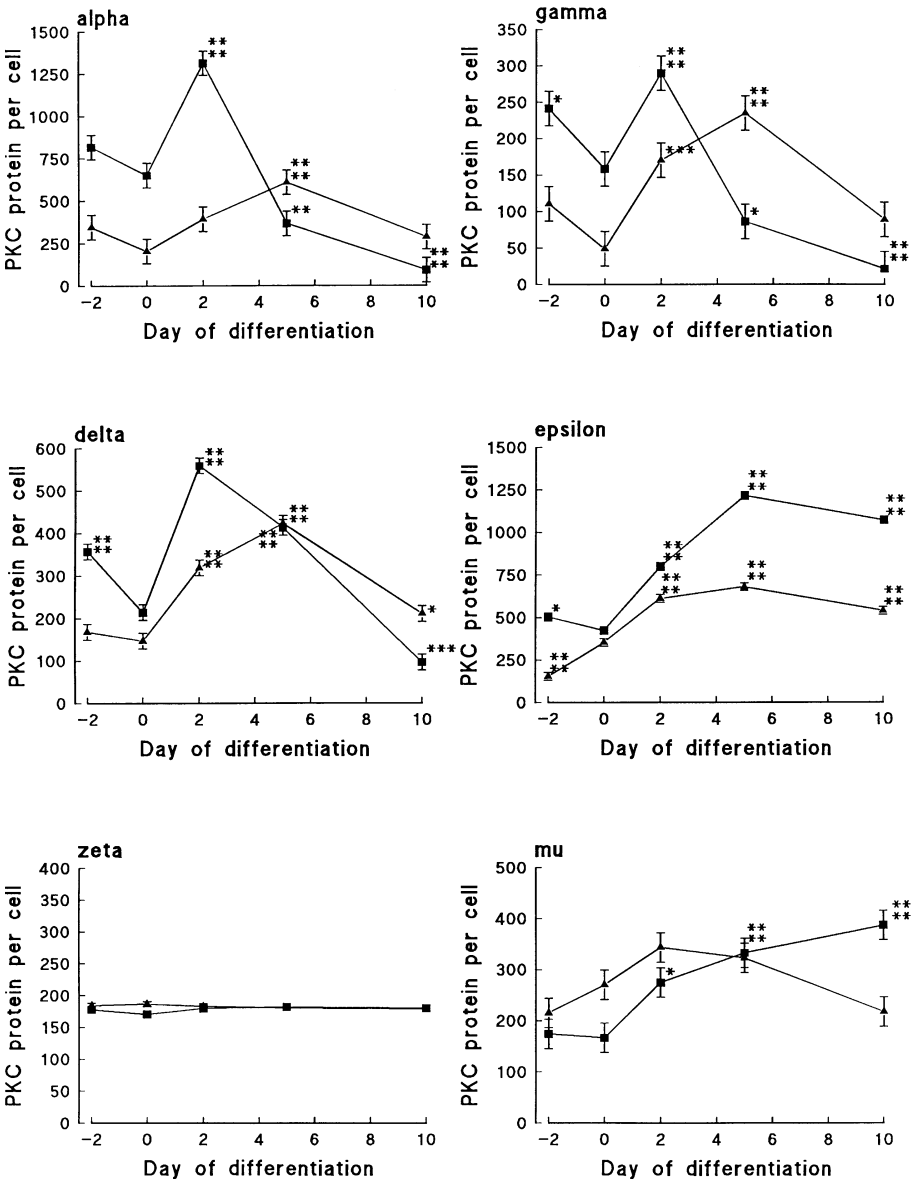

Figure 2 Changes in PKC-isoform expression during differentiation of 3T3-F442A pre-adipocytes and 3T3-C2 cells

Confluent cultures of 3T3-F442A pre-adipocytes (■) or 3T3-C2 (▲) cells were treated with differentiation medium at day 0. Cell lysates were prepared at various times throughout the differentiation period, as indicated, for assessment of PKC subtype levels. Data derived from densitometric analysis of immunoblots have been normalized on a per-cell basis and is presented as means ± S.E.M. for four separate experiments carried out on different cell preparations. *, **, *** and **** indicate that a value differs significantly from that for day 0 fibroblasts, with significance levels of $P < 0.05$, 0.02, 0.01 and 0.001, respectively. Statistical analysis was by analysis of variance.

higher than those in just-confluent (day 0) cells (Figure 2). In contrast, a significantly ($P < 0.01$) smaller 1.92 ± 0.14-fold increase was observed between days 0 and 5 in 3T3-C2 cells treated identically (Figure 2), and no significant increase was observed in 3T3-F442A cells held at confluence for 10 days (Table 1). Therefore the increased expression of PKC-ϵ occurs as a result of adipocyte differentiation. PKC-μ expression also increased during the course of differentiation of 3T3-F442A cells with the maximal increase observed at day 10 (Figure 2). No significant alteration in PKC-μ expression occurred in 3T3-C2 cells over the same period. However, the increase in expression of PKC-μ in differentiating cells was matched in cells maintained at confluence for the same period (Table 1), indicating that PKC-μ expression increases due to prolonged confluency rather than differentiation. Finally, the expression of PKC-ζ was unchanged during treatment of either 3T3-F442A or 3T3-C2 cells with differentiation-inducing agents (Figure 2), but elevated slightly in cells maintained at confluence for 10 days (Table 1).

Effects of depletion of individual PKC isoforms with anti-sense ODNs on 3T3-F442A pre-adipocyte differentiation

To define further the role of PKC subtypes in adipocyte development we used anti-sense ODNs to deplete selectively individual isoforms from 3T3-F442A pre-adipocyte cells prior to the initiation of differentiation. Immunoblot analysis revealed that preincubation of cells with the specific anti-sense ODNs for 48 h resulted in the depletion of over 90% of the appropriate

Table 1 Changes in the expression of PKC subtypes following differentiation of 3T3-F442A cells

Equal quantities of lysate protein from just-confluent 3T3-F442A pre-adipocytes, 10-day-confluent 3T3-F442A pre-adipocytes and fully differentiated 3T3-F442A adipocytes were immunoblotted with isoform-specific PKC antibodies. Following quantification by densitometric analysis, changes in expression of PKC subtypes are shown as a percentage of the expression level in just-confluent fibroblasts (control; 100%). Data were calculated on a per-cell basis and are means ± S.E.M. from eight separate experiments. *, ** and *** indicate that the values are significantly different from that for just-confluent fibroblasts, with significance levels of $P < 0.02$, 0.01 and 0.001, respectively. Statistical analysis was by Student's *t*-test for paired samples.

		PKC protein per cell (% of control)	
PKC isoform	3T3-F442A cell status ...	12 day-confluent undifferentiated pre-adipocytes	12 day-differentiated adipocytes
α		74 ± 21	16 ± 3***
γ		63 ± 18	11 ± 2***
δ		99 ± 9	41 ± 13**
ϵ		138 ± 29	298 ± 54***
ζ		160 ± 3*	112 ± 29
μ		204 ± 34*	220 ± 30

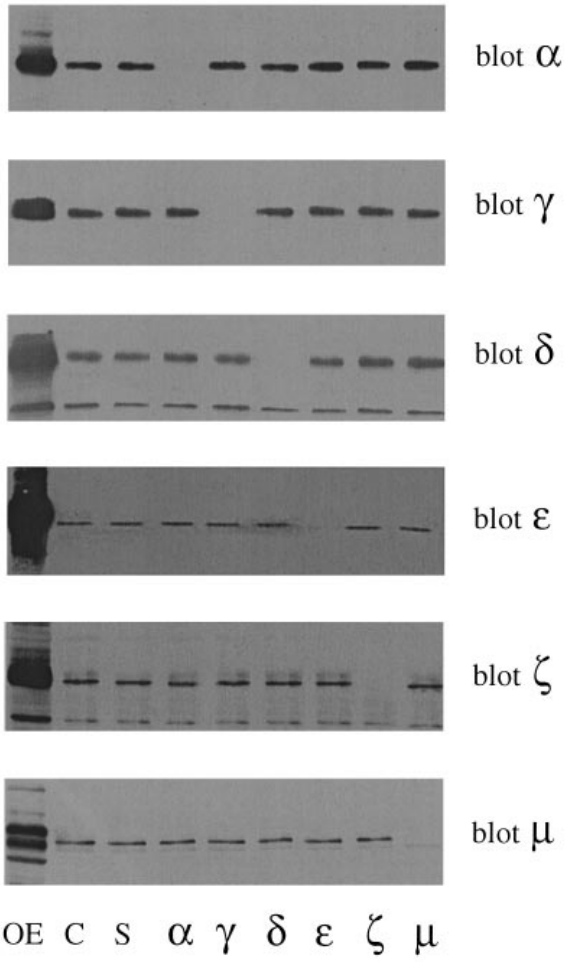

Figure 3 Specific depletion of PKC subtypes by treatment with anti-sense but not sense ODNs

3T3-F442A pre-adipocytes were preincubated with ODNs (10 μM) for 48 h before the preparation of lysates, as described in the Materials and methods section. Immunoblots show the amount of each isoform in 25 μg of cell lysate prepared from NIH-3T3 cells over-expressing the appropriate PKC subtype (OE), control 3T3-F442A pre-adipocytes treated with lipofectin only (C), 3T3-F442A pre-adipocytes treated with the appropriate sense ODN (S) and 3T3-F442A pre-adipocytes treated with anti-sense ODN to the PKC isoform indicated. The immunoblots shown are representative of experiments performed on at least four occasions for each PKC isoform.

PKC subtype (Figure 3). However, in order to achieve the successful depletion of PKC subtypes it was necessary for lipofectin to be present during the first 6 h of the 48 h treatment with anti-sense ODN (results not shown). In contrast, there was no detectable effect following the identical treatment of cells with the appropriate sense ODNs. Importantly, each anti-sense ODN was specific for the appropriate PKC subtype and had no effect on cellular levels of other PKC subtypes (Figure 3).

Cells were treated with the individual anti-sense ODNs for 48 h prior to the addition of differentiation medium. This ensured that the depletion of the relevant PKC subtype was complete at the initiation of differentiation. Immunoblot analysis revealed that maximal depletion of the PKCs persisted for at least the first 3 days of differentiation (results not shown). The aim of these studies was to address the impact of depletion of PKC isoforms during these early stages of differentiation on the attainment of the adipocyte phenotype. Effects on differentiation were assessed qualitatively, by examination of changes in cell morphology, and also quantitatively, by assaying the activity of the adipocyte marker enzyme GPDH.

Treatment of cells with lipofectin alone had no effect upon the differentiation process (results not shown). From morphological examination of cells it appeared consistently that depletion of PKC-α, -δ and -μ enhanced the rate of adipocyte development during the early stages of differentiation (Figure 4; days 2–4). However this effect was no longer detectable at the later stages of the differentiation process. Consistent with these observations, at day 4 of differentiation the specific activity of the adipocyte enzyme marker GPDH was significantly elevated in cells depleted of PKC-α, -δ or -μ, as compared with control cells that had been treated with lipofectin alone (Table 2). However, by day 7 there was no longer any significant difference between GPDH activity in cells depleted of PKC-α as compared with control cells, and only small increases in GPDH activity were detected in cells depleted of PKC-δ and -μ (Table 2). In contrast, depletion of PKC-ϵ or -γ clearly inhibited the differentiation process (Figure 4), resulting in significantly decreased GPDH activity by day 4 and even larger reductions in the activity of this adipocyte marker activity by day 7 of differentiation (Table 2). Depletion of PKC-ζ had no detectable effect upon differentiation (Figure 4, Table 2). Treatment of cells with the corresponding sense ODNs to the different PKC isoforms had no significant effects on differentiation, assessed either qualitatively, or quantitatively by measurement of GPDH activity (results not shown). Thus we conclude that (i) during the early stages of differentiation of these cells, the α, δ and μ isoforms of PKC exert an inhibitory influence

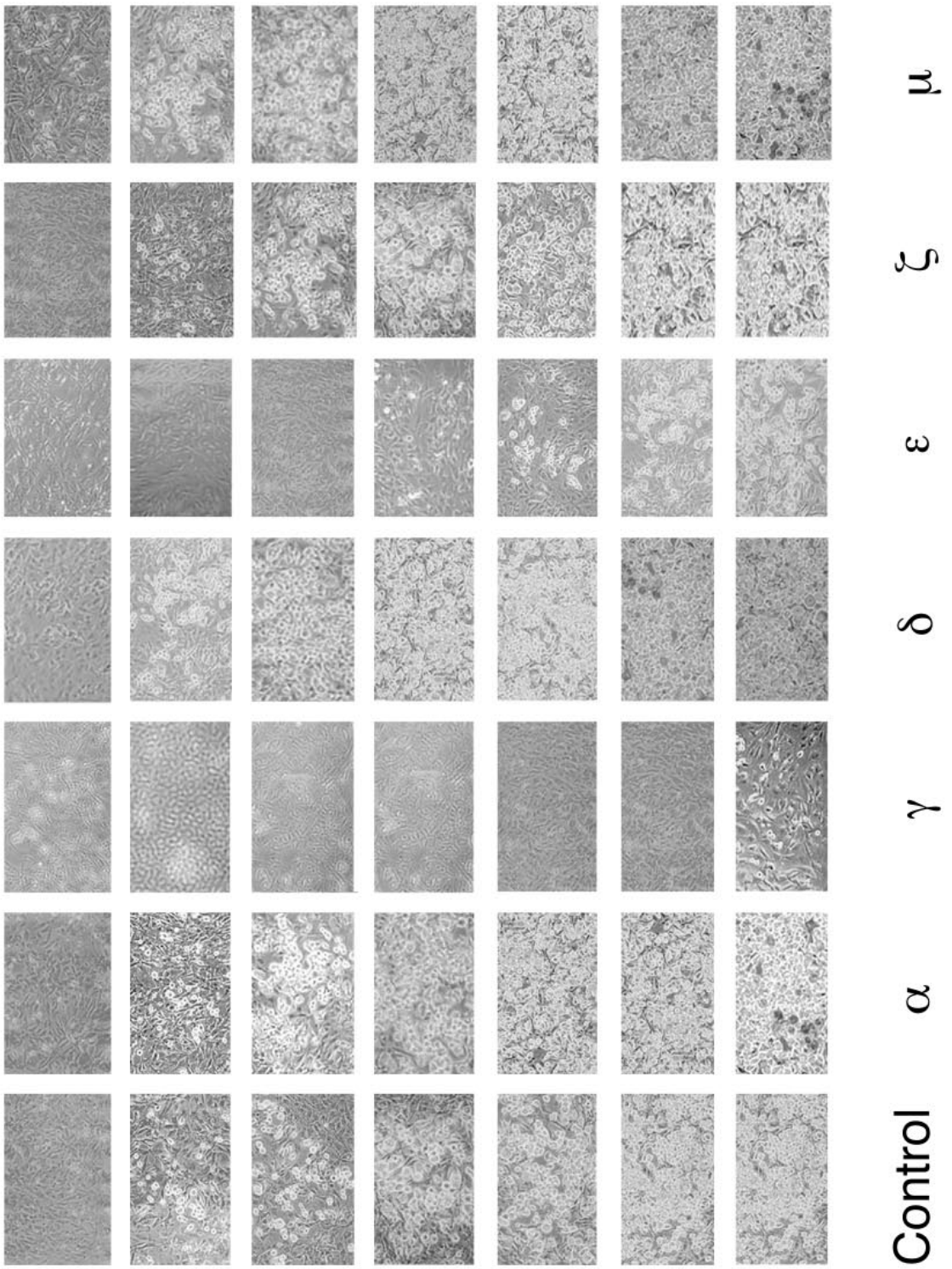

Figure 4 Differential effects of down-regulation of specific PKC isoforms by anti-sense ODNs on differentiation of 3T3-F442A pre-adipocytes

3T3-F442A pre-adipocytes were preincubated with lipofectin alone (control), or anti-sense ODNs to the PKC isoform indicated, prior to the induction of differentiation as described in the Materials and methods section. Changes in morphology were followed by photographing cells daily over the next 7 days. Results shown are representative of experiments performed on at least six occasions.

Table 2 Differential effects of anti-sense on induction of GPDH activity during differentiation of 3T3-F442A preadipocytes

3T3-F442A cells were preincubated with anti-sense ODNs to the PKC subtype indicated prior to the induction of differentiation. Homogenates were prepared from cells at days 4 and 7 of differentiation for determination of GPDH activity. GPDH activity of homogenates is expressed as a percentage of the activity in homogenates prepared from control cultures differentiated following pretreatment with lipofectin alone (100%). Data are means ± S.E.M. of six separate experiments. * indicates that the value differs significantly from that for cells treated with lipofectin alone, $P < 0.001$. Statistical analysis was by Student's *t*-test for paired samples.

		GPDH activity (% of control)	
ODN treatment	Day of differentiation ...	4	7
Anti-sense α		127.0 ± 2.50*	103.0 ± 0.40
Anti-sense γ		87.2 ± 1.30*	11.3 ± 0.20*
Anti-sense δ		162.9 ± 8.00*	108.9 ± 0.50*
Anti-sense ϵ		84.7 ± 1.42*	34.8 ± 0.30*
Anti-sense ζ		101.8 ± 2.40	100.5 ± 0.87
Anti-sense μ		153.2 ± 4.00*	108.5 ± 1.20*

on expression of the adipocyte marker enzyme GPDH and (ii) PKC-ϵ and -γ are necessary for differentiation of 3T3-F442A cells.

PKC-γ, but not PKC-ε, is required for the clonal expansion phase of adipocyte differentiation

Following the induction of differentiation, 3T3-pre-adipocytes undergo several rounds of mitotic clonal expansion, which is necessary for terminal differentiation to occur [1,2]. This is reflected by an approximate doubling of the DNA content of cell cultures which can be observed some two days after exposure to differentiation medium (Table 3). Of particular interest is whether the anti-differentiative effects of depleting PKC-ϵ and -γ reflect a requirement for these subtypes in the process of clonal expansion. The results in Table 3 clearly demonstrate that the increase in DNA content observed in control cultures at day 2 of differentiation was no longer apparent if cells were depleted of PKC-γ prior to exposure to differentiation medium. Indeed, in cultures depleted of PKC-γ the increase in DNA content was not observed until day 7 of differentiation. In contrast, treatment of cells with the corresponding PKC-γ sense ODN or anti-sense depletion of PKC-ϵ had no effect upon clonal expansion (Table 3). Similarly we failed to detect any effect upon clonal expansion, as measured by an increase in the DNA content of cultures, following depletion of the α, δ, μ or ζ subtypes of PKC (results not shown). These results suggest that differential mechanisms account for the anti-differentiative effects of depletion of PKC-γ and -ϵ. Thus PKC-γ appears to be required for the clonal expansion of differentiating pre-adipocytes, whereas PKC-ϵ is not and is likely therefore to influence another aspect of the differentiation process.

DISCUSSION

There is now mounting evidence to show that the multiple members of the PKC family assume distinct functional roles in the regulation of cellular growth and differentiation [3–11]. In particular the PKC family of isoforms has an important role in the cross-talk that serves to integrate multiple signalling pathways and in the regulation of gene expression [43–45]. Clearly adipocyte differentiation is a complex process, requiring the strict ordering of a cascade of multiple and interacting molecular events, leading to the expression of multiple genes required for maintenance of the adipocyte phenotype [1,2]. In this study we have used the 3T3-F442A pre-adipocyte cell model to investigate the role of individual PKC isoforms in the regulation of adipocyte development.

We detected the α, γ, δ, ϵ and ζ isoforms of PKC in rat adipocytes and in 3T3-pre-adipocytes and adipocytes. The presence of these subtypes in rat adipocytes is consistent with previous reports [26,46], and the expression of PKC-α, -ζ and -ϵ has been reported in 3T3-L1 pre-adipocytes and adipocytes [27,28,47]. Although Farese and co-workers [46,47] have reported the presence of PKC-β in both rat adipocytes and 3T3-L1 adipocytes, we have been unable to detect this isoform. The reason for this discrepancy in results is unknown. Others have reported that PKC-β is not expressed in 3T3-L1 pre-adipocytes or adipocytes but that brief, transient, expression of this isoform occurs during differentiation of these cells [28]. However, we were unable to detect PKC-β at any point during the differentiation of 3T3-F442A cells (I. Fleming and E. Kilgour, unpublished work).

Initially, in order to gain an indication of their functional roles, we examined changes in the expression of individual PKC subtypes during differentiation of 3T3-F442A cells. Cellular levels of PKC-ζ were unchanged throughout differentiation, suggesting that it assumes no obligatory role in the regulation of this process. Although PKC-μ levels were elevated in adipocytes, this was attributable to prolonged confluency rather than to the differentiation process itself. Following the induction of different-

Table 3 Depletion of PKC-γ with anti-sense ODN inhibits clonal expansion of differentiating 3T3-F442A cells

3T3-F442A pre-adipocytes were pretreated with anti-sense ODNs to either PKC-γ or PKC-ϵ or sense ODN to PKC-γ for 48 h prior to the induction of differentiation. Cellular homogenates were prepared either just prior to (day 0) or 2, 4 or 7 days after the induction of differentiation for determination of DNA content. Data are means ± S.E.M. of six separate experiments. *indicates that the value differs significantly from that for cells treated with lipofectin alone, $P < 0.001$. Statistical analysis was by Student's *t*-test for paired samples.

		Total DNA per plate (μg/ml of extract)			
ODN treatment	Day of differentiation ...	0	2	4	7
None		7.4 ± 0.41	14.9 ± 1.44	14.9 ± 1.35	15.0 ± 1.51
Anti-sense γ		7.3 ± 0.29	7.4 ± 0.52*	7.2 ± 0.49	14.7 ± 1.43
Anti-sense ϵ		7.4 ± 0.30	14.4 ± 1.71	14.7 ± 1.52	15.0 ± 1.11
Sense γ		7.5 ± 0.49	14.9 ± 1.61	15.2 ± 1.56	15.1 ± 1.61

iation, cellular levels of PKC-α, -γ and -δ increased rapidly (Figure 2), peaking at around day 2, correlating with the time point at which clonal expansion of the differentiating cells was observed (Table 3). Subsequently, cellular levels of the α, γ and δ subtypes decreased such that a significant reduction in their expression was observed as cells attained the adipocyte phenotype. Thus following the clonal expansion stage, these isoforms may exert an inhibitory influence upon the differentiation process. PKC-ϵ expression increased during differentiation, implying a requirement for this isoform for induction and maintenance of the adipocyte phenotype. Consistent with our observations in 3T3-F442A cells, it has been reported that in the related 3T3-L1 cell line differentiation results in no change in the cellular levels of PKC-ζ, but is accompanied by an increase in PKC-ϵ expression and a reduction in expression of PKC-α [27,28].

To define the dependence of adipocyte differentiation on PKC isoforms more precisely, we used anti-sense ODNs to achieve the selective depletion of individual PKC subtypes. Our results showed that we could achieve the complete depletion (assessed by Western blotting) of PKCs by using sequence-specific anti-sense ODNs designed to hybridize with the isoform of interest. Importantly, none of our anti-sense treatments significantly affected the expression of other PKC subtypes. Using this approach we demonstrated that, in agreement with the expression data, depletion of PKC-ζ had no detectable effect upon adipocyte differentiation. In contrast, depletion of PKC-α, -δ or -μ significantly accelerated the differentiation of fibroblasts into adipocytes. Hence these three isoforms each exert an inhibitory influence upon adipocyte differentiation. Therefore the decrease in expression of the α and δ subtypes, which occurs during the later stages of differentiation, may be required for the attainment and maintenance of the adipocyte phenotype.

Depletion of PKC-γ, which displayed a similar temporal pattern of expression to the α and δ PKC subtypes, severely inhibited adipocyte differentiation. Further anti-sense studies revealed that PKC-γ is essential for the clonal expansion phase, which occurs early in the differentiation process. Thus there is an absolute requirement for PKC-γ in the process of clonal expansion. These experiments also show that neither PKC-α nor PKC-δ, whose expression levels follow a similar temporal pattern to those of PKC-γ and are thus elevated during the clonal expansion phase, are able to functionally substitute for PKC-γ.

Depletion of PKC-ϵ also severely attenuated adipocyte differentiation, thus demonstrating that other PKC subtypes are unable to assume the essential functional role that this isoform plays in adipocyte development. Further studies revealed that PKC-ϵ is not required for clonal expansion of differentiating cells, but it is likely to be necessary in the later stages of differentiation, when its expression is markedly elevated, for the attainment and maintenance of the adipocyte phenotype.

In conclusion, we have shown that in 3T3-F442A cells the α, δ and μ isoforms of PKC exert an inhibitory influence upon the process of adipocyte differentiation, whereas PKC-ϵ is essential for the attainment of the adipocyte phenotype and PKC-γ is required for the clonal expansion of differentiating pre-adipocytes. These results demonstrate that PKC subtypes assume differential functional roles in 3T3-F442A cells and are therefore likely to target different cellular substrates. At present the mechanisms by which the PKC family regulate adipocyte development are uncertain and are the subject of further studies. These findings may have relevance to our understanding of the diseased state of obesity, which involves changes in the turnover of adipocyte cells within adipose tissue [48] and involves selective changes in the expression of PKC isoforms [27].

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