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Research Article

Protein kinase C-ε promotes adipogenic commitment and is essential for terminal differentiation of 3T3-F442A preadipocytes

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Abstract. The role of protein kinase C (PKC) isoforms in the commitment of multipotent fibroblasts to the adipocyte lineage and in their terminal differentiation into mature adipocytes was investigated. Ectopic overexpression of PKC-ε, but not other PKC isoforms, committed multipotent NIH-3T3 cells to adipogenic differentiation in the presence of hormonal inducers. In committed 3T3-F442A preadipocytes, PKC-ε protein expression increased during the course of terminal differentiation and cell-permeable PKC-ε inhibitory peptides, which prevent interaction with RACK (receptor for activated C-kinase) proteins, severely inhibited differentiation. PKC-ε accumulated in the nuclei of 3T3-F442A cells shortly after induction of differentiation and exhibited a distinctive punctate speckling immunocytochemical staining pattern. The spatiotemporal aspects of PKC-ε localization and expression coincided with that of C/EBP-β, a transcription factor critically involved in promoting the early phase of adipogenesis. Collectively, these results demonstrate a role for PKC-ε in both adipogenic commitment and preadipocyte terminal differentiation.

Key words. Multipotent; fibroblast; adipogenesis; NIH-3T3; 3T3-F442A; nucleus.

White adipose tissue acts as an important regulator of energy balance by storing energy in the form of triglycerides during periods of nutritional excess and releasing it in the form of free fatty acids in times of nutritional need. Excessive deposition of adipose tissue, as occurs in obesity, is caused by increases in both the size and number of adipocytes. Adipocytes are terminally differentiated cells formed from committed fibroblast-like precursors, known as preadipocytes. Preadipocytes themselves are formed from multipotent fibroblast precursors during embryonic development. The process of adipocyte differentiation has been studied extensively using mouse multipotent stem cell lines such as $10T^{1/2}$ and NIH-3T3 and committed preadipocyte lines such as 3T3-L1, 3T3-F442A and Ob1771. These studies have led to the identification of numerous adipocyte-specific genes, whose expression is induced during differentiation, as well as a detailed understanding of their transcriptional regulation [1–4]. To enter the differentiation program, preadipocytes must first undergo cell cycle arrest. Adipogenic stimuli then induce several rounds of cell division, a process known as clonal expansion. The expanded population of preadipocytes then withdraws from the cell cycle a second time and enters the terminal differentiation program. Adipogenesis is regulated to a large degree by two groups of transcription factors, C/EBP and PPAR-γ. In response to adipogenic stimuli there is rapid induction of C/EBP-β and C/EBP-δ. These factors then promote the transcription of PPAR-γ2 and C/EBP-α, which in turn transactivate the promoters of genes associated with the adipocyte

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phenotype, such as the insulin receptor, glycerophosphate dehydrogenase, acetyl CoA carboxylase and adipocyte-specific fatty acid-binding protein (AP2). Although the transcriptional regulation of adipogenesis is now relatively well understood, less is known about the signalling pathways linking the receptors for adipogenic stimuli with the transcriptional regulators of the process.

Protein kinase C (PKC) is a family of structurally and functionally distinct serine/threonine kinases that play key roles in many cellular processes, including proliferation, differentiation and transformation [5]. The ten identified PKC isoforms are classified according to their activation requirements. The classical PKCs (cPKC: α, β_I, β_{II} and γ) require calcium, diacylglycerol (DAG), and phosphalidylserine (PS); the novel PKCs (nPKC: δ, ε, η and θ) require DAG and PS, but have no requirement for calcium; the atypical PKCs [aPKC: ζ, and ι (λ)] require neither DAG nor PS, but do depend upon PS for activation and are regulated by alternative lipids including ceramide and phosphatidylinositol-3,4,5-trisphosphate [6–8]. A key feature of the process of PKC activation involves their translocation from one cellular compartment to another. Most PKC isoforms reside predominantly in the cytosol in their basal state and translocate to the plasma membrane or other subcellular organelles upon activation. Activated PKCs are further targetted to specific locations in an isoform-specific fashion by a family of proteins known as RACKs [9].

A number of lines of evidence indicate that PKC isoforms play an important and, perhaps, essential role in adipogenesis. Initial studies using phorbol esters, and other non-selective PKC-modulatory agents, suggested both stimulatory and inhibitory roles for PKC in the process [10–13]. More recently, the expression of specific PKC isoforms has been shown to change as preadipocytes differentiate into adipocytes [14–16]. The most notable and consistent of these changes are the reduction in PKC-α expression and the rise in PKC-ε expression. Critical roles for these and other PKC isoforms in the differentiation of 3T3-F442A cells were revealed by our previous studies using isoform-directed antisense oligonucleotides [16]. Thus cellular depletion of PKC-α accelerated differentiation whereas depletion of PKC-ε severely inhibited the process. The purpose of the present study was to further clarify the role of PKC-ε in adipocyte differentiation. We provide evidence that PKC-ε protein expression is not only essential for terminal differentiation, but that its overexpression can commit multipotent fibroblasts to the adipocyte lineage.

Materials and methods

Materials

NIH-3T3 cells stably expressing different PKC isoforms [17] were a kind gift from Dr Mischak (NCI, Bethesda, Md.). PKC-ε inhibitory peptides were obtained from Dr Mochly-Rosen (Stanford, Calif.). Antibodies specific for PKC isoforms were purchased from the following sources: PKC-α and -ζ (Upstate, Botolph Claydon, UK), PKC-δ and -η (Santa Cruz, Biotechnology, Calne, UK), PKC-$\beta_{I/II}$ and -ε (Sigma, Poole, UK). Ciglitazone was obtained from Alexis Biochemicals (Nottingham, UK).

Cell culture

3T3-F442A cells were obtained from Dr H. Green (Harvard Medical School, Cambridge, Mass.) and grown in DMEM (InVitrogen, Paisley, UK) supplemented with 2 mM L-glutamine and 10% newborn calf serum (Sigma). Experiments were conducted on cells between passage 3 and 20. NIH-3T3 fibroblasts were maintained in logarithmic growth phase in DMEM containing 10% fetal calf serum (FCS) and 2 mM glutamine.

To induce adipocyte differentiation, 3T3-F442A preadipocytes were grown to confluence and maintained at confluence for 2 days. Culture medium was then changed to differentiation medium [DMEM supplemented with 2 mM L-glutamine, 10% FCS and insulin (5 µg/ml)] and changed every 2 days thereafter. To induce differentiation in NIH-3T3 fibroblasts, 2-day post-confluent cells were switched to differentiation medium containing dexamethasone (1 µM), isobutylmethylxanthine (500 µM) and the PPARγ agonist ciglitazone (500 nM). After 2 days, cells were switched to differentiation medium, which was then changed every 2 days.

Differentiation was assessed by measurement of glyceraldehyde-3-phosphate dehydrogenase activity, as described previously [18] or by staining of neutral lipids with Nile Red (Sigma), as follows. Cells were grown and differentiated on glass coverslips within six-well cell culture plates. At the desired time point, cells were washed three times with PBS then fixed in 100% methanol for 20 min at –20 °C. Nile Red solution (1 µg/ml in PBS) was then applied at room temperature for 1 h, following which cells were mounted, counterstained with DAPI and viewed by indirect fluorescence microscopy (Axioplan 2; Zeiss, Bicester, UK). Images were captured with a CCD camera and manipulated using KS300 software (Imaging Associated, Bicester, UK).

Immunocytochemistry

Cells were fixed as described above before blocking in 5% serum (Vector, Peterborough, UK) containing 0.3% Triton-X100 for 1 h at room temperature. Following three washes in ice-cold PBS, the primary antibody (diluted in PBS containing 10% FCS and 0.1% sodium azide) was then applied for 1 h. The coverslips were then washed as above, before applying species-specific fluorochrome-conjugated secondary antibodies (Vector) in the dark for 1 h, as detailed in the legends to figures. Finally, coverslips were mounted onto glass microscope slides using

Vectashield containing DAPI, and the edges sealed with clear nail varnish. Slides were stored at 4 °C until viewing by fluorescence microscopy.

For co-localization studies, two-colour immunostaining was carried out as described above with the following modifications. Since the only antibodies available for these studies were all raised in rabbit, the following procedure was devised to eliminate secondary-antibody cross-reactivity. Following incubation with the first primary antibody and requisite fluorescein (FITC)-conjugated secondary antibody, as described above, rabbit serum (5%) was added for 1 h to block remaining antigen-binding sites on the secondary antibody. An excess of anti-rabbit $F(ab)_2$ (Jackson Immuno Research Labs, Soham, UK) was then applied for 1 h to block remaining sites on the primary antibody available for secondary-antibody binding. After extensive washing of the coverslips with ice-cold PBS, the second primary antibody was applied for 1 h, followed by the second Texas Red (TR)-conjugated secondary antibody. The coverslips were then mounted as described above. Control experiments confirmed that this procedure prevented recognition of the first primary antibody by the TR-conjugated secondary antibody (data not shown).

Subcellular fractionation

Cells were grown and differentiated in standard 90-mm dishes and, at the required timepoint, washed three times in ice-cold PBS. Cells were then gently scraped into 1 ml ice-cold PBS and centrifuged (200 g, 5 min, 4 °C). The cell pellet was resuspended in one volume of buffer 1 [10 mM HEPES, pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 10 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 100 µM leupeptin, aprotinin (2 µg/ml), pepstatin A (1 µg/ml)] and placed on ice for 15 min. The cell suspension was then forced through a 23-G needle five times, and centrifuged at 1000 g for 1 min at 4 °C. The resulting pellet was resuspended in 2/3 volume of buffer 2 [20 mM HEPES, pH 8.0, 1.5 mM $MgCl_2$, glycerol (25%); 420 mM NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 100 µM leupeptin, aprotinin (2 µg/ml), pepstatin A (1 µg/ml)] and rotated at 4 °C for 30 min. Debris was pelleted and the supernatant used as the nuclear extract fraction. The supernatant generated from the initial 1000 g spin was centrifuged at 100,000 g for 45 min at 4 °C (TL-100, Beckman, High Wycombe, UK). The resulting supernatant was used as the cytosolic fraction. The pellet was washed once and then resuspended in buffer 1 containing 1% Triton-X100 and placed on ice for 30 min with occasional agitation. The suspension was then centrifuged as before, yielding the membrane extract fraction in the supernatant. After isolation, fractions were denatured by boiling in Laemmli SDS-PAGE sample buffer for 5 min. The purity of the designated fractions was confirmed by Western blotting with antibodies to subcellular markers; cytosol: glucose-6-phosphate dehydrogenase (GPDH) (Biogenesis, Poole, UK), nucleus: lamin A/C (Santa Cruz) and membrane: sodium/hydrogen exchanger (Abcam, Cambridge, UK).

SDS-PAGE and Western blotting

Equivalent amounts of extract protein, determined by the Bradford assay, were subjected to electrophoresis on 10% polyacrylamide gels followed by transfer to nitrocellulose (Hybond-C; Amersham, Little Chalfont, UK). Membranes were then stained with Ponceau S solution (0.1%) to confirm equal loading, and then blocked in 5% non-fat dry milk for 1 h. Monoclonal anti-PKC-ε antibodies (Sigma) were diluted (1 : 1000) in 1% milk and applied to the membranes overnight at 4 °C. After extensive washing, the membranes were reacted with horseradish peroxidase-conjugated secondary antibodies (1 : 10,000 dilution) for 1 h at room temperature and immunoreactive proteins detected by enhanced chemiluminescence (Super Signal WestPico; Pierce, Warrington, UK).

Results

PKC-ε is expressed at low levels in murine preadipocytes and its expression rises steadily during their differentiation into adipocytes. Ectopic expression of genes that regulate adipogenesis, such as *C/EBPα* and *PPARγ*, in non-adipogenic fibroblasts can promote their commitment to the adipocyte lineage [19, 20]. To investigate the ability of PKC-ε to promote adipogenic commitment, we examined the effect of overexpressing this isoform in multipotent NIH-3T3 fibroblasts, which are not committed to the adipocyte lineage. Treatment of 2-day post-confluent NIH-3T3^{PKCε} cells with adipogenic medium resulted in the appearance of cellular lipid droplets within 3–4 days and extensive lipid accumulation after 8–12 days (fig. 1B). By contrast, treatment of parental NIH-3T3 cells, which express low levels of PKC-ε (fig. 1A) did not induce lipid accumulation. The ability of NIH-3T3$^{PKC-ε}$ cells to undergo adipogenic conversion was confirmed by measuring expression of the adipocyte-specific marker enzyme GPDH (fig. 1C). To examine whether the adipogenic property of PKC-ε was restricted to this isoform, the ability of NIH-3T3 cells, stably overexpressing other PKC isoforms (α, $β_{I/II}$, δ, η and ζ), to undergo adipogenesis was tested. Overexpression of the requisite PKC isoform was confirmed by Western blotting with isoform-specific antibodies and did not significantly affect the expression of other PKC isoforms [data not shown and see ref. 17]. Exposure of each of these lines to hormonal inducers resulted in little adipogenic conversion assessed by GPDH activity measurement (fig. 1C) or Nile Red staining (data not shown). Maximal differentiation of

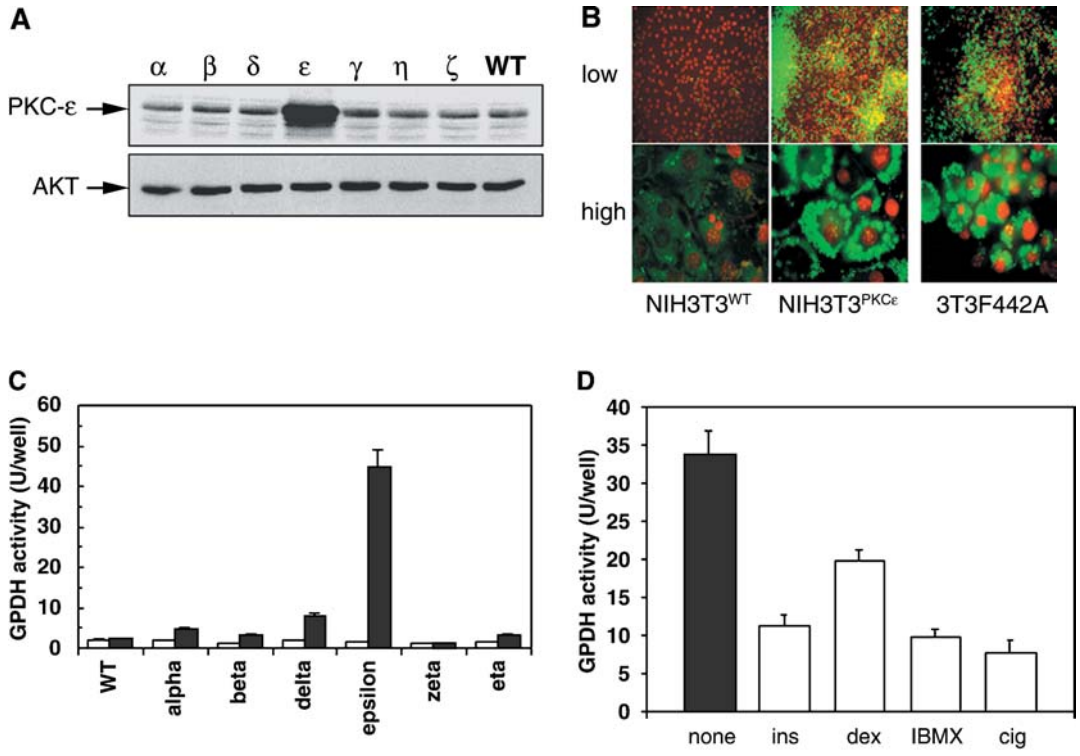

Figure 1. Ectopic expression of PKC-ε commits NIH-3T3 cells to the adipocyte lineage. (*A*) Lysates containing 20 µg protein from NIH-3T3 cells overexpressing PKC-α, -β, -δ, -ε, -γ, -η, -ξ or parental cells with no overexpression (WT) were immunoblotted with anti-PKC-ε antibodies (top panel), or anti-AKT antibodies (lower panel) to demonstrate equal loading of the samples. (*B*) Confluent cultures of NIH-3T3^{WT}, NIH-3T3$^{PKC\text{-}\varepsilon}$ or 3T3-F442A preadipocytes were incubated for 8 days in the requisite differentiation medium, as described in Materials and methods. Cells were then fixed and stained with the neutral lipid stain Nile Red (depicted as green) and the nuclei counterstained with DAPI (red). Two magnified views are shown: × 20 (upper panels) and × 100 (lower panel). (*C*) NIH-3T3 cells overexpressing PKC-α, -β, -δ, -ε, -γ, -η, -ξ or parental cells with no overexpression (WT) were incubated in growth medium (white bars) or differentiation medium (black bars) for 8 days following which cell lysates were prepared and assayed for GPDH activity, as described in Materials and methods. Data are means ± SE from three independent experimental runs for each cell line. (*D*) NIH-3T3$^{PKC\text{-}\varepsilon}$ cells were induced to differentiate for 8 days in complete differentiation medium (none), or with medium lacking insulin (ins), dexamethasone (dex), isobutylmethylxanthine (IBMX) or ciglitazone (cig). GPDH activity was then measured. Data are averages of duplicate determinations from a single experiment, representative of three in total.

NIH-3T3$^{PKC\text{-}\varepsilon}$ cells was dependent upon all of the added components of the differentiation medium, i.e. insulin, dexamethasone, IBMX and the PPARγ agonist ciglitazone (fig. 1D). These results indicate that ectopic overexpression of PKC-ε, but not of five other PKC isoforms, is sufficient to commit NIH-3T3 cells to the adipocyte lineage.

To investigate the role of PKC-ε in adipogenesis further, we returned to the 3T3-F442A preadipocyte system. Our previous work, using antisense oligonucleotides, suggested that expression of PKC-ε was necessary for the differentiation of these cells into adipocytes [16]. To extend these findings we decided to examine whether functional PKC-ε protein was also required for the differentiation of these cells. To do this we employed a peptide derived from the N-terminal region of PKC-ε (PKC-εV1-2), which, when conjugated to a cell-permeable peptide carrier, acts as a specific inhibitor of PKC-ε signalling in various cellular contexts [21, 22], by preventing its translocation and interaction with the PKC-ε-specific RACK protein [23]. Compared to untreated cells, inclusion of PKC-εV1-2 in the differentiation medium severely retarded (by up to 74%) the acquisition of the mature adipocyte phenotype, assessed by Nile Red staining of neutral lipids and measurement of GPDH activity (fig. 2). These effects were dependent upon the concentration of peptide administered and did not occur with control peptides, consisting of either carrier peptide alone or carrier conjugated to a scrambled PKC-εV1-2 sequence. These data support our previous conclusions and further demonstrate a requirement for PKC-ε translocation and activation for the differentiation of 3T3-F442A preadipocytes.

Activation of PKC isoforms occurs in conjunction with their translocation to subcellular sites where specific binding partners and substrates are located. We therefore decided to define the cellular location of PKC-ε during the differentiation of 3T3-F442A cells, using two ap-

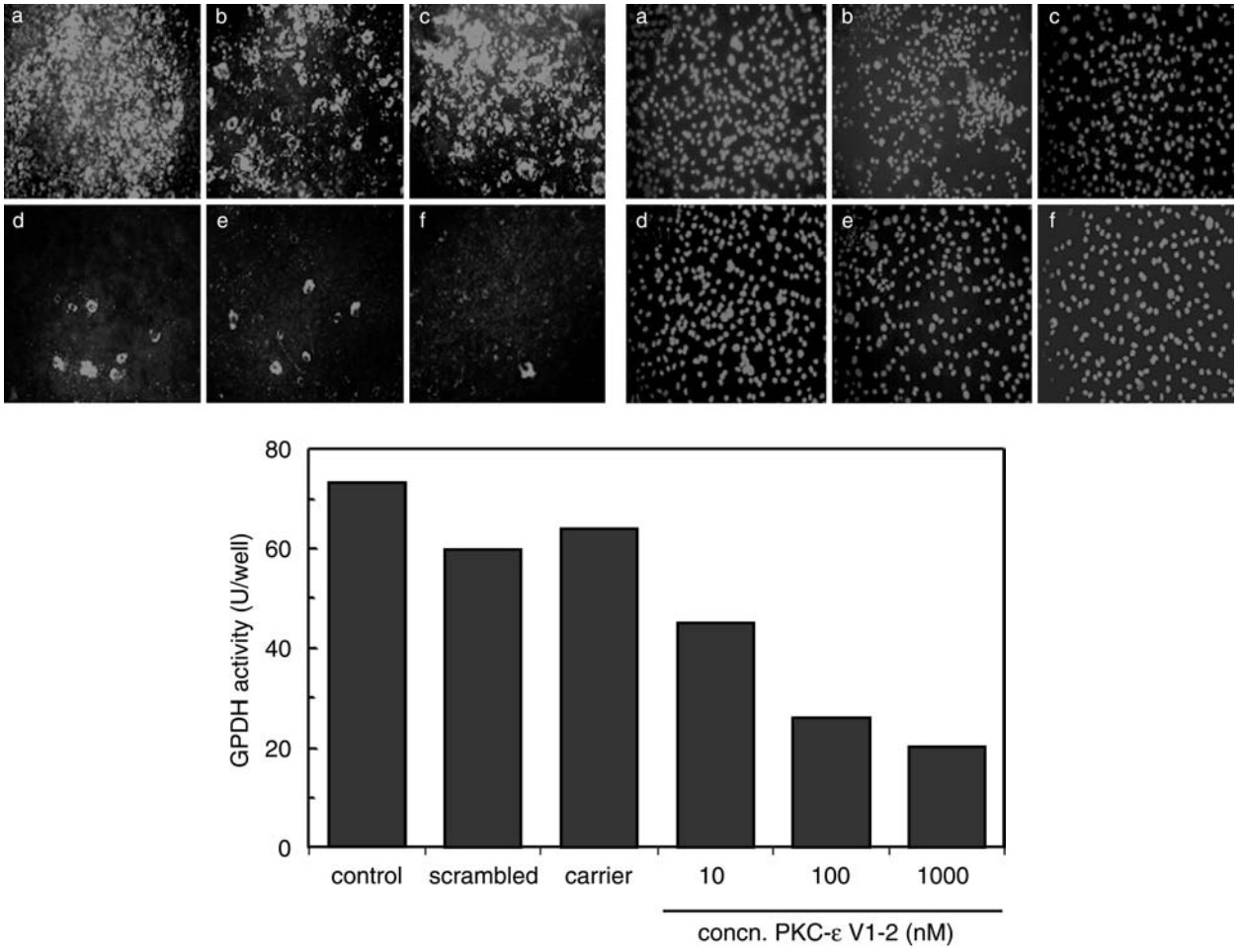

Figure 2. Differentiation of 3T3-F442A preadipocytes requires functional PKC-ε. *Upper panel*: 2-day confluent cells were induced to differentiate under standard conditions (a), in the presence of scrambled peptide (b), antennapedia peptide carrier alone (c) or with PKC-εV1-2 peptide at 10 nM (d), 100 nM (e) or 1000 nM (f). Peptides were replenished every 24 h and the incubations continued for a total of 8 days, following which cells were fixed and stained with Nile Red (green) with DAPI counterstaining (red). *Lower panel*: cells from experiments run in parallel to those depicted above were lysed and assayed for GPDH activity. Data are averages of duplicate determinations and are representative of three similar experiments.

proaches. First, cells were harvested at different stages of differentiation prior to their fractionation into cytosolic, membrane and nuclear fractions by ultracentrifugation. Immunoblotting of these fractions demonstrated alterations in both the expression and localization of PKC-ε during differentiation. In agreement with our previous data [16], the overall expression level of PKC-ε increased as differentiation ensued. The majority of this increase was observed within the cytosolic fraction (fig. 3A). PKC-ε was also found within the membranous fraction at fairly constant levels throughout the timecourse. PKC-ε was detectable in the nuclear fraction at the onset of differentiation but markedly accumulated within this fraction as differentiation proceeded.

We confirmed the nuclear localization of PKC-ε using immunocytochemistry. As shown in figure 3B, PKC-ε was expressed at low levels predominantly within the cytosol in confluent preadipocytes prior to induction. Following treatment of cells with differentiation medium for 8 h, PKC-ε immunoreactivity was detectable in association with the nuclei where it stained with a discrete speckled pattern. As differentiation proceeded, the amount of PKC-ε in both the cytoplasm and nucleus increased. Localization at time points beyond 2 days could not be assessed because cells begin to express surface glycosaminoglycans, which interfere with antibody binding and make immunocytochemical assessments uninterpretable [24]. Parallel experiments revealed that neither PKC-α nor PKC-ζ were detectable in the nucleus within the same time frame (data not shown). Taken together these data demonstrate that nuclear localization of PKC-ε is an early event in the differentiation of 3T3-F442A preadipocytes.

Given the apparent importance of PKC-ε in promoting adipocyte differentiation, PKC-ε is likely to phosphorylate proteins that play a major role in regulating the

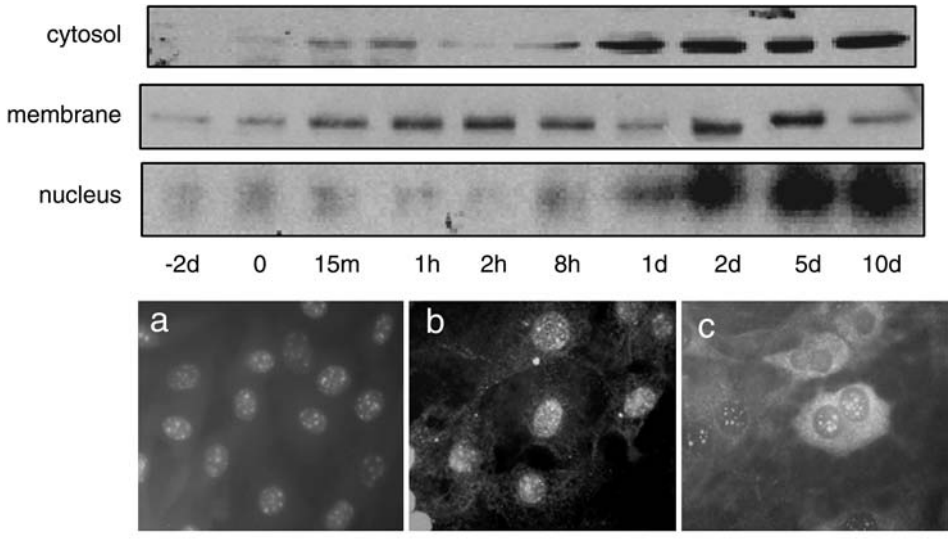

Figure 3. PKC-ε accumulates in the nucleus of differentiating 3T3-F442A preadipocytes. *Upper panel*: cells were induced to differentiate under standard conditions and, at the times indicated, homogenized and fractionated into cytosolic, membranous and nuclear fractions, as described in Materials and methods. Equivalent amounts of each fraction (20 µg of protein) were then immunoblotted with antibodies to PKC-ε. *Lower panel*: cells prior to induction (a) or following induction for 8 h (b) or 2 days (c) were fixed and stained with Nile Red (green) and DAPI counterstaining (red).

process. The temporal and spatial aspects of PKC-ε protein expression during differentiation suggest that such substrates may be present in the nucleus at early stages of the process. The β and δ isoforms of the C/EBP family of transcription factors are expressed early in adipogenic differentiation and play an essential role in the cascade of events that ultimately lead to the expression of adipocyte-specific genes. C/EBPs are also subject to regulation by phosphorylation [25], although the protein kinases involved have, in most cases, not been identified. We therefore sought evidence for a link between PKC-ε and C/EBP isoforms during the early stages of differentiation. At various stages after the induction of differentiation, 3T3-F442A cells were co-stained for PKC-ε along with each of the three C/EBP isoforms, α, β and δ. As expected, C/EBP-α was barely detectable during the very early stages of differentiation and was only observed after 2 days, when it was located within the nucleus (data not shown). Consistent with its earlier role in differentiation, C/EBP-β was detectable in cells prior to induction, with some evidence of nuclear staining. However, following 8 h of exposure to differentiation medium, C/EBP-β stained only in the nucleus, where it exhibited a distinctive speckled pattern similar to that found for PKC-ε (fig. 4). After 2 days, the amount of C/EBP-β in the nucleus had decreased substantially but the staining was still of the same pattern. C/EBP-δ localization was qualitatively similar to that of C/EBP-β, with evidence of nuclear accumulation during the first 2 days of differentiation (data not shown). When cells were co-stained with antibodies to C/EBP-β and PKC-ε, the two

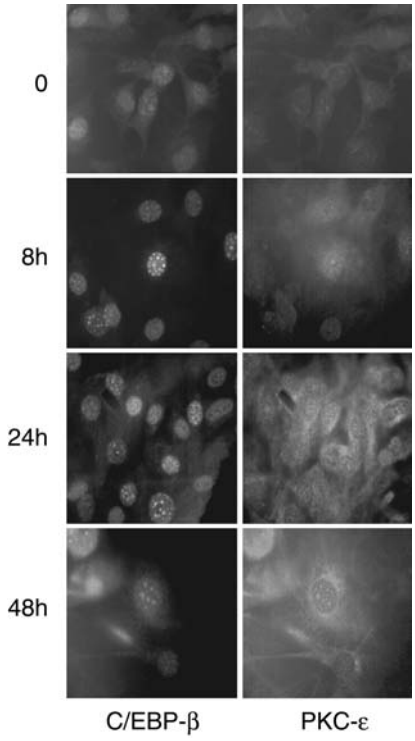

Figure 4. Co-localization of PKC-ε and C/EBP-β during early differentiation of 3T3-F442A preadipocytes. Cells were induced to differentiate under standard conditions and, at the times shown, were fixed and stained first with antibodies to C/EBP-β and then with ones to PKC-ε. Following incubation with C/EBP-β rabbit polyclonal antibodies and FITC-conjugated secondary antibodies (signal depicted green, left-hand panels), sites were blocked as described in detail in Materials and methods. The fixed cells were then reacted with PKC-ε antibodies and the signal revealed with TR-conjugated secondary antibodies (red, right-hand panels).

resultant immunoreactive signals exhibited an identical staining pattern within a majority of cells examined (fig. 4). These results provide evidence that the two proteins may co-localize during the early stages of differentiation.

Discussion

The combined findings of this study provide evidence that PKC-ε promotes adipogenesis at two stages of the pathway leading from multipotent fibroblasts to mature adipocytes. Overexpression of PKC-ε was sufficient to commit multipotent NIH-3T3 cells to the adipocyte lineage; following treatment with adipogenic stimuli and blockade of PKC-ε function, 3T3-F442A preadipocytes were severely restricted in their ability to differentiate into adipocytes.

The adipogenic properties of PKC-ε, when overexpressed in NIH-3T3 cells, appear specific for this isoform, since other PKC isoforms, known to be expressed in preadipocytes or adipocytes, had little or no adipogenic potential. The mechanisms through which overexpression of PKC-ε promotes adipogenesis in NIH-3T3 cells are unknown. NIH-3T3$^{PKC\varepsilon}$ cells express approximately six-fold higher PKC enzyme activity [26] suggesting that targets for phosphorylation by PKC-ε could mediate the adipogenic effect of overexpression. However, increased global PKC activity per se does not trigger adipocyte lineage commitment, since NIH-3T3 cells overexpressing other PKC isoforms also exhibit four- to eightfold increases in PKC activity [26].

Differentiation of NIH-3T3$^{PKC\varepsilon}$ cells was not spontaneous and still required the addition of standard adipogenic hormones to the incubation medium. This suggests that PKC-ε sensitizes otherwise non-committed cells to a state capable of responding to these stimuli. In this regard, NIH-3T3$^{PKC\varepsilon}$ exhibit increased responsiveness to insulin [26]. However, since overexpression of other PKC isoforms led to similar effects, increased insulin sensitivity is unlikely to account for the specific ability of PKC-ε to promote adipogenesis. Indeed, since omission of any one of the inducers (insulin, dexamethasone, IBMX or ciglitazone) from the differentiation medium substantially reduced the ability of NIH-3T3$^{PKC\varepsilon}$ cells to undergo adipogenesis, PKC-ε is likely acting at a point downstream of the convergent actions of these adipogenic stimuli.

Our previous studies had suggested a requirement for the expression of PKC-ε for differentiation of 3T3-F442A preadipocytes. The results reported here confirm the PKC-ε requirement and further provide evidence that functional PKC-ε protein is essential for differentiation. In the absence of available small-molecule inhibitors with sufficient selectivity for PKC-ε we employed peptides targetted to the variable (V1-2) domains of PKC-ε that block the interaction of PKC-ε with its specific RACK-binding protein [23]. A number of studies in various cell types have previously demonstrated both the effectiveness and specificity of these peptides in blocking PKC-ε function [22, 23, 27, 28]. Our work therefore implies that the essential role of PKC-ε in adipocyte differentiation involves its interaction with RACK proteins, such as the recently identified εRACK [29]. The inhibition of adipocyte differentiation by PKC-εV1-2 peptide was dose dependant but incomplete (74% at maximum dose applied). As differentiation takes place over a period of several days, the peptide had to be reapplied a number of times to maintain sufficiently high concentrations to inhibit PKC-ε function. The lack of complete inhibition of the process may therefore have arisen from our inability to block PKC-ε function completely. Alternatively, the requirement for PKC-ε may not be absolute. A similar requirement for PKC-ε in promoting adipogenesis in NIH-3T3 cells could not be demonstrated as the inhibitory peptides are incapable of blocking PKC-ε function when the enzyme is expressed at high levels [D. Mochly-Rosen, personal communication]. The alternative strategy of using selective ATP competitive inhibitors was not possible due to the toxicity of these agents when applied to cells for prolonged periods compatible with assessing adipogenesis.

The functional requirement for PKC-ε during the differentiation of 3T3-F442A preadipocytes suggests that it phosphorylates and regulates proteins critically involved in the control of adipogenesis. The spatiotemporal aspects of its expression during differentiation suggest further that it may regulate nuclear proteins during the initiation of the process. Although nuclear translocation of PKC-ε has been documented in several different cell types [17, 30–32], this is the first demonstration of its nuclear localization in differentiating preadipocytes. Interestingly, Racke et al. [33] recently reported nuclear translocation of PKC-ε during the differentiation of a megakaryocyte precursor cell line. The substrates or binding partners for nuclear PKC-ε during adipocyte differentiation remain to be identified. Although a number of proteins have been tentatively identified as nuclear PKC substrates [32], there is little or no information on the PKC isoform specificity of these interactions. In this study we provide evidence that PKC-ε co-localizes with C/EBP-β in the nucleus during the early stages of differentiation. The subnuclear punctate speckling pattern of C/EBP-β staining that we observed has been reported for other cell types previously [34] and was identical to that found in differentiating 3T3-L1 preadipocytes by Lane and co-workers [24]. The latter study also showed that C/EBP-β localized to centromeres during the entry of preadipocytes into S phase, and speculated that it was involved in coordinating or promoting S phase entry. Al-

though the apparent co-localization of C/EBP-β with PKC-ε suggests that the two proteins may interact, further studies will be necessary to test whether there is any functional relationship. Although C/EBP-β is expressed very early after the induction of differentiation, it does not acquire activity in terms of DNA-binding and transactivation for several hours implying that additional regulatory events must occur. In its basal inactive state, negative regulatory regions mask the trans-activation domains of C/EBP-β [35, 36]. This repression is reversed by phosphorylation [25, 37, 38]. Indeed, phosphorylation of C/EBP-β is concomitant with its acquisition of DNA-binding ability, which occurs as preadipocytes enter S phase during clonal expansion [24]. Although the protein kinases that directly phosphorylate c/EBP-β and regulate its function in vivo have not been fully defined, the fact that all members of the C/EBP family contain several PKC consensus phosphorylation sites indicates that PKC may be involved in its regulation. In this regard, two independent studies have demonstrated phosphorylation of C/EBP-β by PKC in vitro [39, 40], although in each case, phosphorylation by PKC reduced the ability of C/EBP-β to bind DNA. The results of the current work warrant further investigations into the role of specific PKC isoforms in regulating C/EBP-β phosphorylation in vivo.

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