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Nuclear PLC Beta 1 is required for 3T3-L1 adipocyte differentiation and regulates expression of the cyclin D3–cdk4 complex

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ABSTRACT

A phosphoinositide signalling cycle is present in the nucleus, independent of that which occurs at the plasma membrane. The key enzyme involved in this cycle is phospholipase (PLC) β1. This nuclear cycle has been shown to be involved in both cell proliferation and differentiation. Here, we report that nuclear PLCβ1 activity is upregulated during differentiation of 3T3-L1 adipocytes. During differentiation there are two phases of PLCβ1 activity; the first occurs within 5 min of treatment with differentiation media, does not require new PLCβ1 to enter the nucleus and is regulated by pERK and PKC α while the second phase occurs from day 2 of differentiation, requires new PLCβ1 protein to enter the nucleus and is independent of regulation by pERK and PKC α. Over-expression with the PLC mutants, Δmk (which lacks the ERK phosphorylation site) and M2B (which lacks the nuclear localisation sequence), revealed that both phases of PLCβ1 activity are required for terminal differentiation to occur. Inhibition of PLCβ1 activity prevents the upregulation of cyclinD3 and cdk4 protein, suggesting that PLCβ1 plays a role in the control of the cell cycle during differentiation. These results indicate nuclear PLCβ1 as a key regulator of adipocyte differentiation.

1. Introduction

Phospholipase Cβ activity at the plasma membrane is regulated by ligand activation of G protein coupled seven-transmembrane receptors and leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to give rise to the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) [1]. An analogous phosphoinositide (PI) signalling system also exists in the nucleus and is distinct from that at the plasma membrane [2–5]. The key PLCβ isoform involved in this pathway is PLCβ1. It has been shown to exist in two alternatively spliced subtypes, PLCβ1a (150 kDa) and PLCβ1b (140 kDa), which differ in a region of the C-terminus [6]. The nuclear localisation of both PLCβ1 subtypes has been shown to involve a nuclear localisation sequence mapped to a cluster of lysines located between residues 1055 and 1072 [7].

Nuclear PLC β1 plays a key role in regulation of the cell cycle in a number of cell types. Stimulation of quiescent Swiss 3T3 cells with IGF-1 leads to a 2–3 fold increase in the nuclear activity [8,9]. Similar increases are seen when NIH 3T3 cells are stimulated with insulin [10] and after IL-1α stimulation of Saos2 osteosarcoma cells [11]. This increase in activity is mediated by the transactivation of activated MAP kinase from the cytoplasm to the nucleus [10,12] . Inhibition of PLC β1 expression in 3T3 cells using anti-sense mRNA abolishes IGF-1 induced DNA synthesis while over-expression of PLC β1 dramatically increases DNA synthesis and the number of cells in S-phase, after treatment with IGF-1 [13].

Nuclear PLC β1 also appears to play an important role in cell differentiation. It has been demonstrated that nuclear PLC β1 protein levels and activity are upregulated during myogenic differentiation of C2C12 cells with insulin [14].

In this study we report a novel pattern of PLC β1 activity during 3T3-L1 adipocyte differentiation. Adipocytes are formed by a process called adipogenesis from preadipocytes. 3T3-L1 mouse embryonic fibroblasts have been employed as a useful model for the study of adipocyte differentiation [15]. When treated with an inducing cocktail composed of isobutylmethylxanthine, dexamethasone and insulin, the cells firstly undergo mitotic clonal expansion followed by a

Abbreviations: C/EBP, CCAAT/enhancer binding protein; cdk4, cyclin-dependent kinase 4; CREB, cAMP response element binding protein; DAG, diacylglycerol; EGF, epidermal growth factor; ERK, extracellular related kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDAC, histone deacetylase; IBMX, iso-butyl-methyl-xanthine; IGF-1, insulin-like growth factor 1; IL-1α, interleukin 1 alpha; IP_3, inositol 1,4,5-trisphosphate; IRS-1, insulin receptor substrate-1; MAPK, mitogen activated protein kinase; MCE, mitotic clonal expansion; MEK, mitogen activated protein kinase kinase; MKP-1, MAPK phosphatase 1; PCNA, proliferating cell nuclear antigen; pERK, phospho-extracellular related kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC β1, phospholipase C beta1; PKC, protein kinase C; PPAR, peroxisome proliferator activated receptor; pRB, phospho-retinoblastoma; Stat5, signal transducer and activator of transcription 5.

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cascade of gene transcriptional events, leading to the expression of adipocyte-specific genes. CCAAT/enhancer binding protein-β (C/EBPβ), CCAAT/enhancer binding protein-α (C/EBPα), and peroxisomal proliferator activated receptor-γ (PPARγ) are the transcriptional factors which are critical for inducing the expression of adipocyte-specific genes [16,17]. Some signalling molecules such as insulin receptor substrate-1 (IRS-1), Ras, p38 MAP kinase, cAMP response element binding protein (CREB), signal transducer and activator of transcription 5 (Stat5), histone deacetylase (HDAC) have been reported to be important in the regulation of adipogenesis [18–23].

We demonstrate that nuclear PLC β1 activity is up-regulated during 3T3-L1 differentiation. There are two separate phases of up-regulation, both of which are required for differentiation to occur and these two phases appear to be regulated by separate mechanisms. We also demonstrate that nuclear PLC β1 is involved in the regulation of the cell cycle proteins cyclin D3 and cdk4, which are involved in the differentiation process.

2. Materials and methods

2.1. Cell culture

3T3-L1 cells were maintained in Dulbecco's Modified Eagles Medium supplemented with 10% fetal bovine serum and 1 mM glutamine (10% FBS DMEM). Prior to each experiment cells were plated in 10% FBS DMEM and allowed to reach confluency. Two days post confluency cells were differentiated by replacing the media with 10% FBS DMEM supplemented with 10 μM dexamethasone, 0.5 mM isobutyl-methyl-xanthine and 10 μg/ml insulin. After a further 2 days media was replaced with 10% FBS DMEM and changed every 2 days for the period of the experiment. Undifferentiated control cultures were maintained in 10% FBS DMEM with the media being replaced every 2 days. Cultures were treated with various compounds as described in the text and these were replaced when media was changed unless otherwise stated.

2.2. Oil Red O staining

Cells were washed twice with cold PBS and then fixed with 4% PFA for 15 min at room temperature. The plates are washed twice with PBS. Cells were then stained with Oil Red O (0. 3% solution in 60% isopropanol) at RT for 30 min. The plates were then washed gently five times with distilled water. At this point cells were counterstained with Ehrlichs hematoxylin and cell counts were performed. Alternatively, after staining the cells were washed twice with distilled water and then incubated for 10 min with 50% isopropanol to remove non-specific staining and then the Oil Red O was eluted by adding 100% isopropanol and shaking the plate for 5 min at RT. The amount of eluted stain was determined by measuring the absorbance at 510 nm.

2.3. SDS-PAGE and Western blotting

Equal amounts of protein were separated by SDS-PAGE and then transferred to PVDF membrane using the Mini Trans-Blot electrophoretic transfer system (Bio-Rad). The blots were blocked for 30 min with 10% non-fat milk powder in TBS with 0.1% Tween 20 (TBS-T) at room temperature then probed overnight at 4 °C with the primary antibody at the corresponding dilution. The blot was washed and then incubated with a 1:2000 dilution of the appropriate secondary antibody conjugated with horse-radish peroxidase in 5% non-fat milk powder in TBS-T for 2 h at room temperature. The blot was washed as before and immunoreactive bands were visualised using ECLplus detection reagent. The following antibodies were used: anti-PLC β1 (K-92-3) [6], anti-ERK (9102) and anti-pERK (9101) rabbit polyclonal antibodies from Cell Signalling; ant-PKC α rabbit polyclonal antibody from Santa Cruz Biotechnology; anti-phosphoserine (AB1603) rabbit polyclonal antibody from Chemicon and anti-PPAR γ (101700) rabbit polyclonal antibody from Cayman Chemical. Anti-mouse and anti-rabbit HRP conjugated secondary antibodies were from GE Healthcare.

2.4. Isolation of nuclei and analysis of PLC activity

Nuclei were prepared as previously described [9]. Briefly, cells were lysed in 500 μl of nuclear isolation buffer (10 mM Tris–HCl pH7.8, 1% Nonindet P-40, 10 mM β-mercaptoethanol, 2 mM Na_3VO_4, 5 mM NaF and 1x Complete Protease Inhibitors (Roche) for 3 min on ice. 500 μl of MilliQ water was then added to swell the cells for 3 min. The cells were sheared by 8 passages through a 23-gauge hypodermic needle. Nuclei were recovered by centrifugation at 400 ×*g*, 4 °C for 6 min and washed once in 500 μl of washing buffer (10 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, 2 mM Na_3VO_4, 5 mM NaF and 1 x Complete Protease Inhibitors). This method has been shown to yield nuclei which lack both inner and outer nuclear membranes and are free of cytoplasmic contamination. Nuclear preparation purity was confirmed by western blotting for β-tubulin [9,13]. Only preparations that were completely clear of β-tubulin were used. The activity of nuclear PLC was analyzed as described previously [9]. Briefly, 10 μg of nuclear proteins were incubated with 100 mM MES buffer pH 6.7, plus 150 mM NaCl, 0.06% sodium deoxycholate, 3 nmol [^{3}H]PIP_2 (specific activity 30,000 dpm/nmol) for 30 min at 37 °C in a total volume of 100 μl. Hydrolysis was stopped by addition of 750 μl chloroform/methanol/37% HCl (160:80:1), 350 μl 1.8% NaCl, 225 μl chloroform and 2,5 μl 20 mg/ml Folch extract (Sigma). 150 μl of the upper phase was removed and measured in a scintillation counter to determine the amount of inositol 1,4,5-triphosphate.

2.5. Immunoprecipitation

Purified nuclei were solubilised in immunoprecipitation buffer (25 mM HEPES pH 7.5, 5 mM EDTA and EGTA, 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 10% glycerol, 1% Triton-X100 plus Complete™ protease inhibitors (Roche)) for 20 min at 4 °C with shaking. Cell debris was removed by centrifugation at 12 000 g and 4 °C for 5 min. The supernatants were incubated with 50 μl of a 50% slurry of protein A/G agarose beads for 1 h. The cleared lysates were then incubated with 5 μg mouse anti-PLC β1 antibody for 16 h. The immunocomplexes were recovered by adding 50 μl protein A/G agarose beads for another hour. This was washed by adding 1 ml IP buffer and released by boiling in 50 μl of 1 X SDS-PAGE buffer for 5 min. Samples were then separated by 8% SDS-PAGE and probed with specific primary antibodies as described in the text.

2.6. Transfection

Cells were transfected with wild type PLC β1a, PLC β1b and mutants M2b and Δmk cloned into pRc/CMV. The mutant M2b lacks a nuclear localisation sequence with lysine residues 1056, 1063 and 1072 in region 2 of the COOH terminus being substituted with isoleucine [7] and Δmk lacks the MAPK phosphorylation site with a serine at position 982 replaced by a glycine [12]. Transfections were carried out using LipofectAMINE PLUS (Invitrogen) according to manufacturer's instructions. Stable transfectants were generated by selection with 1.2 mg/ml G418.

2.7. Thymidine assay

1×10^4 3T3-L1 cells were plated into 24 well dishes. Two days post confluence differentiation was induced as described above. Two hours prior to the end of each time point, 0. 5 μCi of [^{3}H]-thymidine was added to each well and the cells were incubated at 37 °C, 5% CO_2. At the end of 2 h the media containing the [^{3}H]-thymidine was removed and the cells were washed twice with ice cold PBS. Cells were then incubated on ice with ice cold 10% TCA for 10 min. The 10% TCA was removed and the cells were rinsed again with 10% TCA. Cells were left to air dry for approximately 30 min until completely dry and then lysed by addition of 200 μl 0.1 N