# Translocation of Akt/PKB to the nucleus of osteoblast-like MC3T3-E1 cells exposed to proliferative growth factors

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**Abstract An active phosphatidylinositol 3-kinase (PI3K) has been shown in nuclei of different cell types. The products of this enzyme, i.e. inositides phosphorylated in the D3 position of the inositol ring, may act as second messengers themselves. Nuclear PI3K translocation has been demonstrated to be related to an analogous translocation of a PtdIns(3,4,5)$P_3$ activated PKC, the ζ isozyme. We have examined the issue of whether or not in the osteoblast-like clonal cell line MC3T3-E1 there may be observed an insulin-like growth factor-I- (IGF-I) and platelet-derived growth factor- (PDGF) dependent nuclear translocation of an active Akt/PKB. Western blot analysis showed a maximal nuclear translocation after 20 min of IGF-I stimulation or after 30 min of PDGF treatment. Both growth factors increased rapidly and transiently the enzyme activity of immunoprecipitable nuclear Akt/PKB on a similar time scale and after 60 min the values were slightly higher than the basal levels. Enzyme translocation was blocked by the specific PI3K inhibitor, LY294002, as well as cell entry into S-phase. Confocal microscopy showed an evident increase in immunostaining intensity in the nuclear interior after growth factor treatment but no changes in the subcellular distribution of Akt/PKB when a LY294002 pre-treatment was administered to the cells. These findings strongly suggest that the intranuclear translocation of Akt/PKB is an important step in signalling pathways that mediate cell proliferation.** 

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*Abbreviations:* PKB, protein kinase B; PKC, protein kinase C; PKA, protein kinase A; PI3K, phosphatidylinositol 3-kinase; PDK1, phospholipid-dependent kinase 1; PtdIns(3,4,5)$P_3$, phosphatidylinositol-3,4,5-trisphosphate; DAG, diacylglycerol; IGF-I, insulin-like growth factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; NGS, normal goat serum; FCS, fetal calf serum; BSA, bovine serum albumin; D-MEM, Dulbecco's modified minimum essential medium; PBS, phosphate-buffered saline; CLSM, confocal laser scanning microscope; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; 5-BrdU, 5-bromodeoxyuridine

*Key words:* Akt/PKB; Nucleus; Signal transduction; Platelet-derived growth factor; Insulin-like growth factor; MC3T3-E1 osteoblast-like cell

## 1. Introduction

Akt/PKB is a 57 kDa serine/theronine kinase that shares sequence homology with both PKC and PKA [1]. Akt/PKB has been recognized to be the product of c-Akt, the cellular homologue of the viral oncogene v-Akt [2]. Molecular analysis revealed the existence in Akt/PKB of a pleckstrin homology domain, located at the amino-terminus of the kinase, which mediates interaction of Akt/PKB with other cell proteins [3].

Several growth factors including insulin, PDGF, EGF, FGF and IGF-I are capable of activating Akt/PKB [4]. Akt/PKB is a downstream target of PI3K, as demonstrated by experiments in which PI3K was inhibited either by Wortmannin or LY294002, or by overexpression of a deletion mutant of the regulatory subunit of PI3K [5–7]. It is commonly thought that Akt/PKB is activated following its recruitment to the plasma membrane. This activation requires phosphorylation of threonine and serine residues of Akt/PKB, a reaction which is catalyzed by PDK1 and by a still unidentified PDK2, respectively. In addition PDK1 has been demonstrated to be active only in the presence of PtdIns(3,4,5)$P_3$, the final product of PI3K [8,9].

In addition to its well established role at the plasma membrane, it is now widely accepted that PI3K is also involved in nuclear signal transduction events (for a comprehensive review see [10]). The generation at the nuclear level of lipid messenger molecules, such as DAG or PtdIns(3,4,5)$P_3$, has been demonstrated to be the driving force to recruit to the nuclear compartment PKC α in Swiss 3T3 cells or PKC ζ in HL-60 and PC12 cells, respectively [11–13]. In the latter case the translocation of PKC ζ followed a progressive and sustained nuclear translocation of PI3K. This PI3K nuclear translocation has mainly been linked to cell differentiation [12,13].

In addition there are some reports in the literature that have described an intranuclear migration of Akt/PKB follow-

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ing serum stimulation of starved human fibroblasts or triggering the B cell antigen receptor (BCR) in B lymphocytes [14,15].

In light of these findings we decided to investigate whether or not Akt/PKB translocates to the nucleus of MC3T3-E1 cells in response to stimulatory growth factors such as IGF-I or PDGF and if this event is dependent on PI3K activity. Here, we show that both of these growth factors are capable of promoting a nuclear migration of Akt/PKB and this event is blocked by a selective PI3K inhibitor, LY294002.

## 2. Materials and methods

### 2.1. Materials

D-MEM, FCS, monoclonal antibody to β-tubulin, normal rabbit IgG, NGS, Cy3-conjugated anti-rabbit IgG, peroxidase-conjugated anti-rabbit IgG, and BSA were from Sigma, St. Louis, MO, USA. IGF-I, PDGF (B/B), histone H2B, and the Lumi-Light$^{Plus}$ enhanced chemiluminescence detection kit were from Roche Molecular Biochemicals, Milan, Italy. Protein A-agarose was purchased from Transduction Laboratories, Lexington, KY, USA. [γ-$^{32}$P]ATP was from Amersham Pharmacia Biotech, Uppsala, Sweden. LY294002 was from Calbiochem, La Jolla, CA, USA. The Protein Assay kit (detergent compatible) was from Bio-Rad, Hercules, CA, USA. cAMP-dependent protein kinase inhibitor peptide was from Bachem (Bubendorf, Switzerland). P-81 paper was from Whatman (Maidstone, UK).

### 2.2. Cell culture

MC3T3-E1 mouse calvaria fibroblasts were cultured in D-MEM containing 10% FCS. Prior to stimulation, cells, seeded at $3\times10^3$/cm$^2$, were subcultured for 24 h and then cultured for an additional 36 h in serum-free medium containing 0.5% BSA. Quiescent cultures were washed twice with serum-free medium containing 0.2% BSA, then incubated in the same medium for the indicated times in the presence of 30 ng/ml of either IGF-I or PDGF. For treatment with LY294002, the drug was added at 25 μM to serum-free medium for 1 h prior to stimulation with growth factors.

For 5-BrdU incorporation, to detect cells in S-phase, cells were labelled with 100 μM 5-BrdU for 10 min and treated as previously described [16].

### 2.3. Isolation of nuclei

This was accomplished as previously reported, with minor changes [17]. Briefly, cells ($5\times10^6$) were resuspended in 500 μl of 10 mM Tris–HCl, pH 7.8, 1% Nonidet P-40, 10 mM β-mercaptoethanol, 0.5 mM phenylmethyl fluoride (PMSF), 10 μg/ml soybean trypsin inhibitor, 1 μg/ml of leupeptin and aprotinin, 1.0 mM $Na_3VO_4$, 20 mM okadaic acid. They were incubated at 0°C for 2 min, then 500 μl of double-distilled $H_2O$ was added and the cells allowed to swell for 2 min. Cells were sheared by eight passages through a 22-gauge needle. Nuclei were recovered by centrifugation at $400\times g$ for 6 min and washed once in 10 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, plus protease and phosphatase inhibitors as above. The purity of nuclear preparations was evaluated by Western blot analysis using a monoclonal antibody to β-tubulin, as previously described [18]. The absence of immunoreactivity to the cytoskeletal protein in the isolated nuclear preparations confirmed that the isolation procedure produced nuclei of high purity that were free of cytoplasmic contaminants (data not shown).

### 2.4. Preparation of whole cell homogenates and cytosolic extracts

To obtain homogenates, cells were resuspended in 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 0.5 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml of leupeptin and aprotinin, 1.0 mM $Na_3VO_4$, 20 mM okadaic acid, and homogenized by 30 passages through a 25-gauge needle.

### 2.5. Protein assay

This was performed according to the instruction of the manufacturer using the Bio-Rad Protein Assay (detergent compatible).

### 2.6. Western blot analysis

Proteins (from $3\times10^6$ nuclei) separated on 7.5% polyacrylamide gels were transferred to nitrocellulose sheets using a semi-dry blotting apparatus. Sheets were saturated in PBS containing 5% normal goat serum and 4% BSA for 60 min at 37°C (blocking buffer), then incubated overnight at 4°C in blocking buffer containing a polyclonal antibody diluted 1:8000, directed to the C-terminus region of Akt1 and Akt2 (PKB α and β) [7]. After four washes in PBS containing 0.1% Tween-20, they were incubated for 30 min at room temperature with peroxidase-conjugated anti-rabbit IgG, diluted 1:3000 in PBS–Tween-20, and washed as above. Bands were visualized by the enhanced chemiluminescence method. Densitometric analysis was performed on the Molecular Analyst GS670 (Bio-Rad, Hercules, CA, USA).

### 2.7. Immunoprecipitation of Akt/PKB

Nuclei (from $5\times10^6$ cells) were lysed for 30 min at 4°C in 50 mM HEPES, pH 7.9, 100 mM NaCl, 10% glycerol, 10 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.0 mM $Na_3VO_4$, 1 mM PMSF, 1 μg/ml leupeptin and aprotinin. The suspension was passed several times through a 26-gauge needle, then centrifuged at $12\,000\times g$ for 15 min at 4°C. Lysates (1 ml, containing 500 μg of protein) were pre-cleared by adding 5 μg of normal rabbit IgG and 10 μg of 50% protein A-agarose, followed by incubation for 1 h at 4°C and centrifugation at $12\,000\times g$ for 10 min at 4°C. Cell lysates were incubated for 2 h at 4°C under constant agitation with 5 μg of a polyclonal antibody to Akt/PKB. 10 μg of 50% protein A-agarose was added and incubation proceeded for 1 h at 4°C under constant agitation.

### 2.8. Akt/PKB activity assay

The immunoprecipitates were washed twice in lysis buffer, once in distilled water and twice in the Akt/PKB kinase buffer (20 mM HEPES–NaOH, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT) as previously reported [19]. Assays (100 μl) contained 20 mM HEPES–NaOH, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 1 μM cAMP-dependent protein kinase inhibitor peptide, 5 μg histone H2B as exogenous substrate, 2 μM ATP, 10 μCi [γ-$^{32}$P]ATP (3000 Ci/mmole). Samples were incubated for 30 min at 30°C and the reaction was then stopped by spotting 80 μl onto P-81 filter papers and immersing in 1% (v/v) orthophosphoric acid. The papers were washed several times, rinsed in ethanol, and air-dried, and the radioactivity was determined by scintillation counting. Background values, obtained by samples in which the anti-Akt/PKB antibody was replaced by normal rabbit serum, were subtracted from all values.

### 2.9. In situ immunofluorescence

Cultures of MC3T3-E1 cells (control and growth factor-treated), grown on glass coverslips, were washed twice in cold PBS, pH 7.2, fixed with freshly prepared 4% paraformaldehyde (30 min at room temperature) and permeabilized with 0.2% Triton X-100 in PBS (10 min). Samples were reacted with the polyclonal antibody (diluted 1:500) directed against Akt/PKB and used also for Western blotting and immunoprecipitates [7]. The pictures presented in this article have been obtained by means of the monoclonal antibody. The anti-Akt/PKB antibodies were diluted in 2% BSA, 3% NGS in PBS. The secondary antibody was either a Cy3-conjugated anti-rabbit or anti-mouse IgG, diluted 1:1000. All incubations were carried out at 37°C. Samples were subsequently washed three times in PBS and mounted as previously described [20]. Confocal laser scanning microscope (CLSM) and image processing analysis were performed as described previously [21]. The quantitative analysis of fluorescence intensity was carried out on confocal as reported elsewhere [22,23].

### 2.10. Statistical analysis

Data are the means from three different experiments and are expressed as mean ± S.D. The asterisk indicates significant differences ($P<0.01$) in a Student's paired $t$-test. All of the other differences were found to be not significant with $P>0.01$.

## 3. Results

### 3.1. IGF-I and PDGF induce nuclear translocation of an active Akt/PKB

Nuclei prepared from quiescent cells showed by means of Western blotting the constitutive presence of Akt/PKB, which migrated at 57 kDa. IGF-I treatment of MC3T3-E1 cells in-

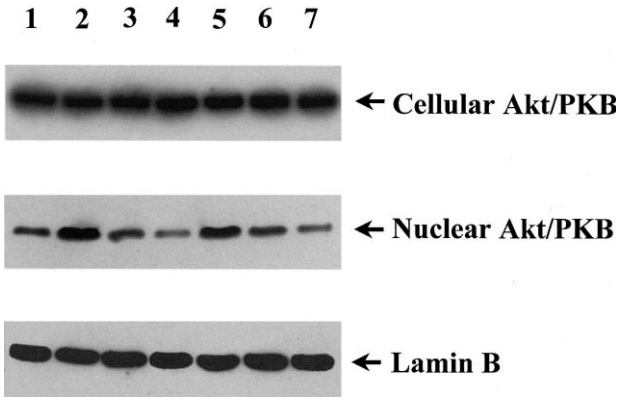


Fig. 1. IGF-I and PDGF induce nuclear translocation of Akt/PKB in MC3T3-E1. Western blotting analysis showing the presence of Akt/PKB in whole cells and isolated nuclei. 80 μg of nuclear protein was blotted to each lane, and the blots were also normalized to an endogenous nuclear protein (lamin B). Lane 1, unstimulated cells; lane 2, samples from cells exposed to IGF-I alone for 20 min; lane 3, samples from cells exposed to IGF-I alone for 60 min; lane 4, samples from cells exposed to IGF-I plus LY294002 for 20 min; lane 5, samples from cells exposed to PDGF alone for 30 min; lane 6, samples from cells exposed to PDGF alone for 60 min; lane 7, samples from cells exposed to PDGF plus LY294002 for 30 min.

duced an increase in intranuclear amount of Akt/PKB, which was maximal after 20 min of stimulation (Fig. 1). At 60 min of treatment nuclear Akt/PKB protein was still higher than at time 0, as evidentiated also by densitometric analysis of Western blots (Fig. 1 and Table 1). At variance, maximal nuclear translocation of Akt/PKB in PDGF-treated samples occurred at 30 min. Also in this case, at 60 min since the beginning of stimulation we observed a decrease in immunochemical reactivity that, however, remained at a level higher than the control (Fig. 1 and Table 1). In both cases, pre-treatment for 1 h of cells with a PI3K-specific pharmacological inhibitor, LY294002 (25 μM), suppressed the growth factor-dependent nuclear translocation of Akt/PKB in MC3T3-E1 osteoblast cells (Fig. 1 and Table 1). In contrast, no marked changes in the amount of Akt/PKB protein were detected in whole cell homogenates (Fig. 1).

Changes in nuclear Akt/PKB activity were measured by means of immunoprecipitation of the enzyme followed by in vitro phosphorylation of exogenous histone H2B. Exposure to IGF-I resulted in a rapid increase (nearly 5-fold) in nuclear Akt/PKB activity, that reached its peak at 20 min of treatment (Fig. 2). Starting from this time point, it was observed a slow and progressive decrease of the enzymatic activity, that at 60 min after stimulation was about 2-fold above basal activity.

On the other hand, the increase in nuclear Akt/PKB activity induced by PDGF rose in a more regular manner, with the

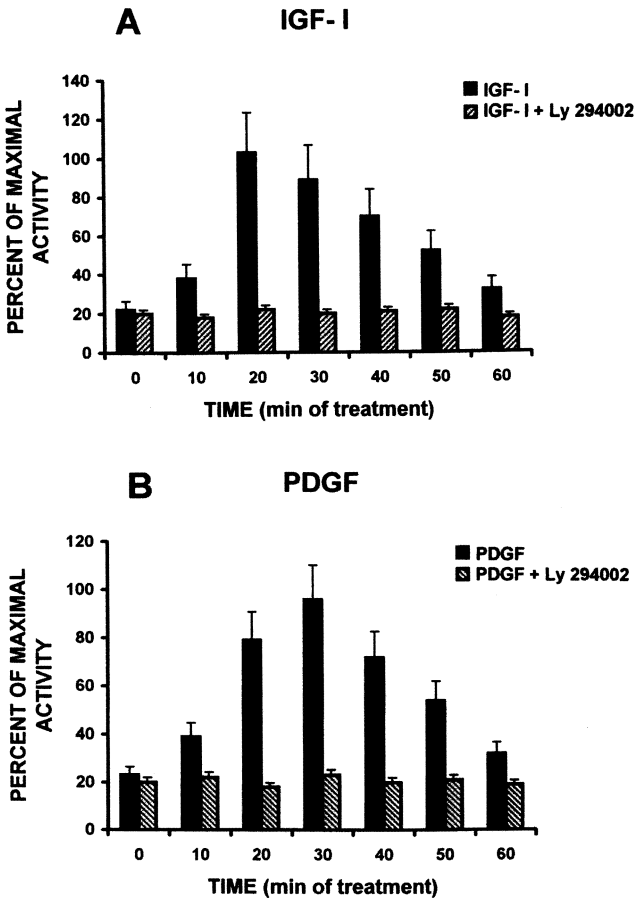


Fig. 2. Exposure of MC3T3-E1 cells to either IGF-I or PDGF increases immunoprecipitable nuclear Akt/PKB activity. Black bars: cells treated with either IGF-I or PDGF. Hatched bars: cells treated with either of the two growth factors plus LY294002. Basal levels measured in the presence or in the absence of LY294002 were 954 ± 123 dpm and 829 ± 133 dpm, respectively. Results are the means of three separate experiments. Standerd deviation is shown.

highest activity at 30 min (Fig. 2). Also in this case, the maximal activity resulted in an about 5-fold rise. Afterwards, a decrease similar to that observed for IGF-I-treated samples was detected (Fig. 2).

If the cells had been exposed to LY294002 for 1 h prior to stimulation with growth factors, no increase in nuclear Akt/PKB activity was measured in immunoprecipitates derived from cells treated with either IGF-I or PDGF.

In parallel we wanted to assay if the nuclear recruitment of an active Akt/PKB could be related to the capacity of IGF-I or PDGF to induce S-phase entry in serum starved osteoblasts. As shown in Table 2, prior to mitogenic stimulation

Table 1
Densitometric analysis of Western blots showing intranuclear translocation of Akt/PKB

| Agonist | 0 min (control) | 20 min | 30 min | 60 min | 20 min+LY294002 | 30 min+LY294002 |
|---|---|---|---|---|---|---|
| None | 12 ± 2.4 | – | – | – | – | |
| IGF-I | – | 43.1 ± 4.7* | – | 20 ± 3.2* | 10 ± 2.3 | – |
| PDGF | – | – | 39 ± 4.3* | 23 ± 3.7* | – | 11 ± 2.5 |

Nuclear protein (80 μg) from quiescent cells, or cells stimulated for various times with IGF-I or PDGF (either in the presence or in the absence of LY294002) was separated by polyacrylamide gel electrophoresis and blotted to nitrocellulose paper. Blots were probed with a polyclonal antibody to PI3K which was then revealed by enhanced chemiluminescence. Films were scanned with a Molecular Analyst GS670. Data are expressed as arbitrary units. Asterisks indicate values that are significantly different ($P < 0.01$) from control.

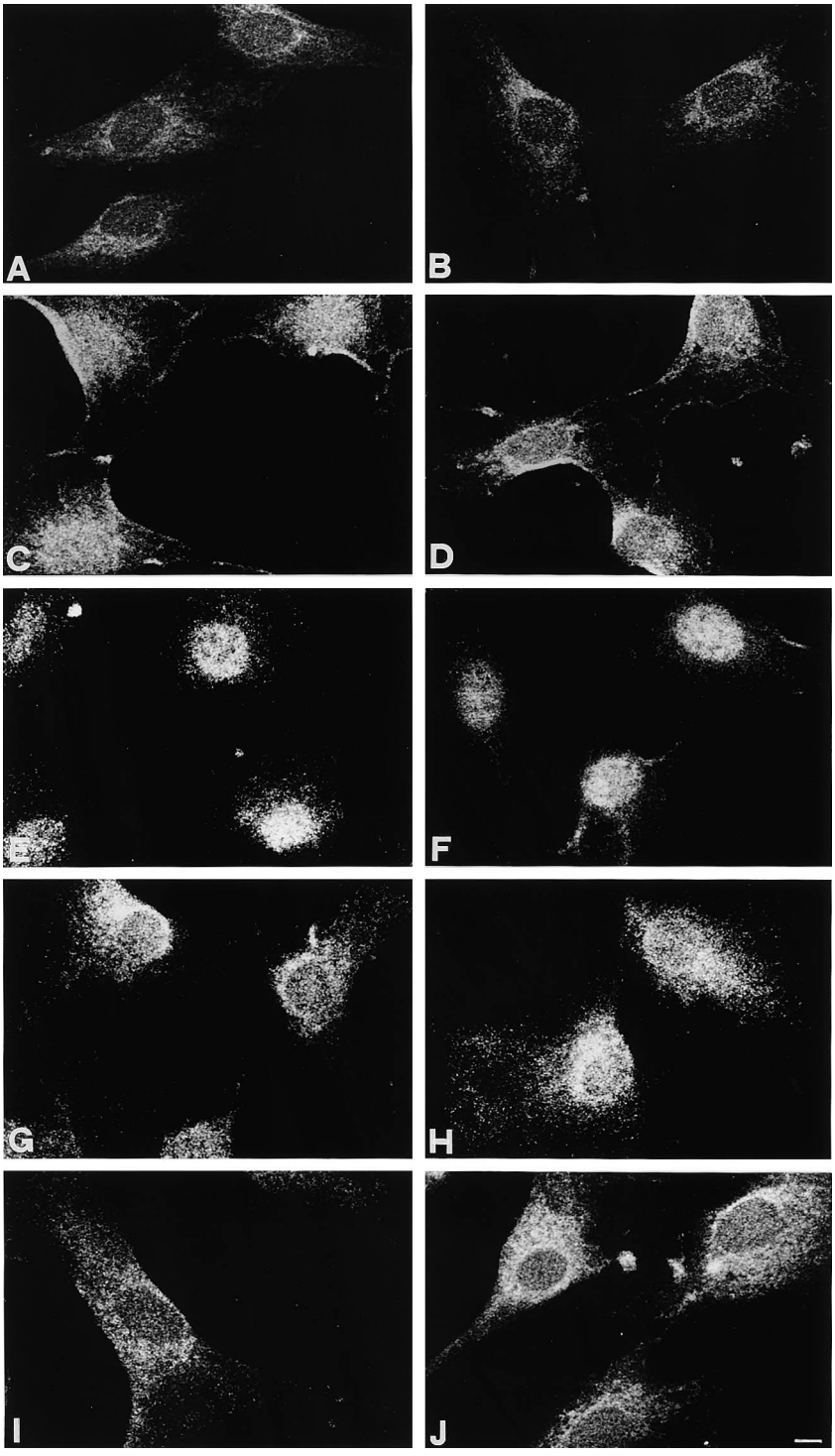


Fig. 3. CLSM analysis of Akt/PKB distribution in MC3T3-E1 cells. A: Unstimulated cells. B: Unstimulated cells pre-treated with LY294002 (25 μM) for 1 h. C: Cells stimulated with IGF-I for 10 min. D: Cells stimulated with PDGF for 20 min. E: Cells stimulated with IGF-I for 20 min. F: Cells stimulated with PDGF for 30 min. G: Cells stimulated with IGF-I for 60 min. H: Cells stimulated with PDGF for 60 min. I: Cells pre-treated with LY294002 and then exposed to IGF-I for 20 min. J: Cells pre-treated with LY294002 and then exposed to PDGF for 30 min. Scale bar: 5 μm.

almost all cells were quiescent. Treatment with either IGF-I or PDGF was able to induce DNA synthesis that starting from 12 h after growth factor administration increased progressively toward 20 h of treatment. Interestingly, pre-treatment of cells with LY294002 blocked DNA synthesis, that remained at control levels at all the examined times.

### *3.2. Subcellular redistribution of AKT/PKB after IGF-I and PDGF stimulation*

CLSM analysis of control cells immunostained with anti-Akt/PKB antibody showed the enzyme to be predominantly located in the cytoplasm. Nevertheless, nuclei, identified by means of phase-contrast microscopy (data not shown),

Table 2
Percent distribution of S-phase cells in MC3T3-E1 cells after stimulation with either IGF-I or PDGF

| Cell treatment | Percentage of 5-BrdU positive cells (time from treatment) | | |
|---|---|---|---|
| | 12 h | 16 h | 20 h |
| Serum starved | 2.7 ± 2.1 | 2.5 ± 1.9 | 2.2 ± 1.8 |
| IGF-I 20 h | 8.7 ± 1.9 | 14.5 ± 2.0 | 18.7 ± 2.4 |
| IGF-I+LY294002 | 2.8 ± 1.5 | 2.9 ± 1.8 | 3.4 ± 1.6 |
| PDGF | 16.9 ± 2.2 | 27.4 ± 2.9 | 32.1 ± 3.0 |
| PDGF+LY294002 | 3.1 ± 1.4 | 4.4 ± 1.7 | 4.6 ± 1.9 |

MC3T3-E1 mouse calvaria fibroblasts were cultured in D-MEM containing 10% fetal calf serum. Prior to stimulation, cells, seeded at $3\times10^3/cm^2$, were subcultured for 24 h and then cultured for an additional 36 h in serum-free medium containing 0.5% BSA. Quiescent cultures were washed twice with serum-free medium containing 0.2% BSA, then incubated in the same medium for the indicated times in the presence of 30 ng/ml of either IGF-I or PDGF. For evaluation of S-phase cells, osteoblast were labelled with 100 μM 5-BrdU for 10 min. The results are the means from three different experiments ± S.D. Asterisks indicate values that are significantly different ($P < 0.01$) from control.

showed also in control cells the presence of some immunostaining, in agreement with both Western blotting analysis and enzymatic assays (Fig. 3A). Pre-treatment of cells with LY294002 did not influence the subcellular distribution of Akt/PKB (Fig. 3B). After 10 min of IGF-I stimulation, we detected in both the plasma membrane and the nuclear interior an evident increase in immunostaining intensity (Fig. 3C). Similar results were obtained with PDGF-treated samples, but after 20 min of stimulation (Fig. 3D). Maximal intranuclear translocation in IGF-I-exposed samples was observed after 20 min. The nuclear fluorescent pattern was characterized by a very brilliant fibrogranular distribution (Fig. 3E). In the case of PDGF stimulation, the nuclear interior was immunostained with a brilliant fluorescence after 30 min of exposure (Fig. 3F).

60 min after the beginning of stimulation with either IGF-I or PDGF, Akt/PKB immunoreactivity was again dispersed throughout the cytoplasm while intranuclear positivity was still higher than in control cells (Fig. 3G and H). Cells pretreated with 25 μM LY294002 and then exposed to either IGF-I or PDGF up to 20 or 30 min, respectively, did not show any intranuclear migration or subcellular modification of Akt/PKB (Fig. 3I and J).

CLSM quantitative evaluation of Akt/PKB nuclear translocation (Table 3) in MC3T3-E1 cells gave results in agreement with activity assays and Western blots, indicating a peak of nuclear fluorescence after 20 or 30 min of IGF-I or PDGF treatment respectively.

The percentage of cells exhibiting the nuclear translocation of Akt/PKB in response to growth factors shows that in both cases nearly 60% of the cells responded to growth factors with a nuclear translocation of the enzyme (Table 4).

## 4. Discussion

In this study we have employed several techniques to follow the intranuclear localization of Akt/PKB during growth factor stimulation of MC3T3-E1 osteoblasts. Akt/PKB is present in the nucleus of these cells also when they are quiescent and in response to stimulation with either IGF-I or PDGF, there is a striking increase in the amount of intranuclear Akt/PKB. This increase occurs more rapidly in the case of IGF-I in comparison with PDGF. The enhanced intranuclear amount of Akt/PKB is paralleled by an increase of its enzymatic activity.

Activation of Akt/PKB has previously mainly been linked to growth factor-induced events occurring at the plasma membrane level [24], in a PI3K-dependent membrane translocation step that requires the phosphorylation of the protein at T308 and S473 [3].

Intranuclear presence of Akt/PKB was first reported in PC12 cells [25]. Subsequently several stimuli have been shown to be capable of inducing intranuclear migration of Akt/PKB, such as serum, okadaic acid and IGF-I [14,26]. It should be underscored however that this study demonstrating IGF-I nuclear translocation of Akt/PKB was carried out in human embryonic kidney cells overexpressing the human β isoform of Akt/PKB. We used a polyclonal antibody directed against the C-terminus region of PKB α and β (Akt1 and Akt2) [7]. Even if PKB γ has a truncated form of C-terminus region, the three isoforms display a high homology and similarity in function [1], and the observed translocation could be considered highly representative of Akt/PKB behavior. In this report we have confirmed the capability of IGF-I to induce the nuclear migration of Akt/PKB, but we have employed a different line and moreover we have investigated the behavior of the endogenous protein. Furthermore we have identified PDGF as an additional growth factor which promotes the intranuclear migration of Akt/PKB.

Very recently it has been reported a possible mechanism regarding the nuclear translocation of Akt1. It has been described that the TCL1 oncogene, involved in the development of human mature T-cell leukemia, participates in PI3K-dependent Akt1 signalling pathway by enhancing Akt1 kinase activity and mediating, through a cytoplasmic interaction, Akt1 nuclear translocation [27].

Akt/PKB is known to be regulated by PI3K products [6]. In MC3T3-E1 cells, induced by the same stimuli, we have detected an intranuclear translocation also of an active PI3K, that on a time scale precedes migration to the nucleus of an active Akt/PKB [28].

Concerning PI3K, its intranuclear accumulation is depen-

Table 3
Quantitative analysis by CLSM of the amount of nuclear Akt/PKB in MC3T3-E1 cells in response to either IGF-I or PDGF

| Condition | 10 min | 20 min | 30 min | 60 min |
|---|---|---|---|---|
| IGF-I | 103 ± 11* | 235 ± 19* | 201 ± 14* | 64 ± 8 |
| IGF-I+LY294002 | 50 ± 5 | 48 ± 7 | 52 ± 5 | 53 ± 4 |
| PDGF | 82 ± 7 | 156 ± 14* | 220 ± 17* | 88 ± 9 |
| PDGF+LY294002 | 46 ± 6 | 49 ± 7 | 51 ± 5 | 49 ± 6 |

Growth factors were applied for the indicated times. Results are expressed as arbitrary fluorescence units. Basal levels measured in the absence or in the presence of LY294002 were 45 ± 6 and 41 ± 5, respectively. Asterisks indicate values that are significantly different ($P < 0.01$) from control.

Table 4
Percentage of cells showing nuclear translocation of Akt/PKB

| Cell treatment | 10 min | 20 min | 30 min |
|---|---|---|---|
| IGF-I | 25 ± 4* | 61 ± 8* | 52 ± 7* |
| IGF-I+LY294002 | 5 ± 2 | 8 ± 1 | 6 ± 2 |
| PDGF | 17 ± 3* | 39 ± 6* | 57 ± 6* |
| PDGF+LY294002 | 6 ± 1 | 5 ± 3 | 8 ± 2 |

Quiescent cell values were 3 ± 2. For each experiment 200 cells were counted. The data are the means ± S.D. from three separate experiments. Asterisks indicate values that are significantly different ($P < 0.01$) from control.

dent on its enzymatic activity. In fact, pre-treatment of MC3T3-E1 cells with LY294002 abolished intranuclear translocation of PI3K, as also demonstrated in SaoS-2 cells [23] or in HL-60 cells [12,29]. Because intranuclear Akt/PKB is active, it must be phosphorylated on threonine and serine residues. The phosphorylation on Thr-308 is catalyzed by PDK1. Since so far intranuclear migration of PDK1 has been ruled out [30], it is currently thought that Akt/PKB could be activated in the cytoplasm and then it migrates to the nucleus [15]. However we cannot exclude the possibility that for sustained intranuclear permanence and activation of Akt/PKB, as induced by IGF-I or PDGF, there is also the need for the presence of an active PI3K within the nuclear compartment. In addition nuclear Akt/PKB activity appears to be important for S-phase entry of the cells. Akt/PKB may be, at the nuclear level, one of the transducers of the PI3K activity required for progression through G1-phase of PDGF-treated fibroblasts [31].

Activated nuclear Akt/PKB is involved in several functions such as the phosphorylation of the winged helix transcription factor FKHR1 (which is induced by IGF-I, see for example [32,33]) and the phosphorylation of the Forkhead transcription factor AFX [34].

Our knowledge of lipid second messenger signalling pathway has strikingly advanced recently due to the recognition that Akt/PKB is one of the major targets of tyrosine kinase receptor-stimulated lipid kinases. The results presented in this paper strengthen the contention that Akt/PKB besides the roles it plays at the plasma membrane is involved in transducing key signals to the nucleus, such as proliferative stimuli.

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