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Effect of Overexpression and Nuclear Translocation of Constitutively Active PKB-α on Cellular Survival and Proliferation in HepG2 Cells

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Abstract Protein kinase B (Akt/PKB) is a key component in the PI 3-kinase mediated cell survival pathway and has oncogenic transformation potential. Although the over-expression of PKB-α can prevent cell death following growth factor withdrawal, the long-term effects of stable over-expression of PKB-α on cell survival in the absence of growth factors remain to be resolved. In the present study, we generated HepG2 cells with stable expression of active PKB-α and compared its characteristics with HepG2 cells. Basal as well as insulin-stimulated levels of Ser^{473} and Thr^{308} phosphorylation in PKB-α transfected HepG2 cells were much higher than HepG2 cells. Constitutive expression of active PKB-α enabled HepG2 cells to survive up to 96 h without serum in growth media while HepG2 cells fail to survive after 48 h of serum withdrawal. A strong positive correlation ($R^2 = 0.71$) between cell proliferation and phosphorylated form of PKB-α at Thr^{308} was observed along with higher levels of phosphorylated 3′-phosphoinositide-dependent kinase-1 (PDK-1). HepG2 cells with constitutive expression of active PKB-α also showed higher levels of phosphorylated p65 subunit of nuclear factor-κB (NFκB) in comparison with HepG2 cells. Predominant nuclear localization of phosphorylated PKB-α was observed in stably transfected HepG2 cells. These results indicate that constitutive expression of active PKB-α renders HepG2 cells independent of serum based growth factors for survival and proliferation. J. Cell. Biochem. 93: 513–525, 2004.

Key words: Akt/PKB-α; insulin signaling; phosphorylation; translocation; serum; cell survival; HepG2 liver cells

Akt/PKB, a protein kinase with sequence homology with protein kinase A and C, is a cellular homologue of viral oncogene v-Akt [Bellacosa et al., 1991; Coffer and Woodgett, 1991; Jones et al., 1991]. Among three mammalian isoforms of PKB (α, β, γ), PKB-α is the most studied isoform for its regulation and substrate specificity. PKB-α is a 57 kD Ser/Thr kinase which has a Pleckstrin Homology (PH) domain and is one of the major downstream components of the phosphatidylinositol 3-kinase (PI 3-kinase) pathway [Andjelkovic et al., 1996; Kohn et al., 1996a]. Previous studies in the last decade have established a pivotal role for PKB-α in various biological processes, including intermediary metabolism, cell proliferation, differentiation, and cell survival [reviewed by Kandel and Hay, 1999; Vanhaesebroeck and Alessi, 2000; Luo et al., 2003].

PKB-α is activated by serum, insulin, insulin like growth factor-1 (IGF-1), platelet derived growth factor (PDGF), and epidermal growth factor (EGF) through a phosphorylation mechanism [Kohn et al., 1995; Alessi et al., 1996; Andjelkovic et al., 1996]. After stimulation through PI-3 kinase pathway, PKB-α is recruited to the plasma membrane through its PH domain, followed by its phosphorylation at Thr^{308} in the activation loop of the kinase domain and at Ser^{473} in the *C*-terminus regulatory region domain. The phosphorylation of Thr^{308} is catalyzed by a 3′-phosphoinositide-regulated kinase-1 (PDK-1), whereas the phosphorylation of Ser^{473} appears to be catalyzed by a yet uncharacterized 3′-phosphoinositide-

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regulated kinase-2 (PDK-2) [Alessi et al., 1997; Stephens et al., 1998; Hresko et al., 2003].

The PI 3-kinase/PKB pathway is a key regulator of cell survival through its ability to regulate multiple downstream targets including, the Bcl-2 family member BAD (Bcl2/Bcl_x antagonist, causing cell death), human caspase-9, forkhead transcription factors and under some conditions, indirectly by activating pro-survival nuclear factor-κB (NFκB) through phosphorylation of I-κB kinase (IKK) [Kandel and Hay, 1999; Ozes et al., 1999; Vanhaesebroeck and Alessi, 2000]. Different strategies have been employed in order to understand the role of PKB in intermediary metabolism as well as in cell survival/proliferation, such as establishing cell lines that conditionally or constitutively overexpress PKB [Kohn et al., 1996b, 1998; Bernal-Mizrachi et al., 2001].

In recent years, many research groups have focussed their attention to understand the molecular mechanism of PKB activation and its translocation, along with searching for new potential phosphorylation targets of this enzyme. Andjelkovic et al. [1997] reported that in human embryonic kidney 293 cells, IGF-1-induced activation of PKB is followed by its translocation to the plasma membrane and then to the nucleus. Similarly, Borgatti et al. [2000] reported that in the osteoblast-like clonal cell line MC3T3-E1, treatment with IGF-1 and PDGF led to nuclear translocation of active PKB. We have shown that HepG2 cells, stimulated with insulin show a distinct nuclear localization of PKB-α as compared to unstimulated HepG2 cells as well as normal hepatocytes [Syed et al., 2002].

In the present study, we generated HepG2 cells with constitutive expression of active PKB-α (HepG2-PKB-CA) to analyze the survival characteristics of the stable transfectants. Constitutive expression of active PKB-α facilitated long-term growth factor independent cell survival. Basal as well as insulin stimulated levels of Ser^{473} and Thr^{308} phosphorylation in HepG2-PKB-CA cells were much higher than in HepG2 cells. HepG2-PKB-CA cells had higher levels of phosphorylated PDK-1 and phospho-p65 subunit of NFκB in comparison with HepG2 cells. Predominantly, nuclear localization of activated PKB-α phosphorylated at both Ser^{473} and Thr^{308} residues was observed in the stably transfected HepG2 cells, reflecting a localization pattern similar to insulin activated cells.

MATERIALS AND METHODS

Cell Culture and Transfection of HepG2 cells

HepG2 cells were grown in DMEM supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic solution (Invitrogen/GIBCO, Burlington, Ontario, Canada) containing penicillin G sodium, streptomycin sulphate, and amphotericin B in a humidified air and 5% CO_2 at 37°C. Cell culture reagents, DMEM, and fetal bovine serum were purchased from Invitrogen/GIBCO (Burlington, Ontario, Canada). Disposable plastic tissue culture plates and other supplies were from VWR Canlab. (Mississauga, Ontario, Canada). The constitutively active PKB-α plasmid, pcDNA3 myri-flag-PKB was kindly provided by Dr. Jim Woodgett (Ontario Cancer Institute, Toronto, Canada). HepG2 cells were seeded 1 day before transfection in 60 mm plates to achieve up to 40–50% confluence. Transfection was performed by incubating HepG2 cells with 2 μg of pcDNA3 myri-flag-PKB plasmid in 2.5 ml of serum free transfection medium for 3 h using Lipofectamine-Plus reagent purchased from Invitrogen/GIBCO. In parallel, HepG2 cells were also transfected with empty vector. After 3 h of incubation, normal media supplemented with serum replaced transfection medium and cells were grown for 2 days before starting selection on 0.6 mg/ml Geneticin (GIBCO). The selection pressure was maintained for 28 days and around 75 well-formed Geneticin resistant HepG2 colonies were picked up using cloning cylinders from VWR Canlab. The colonies were screened by Western blot analysis using anti-phospho Ser 473 PKB-α antibody purchased from BD Biosciences (Mississauga, Ontario, Canada) and anti-phospho Thr 308 PKB-α antibody purchased from Upstate Biotech (Lake Placid, NY). The positive HepG2-PKB-CA clones were kept under 0.1 mg/ml Geneticin to maintain the selection pressure.

3-(4,5 Dimethylthiazol-2-yl)-2,5-Diphenyl-2H-Tetrazolium Bromide (MTT) Assay

The MTT assay was used to measure the proliferation of HepG2 cells in the presence and absence of serum in medium. The assay provides a simple way to detect live and growing cells. The procedures employ the pale yellow tetrazolium salt [3-(4,5-dimethylthiazol)-2-yl-2,5-diphenyl-2H-tetrazolium bromide] (MTT), which is cleaved by active mitochondria to form a dark blue formazon product that can be

completely solubilized in acidic isopropanol [Mosmann, 1983]. Briefly, 5×10^4 HepG2 cells were plated in triplicate in 96-well flat bottom tissue culture plates in the presence and absence of serum for 24–96 h. The MTT reagent (5 mg/ml in PBS; 20 μl/well) was added to the cell culture and incubated for 4 h in a 37°C, 5% CO_2 humidified incubator. The formazon crystals formed during the reaction were dissolved in 100 μl of 0.4°N HCl in isopropanol and absorbance was read at 570 nm.

Western Blot Analysis

HepG2 cells were grown on 100 mm plates to ~80–85% confluence before starting the desired treatments. Overnight-starved cells were washed twice with PBS and then treated with insulin (human recombinant; Sigma, Oakville, Ontario, Canada) in serum free media. The incubation conditions and concentrations of insulin are indicated in figure legends. After the treatments, cells were washed with ice cold PBS and cells were lysed on ice using 1ml lysis buffer containing 50 mM HEPES (pH 7.4), 150 mM sucrose, 2 mM sodium orthovanadate, 80 mM β-glycerophosphate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium EGTA, 2 mM sodium EDTA, 1% Triton X-100, 0.1% SDS, 1 mM phenyl methyl sulphonyl fluoride, 100 μl of protease inhibitor cocktail containing 8.0 μM aprotinin, 0.2 mM leupeptin, 0.4 mM bestatin, and 0.15 mM pepstatin. Cells were scraped off the plates and kept on ice for 30 min. Samples were then centrifuged at 13,000*g* for 2 min at 4°C and supernatants were stored at −80°C until use. Aliquots (20 μg protein) were mixed with SDS–PAGE sample buffer and resolved by SDS–PAGE. Proteins were transferred on to a blotting grade PVDF membrane using a mini transblot apparatus and visualized by Ponceau S reagent. The blots were blocked with 5% non-fat dry milk/0.02% PBS-Tween 20 for 1 h at room temperature followed by 2 h in cold room. The blots were probed with Anti-PKB-α, anti-phospho Ser^{473} PKB-α, anti-phospho Thr^{308} PKB-α, anti-phospho p65 subunit of NFkB (Ser^{536}) or anti-phospho PDK-1 (Ser^{241}), 1:1,000 overnight in cold room. The membranes were washed and incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase (1:10,000) for 1 h at room temperature. After further washing steps, visualization was carried out using a chemiluminescence reagent, Amersham Biotech (Boston, MA). Protease inhibitor cocktail for mammalian cell culture and other analytical grade reagents were purchased from Sigma. Anti-phospho p65 subunit of NFkB (Ser^{536}) or anti-phospho PDK-1 (Ser^{241}) antibodies were purchased from Cell Signaling Tech. (Mississauga, Ontario, Canada). Electrophoresis and Bradford protein assay reagents were from Bio-Rad Lab (Mississauga, Ontario, Canada).

Immunofluorescence Analysis

For immunofluorescence analysis, HepG2 cells were grown on 18 mm round cover slips. This was followed by fixation and permeabilization, as described by Loh et al. [1999]. The cells were then incubated for 30 min with 1:100 dilutions of Chromopure IgG sheep whole molecule (negative control) (BD Biosciences) or anti-phospho rat PKB-α (Ser^{473}) (sheep polyclonal IgG), or anti-phospho rat PKB-α (Thr^{308}) (sheep polyclonal IgG) (UBI, Lake Placid, NY). The cells were then incubated for 45 min with FITC-labeled secondary antibody (1:200) (BD Biosciences). These samples were mounted onto glass slides using mounting media Citifluor™. Indirect immunofluorescence was performed using a Zeiss Axidkop Routine microscope for transmitted and incident-light fluorescence equipped with Northern Eclipse image software, Empix Imaging, Inc. (Mississauga, Ontario, Canada).

Other Methods

Protein was determined by the method of Bradford [1976] using bovine serum albumin as the standard. Relative band intensities of different Western blots were measured by NIH-image software. Statistical analyses were performed by use of the Student's *t*-test.

RESULTS

Stable and Constitutive Expression of PKB-α in HepG2 Cells and Insulin Effect

PKB-α can be rendered constitutively active by targeting it to the plasma membrane through attachment of a myristoylation signal at the *N*-terminus [Andjelkovic et al., 1997]. The positive clones expressing constitutively active PKB-α plasmid (pcDNA3 myri-Flag-PKB) in HepG2 cells (HepG2-PKB-CA) were selected by screening of cell lysates with anti-phospho Ser^{473} PKB-α, anti-phospho Thr^{308} PKB-α, and

anti-PKB-α antibodies. The positive HepG2-PKB-CA cells showed up to 5-fold higher basal phosphorylation levels at Ser^{473} and Thr^{308} of PKB-α and 2-fold higher expression of basal PKB-α levels as compared to the HepG2 cells (Fig. 1a,b). The effects of insulin treatments on PKB phosphorylation in the HepG2-PKB-CA cells and the HepG2 cells are shown in Figure 2. Although the basal level of Ser^{473} and Thr^{308} phosphorylation was higher in the HepG2-PKB-CA cells (Fig. 1), this was further increased up to 3.5-fold by 10 nM insulin in 5 min (Fig. 2a,b). In HepG2 cells, insulin stimulated Ser^{473} and Thr^{308} phosphorylation of PKB-α up to 6-fold (Fig. 2c,d). In both types of cells, no change in total PKB-α was observed. A comparison of HepG2-PKB-CA and HepG2 cells

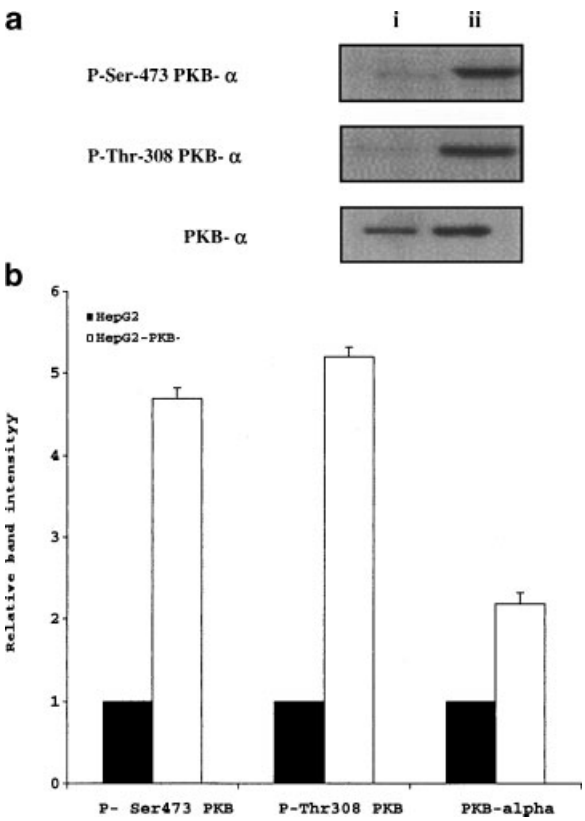

Fig. 1. Basal levels of phosphorylated and total PKB-α in HepG2 and myrPKB-α transfected HepG2 cells. Overnight starved and PBS washed HepG2 cells and HepG2-PKB-CA cells were lysed as described in ''Materials and Methods.'' Whole cell lysates were used for Western blots using anti-bodies against P-Ser^{473} PKB-α, P-Thr^{308} PKB-α, or PKB-α. A typical Western blot is shown in (**a**) **lane** (**i**) HepG2; **lane** (**ii**) HepG2-PKB-CA cells. The relative intensities of bands were determined using NIH-image software and the control band was given an arbitrary value of 1. The results shown in (**b**) are the average ± SEM of three experiments.

indicated that the total phosphorylation state at Ser^{473} and Thr^{308} of PKB in HepG2-PKB-CA cells was higher than the total phosphorylation state observed in HepG2 cells. This observation suggests that transfected HepG2-PKB-CA cells show attributes similar to insulin-mediated activation of PKB-α in HepG2 cells, consistent with previous observations in 3T3-L1 fibroblast and adipocytes [Kohn et al., 1996].

Growth Factor Independence of HepG2 Cells With Constitutive Expression of PKB-α

It has been reported PKB provides cell survival signals to protect cells from cell death induced by either UV light [Kulik et al., 1997], by withdrawal of the survival factor IGF-1 in neuronal cells [Dudek et al., 1997] or by withdrawal of serum factor from cells which overexpress c-myc [Kauffmann-Zeh et al., 1997]. Therefore, it was of interest to study survival of the HepG2 cells constitutively expressing PKB-α in presence and absence of serum. For this, we plated equal numbers of HepG2 cells or HepG2-PKB-CA cells in the presence and absence of serum in 96-well plates and assessed cell survival by MTT assay after 0–96 h. HepG2 cells proliferated normally in the presence of serum, reaching a peak at 72 h. In the absence of serum, however, the HepG2 cells divided up to 48 h and then underwent cell death (Fig. 3a). In comparison, the HepG2-PKB-CA cells both in the presence and absence of serum kept on proliferating and achieving a peak at 72 h (Fig. 3b). Unlike HepG2 cells, cellular proliferation was normal in the absence of serum in HepG2-PKB-CA cells. The proliferation in the HepG2-PKB-CA cells was higher than HepG2 in the presence of serum (Fig. 3b).

Under similar experimental conditions, the profiles of PKB-α and its phosphorylation status at Ser^{473} and Thr^{308} residues were further compared with observations made on HepG2 cell proliferation. As shown in Figure 4a, in the presence of serum, the basal level of Ser^{473} phosphorylation of PKB-α in HepG2-PKB-CA cells was much higher than in HepG2 cells (Fig. 5a) and reached maximum peak at 48 h. In the absence of serum, the higher phosphorylation at Ser^{473} of PKB-α was still maintained at 0 h and gradually increased up to 96 h in HepG2-PKB-CA cells, Figure 4c,d) whereas in HepG2 cells, 0–24 h showed a lower level of phosphorylation at Ser^{473} of PKB-α that increased up to 96 h (Fig. 5a,b). The phosphorylation at Thr^{308}

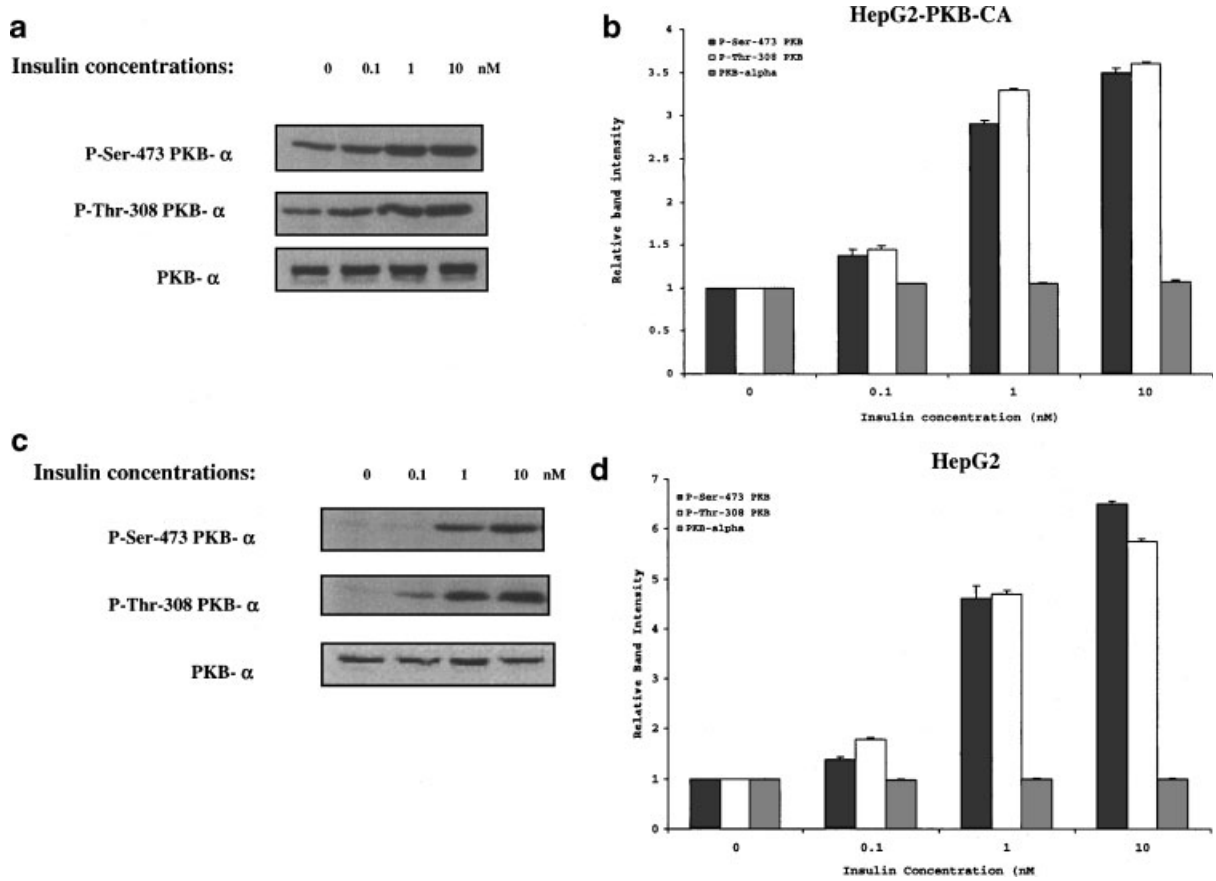

Fig. 2. Effects of varying concentrations of insulin on phosphorylation of protein kinase B in HepG2-PKB-CA and HepG2 cells. Overnight starved and PBS washed HepG2-PKB-CA and HepG2 cells were incubated with varying concentrations (0–10 nM) of insulin in serum free media for 5 min at 37°C. The conditions for washing of cells and preparation of lysates are as described in ''Materials and Methods.'' Whole cell lysates were used for Western blots using anti-bodies against P-Ser^{473} PKB-α, P-Thr^{308} PKB-α, or PKB-α. A typical Western blot is shown in (**a** & **c**) for HepG2-PKB-CA and HepG2 cells. The relative intensities of bands were determined using NIH-image software and the control band was given an arbitrary value of 1. The results shown in (**b** & **d**) for HepG2-PKB-CA and HepG2 cells, respectively, are the average ± SEM of three experiments.

residue of PKB-α in the HepG2-PKB-CA cells showed a consistent increase over the 96 h period in the presence of serum and reached a peak at 48 h in the absence of serum (Fig. 4c,d). In HepG2 cells, phosphorylation at Thr^{308} residue of PKB-α followed the similar increasing trend as observed with phosphorylation at Ser^{473} residue in the presence of serum (Fig. 5a,b). The basal levels of PKB-α also increased with time in the HepG2-PKB-CA cells in the presence and absence of serum. In the presence of serum, there was also an increase in basal level of PKB-α HepG2 cells, albeit at lower levels in comparison to HepG2-PKB-CA cells grown both in the presence and absence of serum in growth medium. In the absence of serum, there was a reduction in phosphorylation at Ser^{473} and Thr^{308} and in levels of PKB-α with time in HepG2 cells (Fig. 5c,d). The data in Figures 3–5 were further analyzed to determine whether cell proliferation was correlated with the amount of total and phosphorylated forms of PKB-α. There was a positive correlation between cell proliferation and the total amount of PKB-α ($R^2 = 0.44$) and phosphorylated Ser^{473} form of PKB-α. However, there was a very strong correlation between cell proliferation and phosphorylated Thr^{308} form of PKB-α ($R^2 = 0.71$).

After being targeted to the membrane, PKB-α undergoes phosphorylation at Thr^{308} by PDK-1 [Alessi et al., 1997; Stephens et al., 1998]. Our initial observation was that HepG2-PKB-CA cells showed a gradual increase in phosphorylation at Thr^{308} of PKB-α up to 96 h in the presence and absence of serum and that there was no

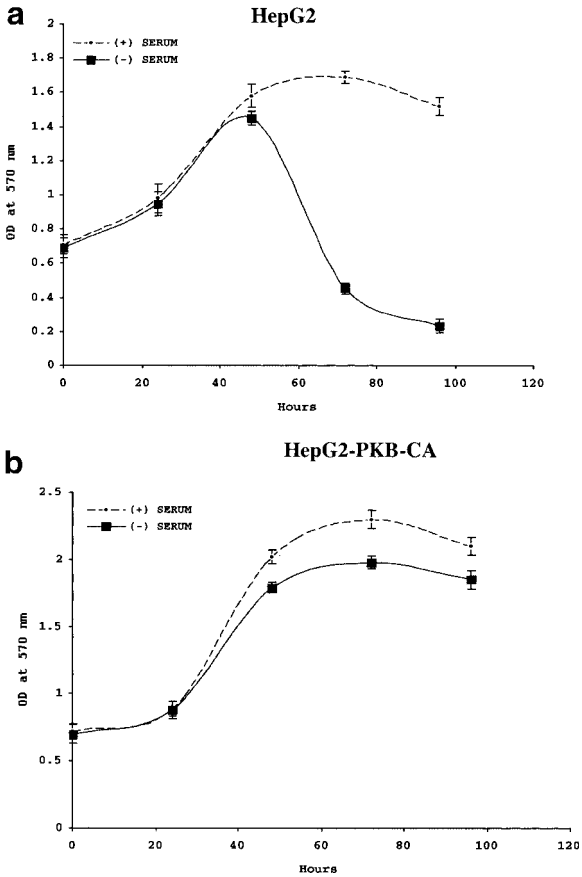

Fig. 3. Effect of the presence and absence of serum in growth medium on cell survival of HepG2 and HepG2-PKB-CA cells. Equal number of HepG2 and HepG2-PKB-CA cells were cultured in 96-well plate. The cell survival was estimated by MTT assay as described in ''Materials and Methods.'' **a**: Represents survival curve for HepG2 cells in the presence and absence of serum. **b**: Represents survival curve for HepG2-PKB-CA cells. Error bars signify the SEM for six replicates in three experiments.

corresponding increase in phosphorylation of Ser^{473} of PKB-α (Fig. 4). We were, therefore, interested to know whether there was an increase in the level of phosphorylated PDK-1. As shown in Figure 6a there was 50% higher phosphorylated PDK-1 in transfected HepG2-PKB-CA cells as compared to HepG2 cells.

Elevated Levels of Phosphorylated p65 Subunit of NFκB With Constitutive Expression of PKB-α

Several studies have demonstrated that NFκB and PKB signaling pathways can converge and activation of the PI-3 kinase/PKB pathway by tumor necrosis factor-α (TNF-α) and PDGF indeed stimulate NFκB in different cell types [Ozes et al., 1999; Romashkova and Makarov, 1999]. Phosphorylation of the p65 subunit at Ser^{536} has been correlated to the nuclear translocation of the NFκB complex [Sakurai et al., 1999]. Since, HepG2-PKB-CA cells showed characteristics of constitutively active PKB-α, it was of interest to look for NFκB activation in these cells. The amount of phosphorylated p65 subunit of NFκB was determined in whole cell lysate of HepG2-PKB-CA cells and HepG2 cells. As shown in Figure 6b,d, there was approximately 2-fold higher level of phosphorylated p65 subunit of NFκB in the HepG2-PKB-CA cells as compared to HepG2 cells.

Localization of PKB-α in HepG2 Cells With Constitutive Expression of PKB-α

Since our initial observation that HepG2-PKB-CA cells showed characteristics similar to insulin activated PKB-α in HepG2 cells, it was of interest to determine the localization of active PKB-α to get an insight into the potential site of its actions. In unstimulated HepG2 cells, PKB-α was mostly present in cytoplasm with a trace and scattered presence in the nucleus (Fig. 7 panel (i) A–D). Similarly, phospho-Thr^{308} PKB-α and phospho-Ser^{473} PKB-α were mostly present in cytoplasm with a trace amount in the nucleus (Fig. 7 panels (ii & iii) A & D). In comparison, a different localization pattern was observed in HepG2-PKB-CA cells (Fig. 8). First, as expected, the amount of enzyme in HepG2-PKB-CA was higher as compared to non-transfected HepG2 cells (Fig. 8 panel (i) A) as evidenced by higher green fluorescence levels. PKB-α was present mostly in cytoplasm and around nuclear membrane as well as in trace amount inside the nucleus (Fig. 8 panel (i) A, C, & D). Both Phospho-Ser^{473} as well as phospho-Thr^{308} PKB-α showed a predominant nuclear along with some cytoplasmic presence (Fig. 8 panels (ii & iii) A & D). These results indicate a different localization of PKB-α and its phospho-specific isoforms in the HepG2-PKB-CA cells as compared to HepG2 cells.

DISCUSSION

The phosphorylation and activation of PKB-α is one of the critical steps for metabolic regulation, cell survival, and proliferation. Hyperactivation of the PI 3-kinase/PKB pathway has been implicated in oncogenesis and over-expression

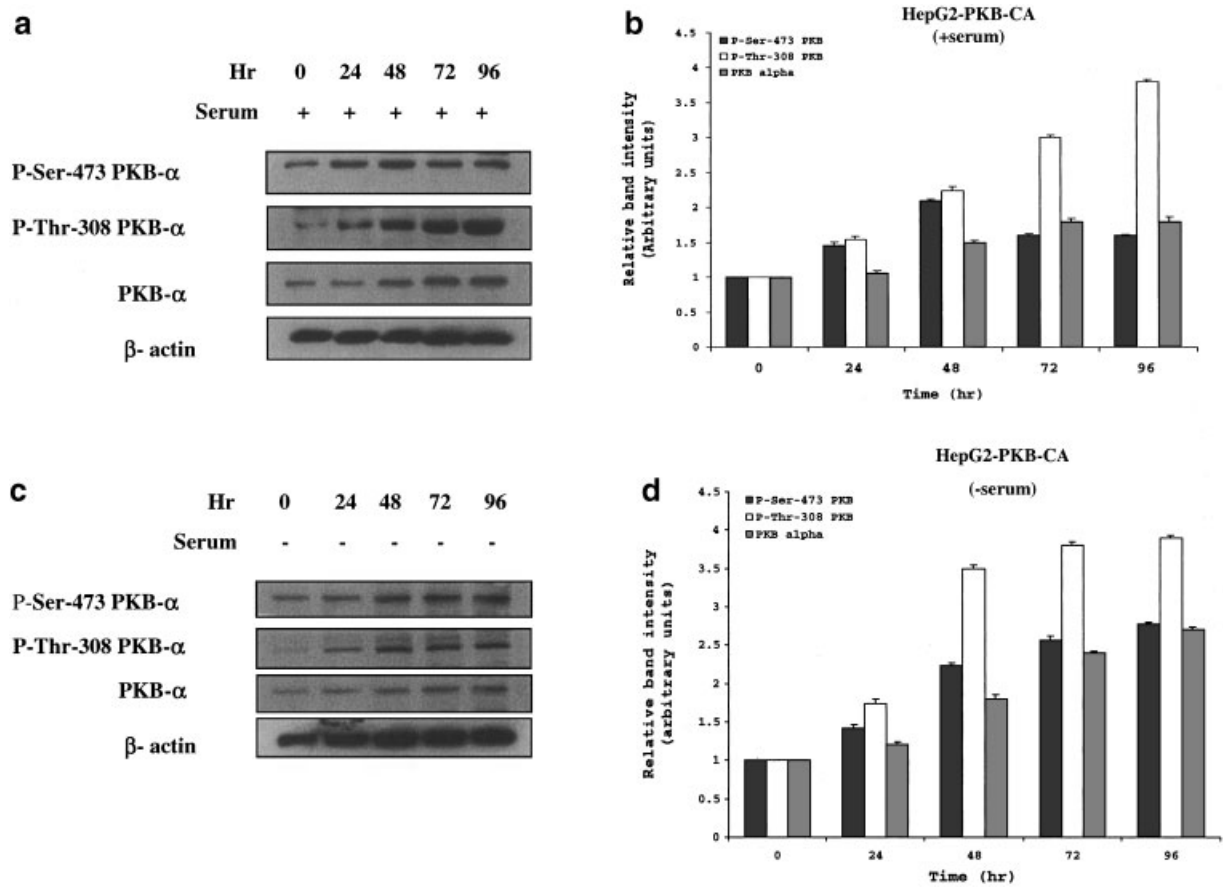

Fig. 4. Effect of the presence and absence of serum in growth medium on basal levels of phosphorylated and total PKB-α in HepG2-PKB-CA cells. Equal number of HepG2-PKB-CA cells were cultured in 6-well plates for 0–96 h. The conditions for washing of cells and preparation of lysates are as described in ''Materials and Methods.'' Whole cell lysates were used for Western blots using anti-bodies against P-Ser^{473} PKB-α, P-Thr^{308} PKB-α, or PKB-α and β-actin. Typical Western blots are shown in (**a**), for P-Ser^{473} PKB-α, P-Thr^{308} PKB-α, PKB-α and β-actin in presence of serum. Typical Western blots are shown in (**c**), for P-Ser^{473} PKB-α, P-Thr^{308} PKB-α , PKB-α and β-actin in absence of serum. The relative intensities of bands were determined using NIH-image software and the control band was given an arbitrary value of 1. The results shown in (**b** & **d**) are the average ± SEM of three experiments.

of PKB has been linked with several cancers [Luo et al., 2003]. Constitutive over-expression of PKB-α in mouse liver results in hepatomegaly [Ono et al., 2003]. Several growth factors including insulin, PDGF, EGF, FGF, and IGF-I are capable of activating Akt/PKB. PKB-α regulates several aspects of glucose uptake, metabolism, and storage, as well as cell survival in response to insulin stimulation. In order to understand the role of the PI 3-kinase/PKB pathway in hepatocarcinoma cells, we carried out the stable over-expression of a constitutively active PKB-α construct in HepG2 cells. HepG2 cells are a human hepatocellular carcinoma derived cell line and has been used extensively to understand liver associated functions [Javitt, 1990]. The constitutive activation of PKB-α is reflected in higher basal levels of phosphorylation, which was further increased up to 3.5-fold in the presence of insulin. The levels of Ser^{473} and Thr^{308} phosphorylation were significantly higher in HepG2-PKB-CA cells than in HepG2 cells (Figs. 1 and 2). Kohn et al. [1996] have previously reported that constitutive expression of PKB in 3T3-L1 fibroblast cells led to activation of this kinase in the absence of insulin and there was no further stimulation by insulin. Similarly, Andjelkovic et al. [1997] have demonstrated that the transient expression of membrane targeted PKB with myristolation/plamitylation signal led to constitutive activation of PKB-α. As indicated

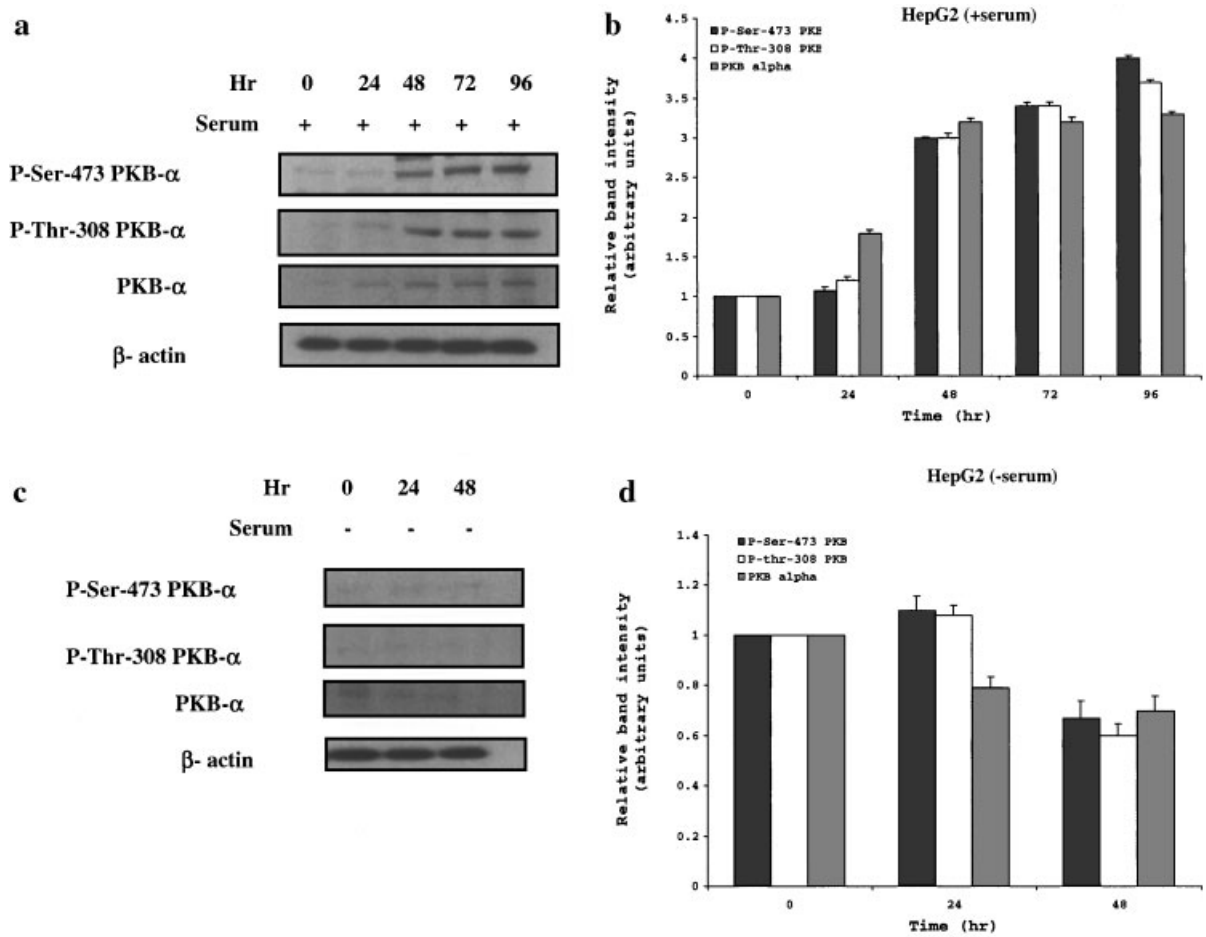

Fig. 5. Effect of the presence and absence of serum in growth medium on basal levels of phosphorylated and total PKB-α in HepG2 cells. Equal number of HepG2 cells were cultured in 6-well plates from 0–96 h in presence and absence of serum in growth medium. The conditions for washing of cells and preparation of lysates are as described in ''Materials and Methods.'' Whole cell lysates were used for Western blots using anti-bodies against P-Ser^{473} PKB-α, P-Thr^{308} PKB-α, or PKB-α and β-actin. Typical Western blots are shown in (**a**), for P-Ser^{473} PKB-α, P-Thr^{308} PKB-α, PKB-α and β-actin in presence of serum. Typical Western blots are shown in (**c**), for P-Ser^{473} PKB-α, P-Thr^{308} PKB-α, or PKB-α and β-actin in absence of serum. The relative intensities of bands were determined using NIH-image software and the control band was given an arbitrary value of 1. The results shown in (**b** & **d**) are the average ± SEM of three experiments.

above that in HepG2 cells, transfection increased the active-form of PKB-α and this activity was further stimulated in the presence of insulin. It is possible that further phosphorylation of PKB-α at Ser^{473} and Thr^{308}, in the presence of insulin, is due to activation of the endogenous inactive PKB-α.

As shown in Figure 3a,b, both HepG2 and HepG2-PKB-CA cells could sustain growth and proliferation for 96 h when grown in the presence of serum. However, in the absence of serum, non-transfected cells undergo apoptosis after 48 h, whereas, the HepG2-PKB-CA cells survive even up to 96 h after serum withdrawal. The serum is a source for exogenous growth factors and is a known activator of PKB-α [Andjelkovic et al., 1996]. The presence of serum, therefore, keeps PKB-α in the active form in these cells (Figs. 4a and 5a). Similarly, constitutive expression of the active form in HepG2 cells rendered them independent of serum-based growth factors (Fig. 4c). This is a significant observation since it provides insight into PKB-α mediated cellular survival in nutrition-deprived conditions, and may be one of the reasons why tumors can progress in hypovascular conditions [Izuishi et al., 2000]. Dudek et al. [1997] have previously reported that

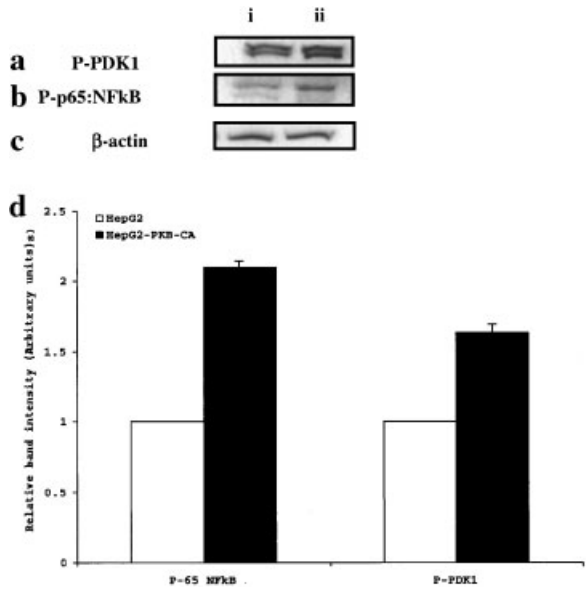

Fig. 6. Levels of phosphorylation of p65 subunit of NFκB and PDK-1 in HepG2 and HepG2-PKB-CA cells. Overnight starved and PBS washed HepG2 cells and HepG2-PKB-CA cells were lysed as described in ''Materials and Methods.'' Whole cell lysates were used for Western blots using phospho-specific antibodies against phosho-p65 subunit of NFkB and phospho-PDK-1 and after deprobing same blots were probed with anti-β-actin. A typical Western blot is shown in (**a**) **lane** (**i**) HepG2; **lane** (**ii**) and HepG2-PKB-CA cells. The relative intensities of bands were determined using NIH-image software and the control band was given an arbitrary value of 1. The results shown in (**b**) are the average ± SEM of three experiments.

withdrawal of survival factors led to synchronous apoptosis in cerebellar neurons and this was overcome by a transient expression of PKB-α along with supplementation with serum and IGF-1. Similarly, Edinger and Thompson [2001] have reported that PKB can maintain cell size and cellular survival by maintaining normal function of cell surface transporters through mTOR (mammalian target of rapamycin) dependent pathways in FL5.12 cells. All of these studies support the notion that the active PKB-α can sustain growth and proliferation of the cells in the absence of exogenous growth factors.

PDK-1 is the protein kinase responsible for phosphorylating PKB-α on Thr^{308} [Stephens et al., 1998]. Scheid et al. [2002] have shown that PI 3-kinase mediated translocation of PKB to the plasma membrane promotes Ser^{473} phosphorylation that in turn is conditional for PDK-1 mediated phosphorylation at Thr^{308} and full activation of PKB. The HepG2-PKB-CA cells also showed higher basal level of PDK-1 phosphorylation in comparison to HepG2 cells Figure 6a,d. This suggests that there is some cross talk between PDK-1 and its substrate, PKB-α. This could be due to higher levels of plasma membrane targeted PKB-α. The higher phosphorylated levels of PDK-1 in HepG2-PKB-CA cells could also explain the gradual rise in Thr^{308} phosphorylation of PKB-α up to 96 h (Fig. 4a,b). Previously, Casamayor et al. [1999] have reported that phosphorylation of PDK-1 at Ser^{241} on the activation loop is necessary for its activation and mutation of this residue to Ala abolishes PDK-1 activation. Increased basal levels of phosphorylated PDK-1 in HepG2-PKB-CA cells could lead to enhanced PDK-1 activity that in turn leads to rise in Thr^{308} phosphorylation of PKB-α Moreover, it is also possible that membrane targeted PKB has sufficient priming by phosphorylation at Ser^{473} residue and this facilitates higher phosphorylation at Thr^{308} residue.

As shown in Figure 6b,d, the 2-fold higher basal level of phosphorylation of p65 subunit of NFκB in HepG2-PKB-CA cells was observed in comparison to HepG2 cells. This could lead to higher level of transcriptional activity associated with the p65/Rel A component of heterodimer of NFkB. The NFκB complex consists of a group of binary complexes of proteins with related promoter-binding and transactivation activities. The prototypical NFκB consists of a p65-p50 heterodimer [Verma et al., 1995]. After activation of the NFκB pathway, phosphorylated p65/Rel A stimulates transcription and p50 serves mainly to bind DNA [Liou and Baltimore, 1993]. Previously, it has been reported that PI 3-kinase/PKB pathway stimulation by TNF-α and PDGF, activate NFκB [Ozes et al., 1999; Romashkova and Makarov, 1999]. Ozes et al. [1999] also reported that there was a higher DNA binding by NFκB in HeLa cells expressing constitutively active PKB. It is possible that the higher basal level of p65/Rel A phosphorylation can be responsible for sustaining proliferation of PKB transfected HepG2 cells in absence of serum through activation of the pro-survival pathway by NFκB. Recently, Meng and D'Mello [2003] have demonstrated that NFκB is capable of activating PKB-α in a transcription dependent feedback loop, that appears to involve PDK-1 and PI-3 kinase, which may provide a clue to our observation of activated PDK-1 in the

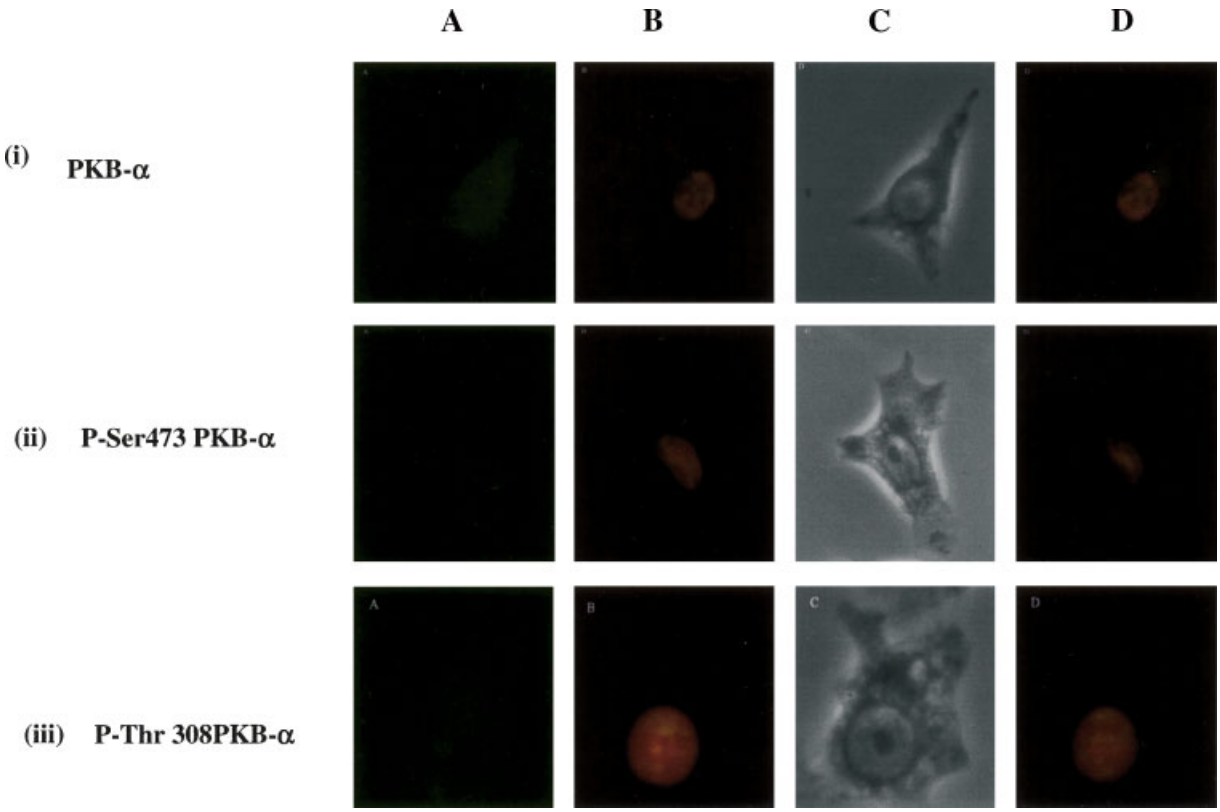

Fig. 7. Fluorescent microscopy and subcellular localization of PKB in HepG2 cells. Overnight starved cells were grown on glass cover slips and prepared for immuno-florescence as described in ''Materials and Methods.'' The subcellular localization of PKB-α and phosphorylated PKB-α at Ser^{473} and Thr^{308} residues were carried out using primary anti-bodies against PKB-α and P-Ser^{473} and P-Thr^{308} PKB-α probed with FITC-conjugated secondary anti-body. Propidium iodide was used to visualize the nucleus. Images in **panels** (**i–iii**) represent (**A**) as a green fluorescence, (**B**) as a red fluorescence, (**C**) as a phasecontrast, (**D**) as a overlay of image (A), (B), and (C). Panels represent, (i) subcellular localization of PKB-α (ii) subcellular localization of P-Ser^{473} PKB-α, and (iii) subcellular localization of P-Thr^{308} PKB-α. The images presented are representative of rest of the cells grown on cover slips. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

constitutively active PKB expressing HepG2-PKB-CA cells (Fig. 6b,d).

In a previous study [Syed et al., 2002], we observed that insulin stimulation of HepG2 cells cause a predominant localization of phosphorylated forms of PKB-α inside the nucleus. The present study reveals the localization pattern of active PKB-α in HepG2-PKB-CA cells. Our observations suggest that these cells mimic nuclear localization pattern of the insulin stimulated state of HepG2 cells. The nuclear localization of active PKB-α is significant considering that PKB-α can also interact with oncogenes such as Tcl1 (involved in development of human mature T-cell leukemia) after nuclear translocation [Pekarsky et al., 2000]. Moreover, many components of the PI 3-kinase pathway, such as the insulin receptor, insulin receptor substrates 1/2 (IRS-1/2), and PDK-1 are capable of nuclear shuffling [Podlecki et al., 1987; Meier et al., 1997; Kim, 1998; Lim et al., 2003; Wu et al., 2003]. The reconstitution of PI-3 kinase pathway takes place inside the nucleus and activates the nuclear transcription events. In the HepG2-PKB-CA cells, the predominant nuclear presence of active PKB-α suggests the constitutive activation of PI 3-kinase pathway inside the nucleus. This could be one of the factors for higher phosphorylation levels of p65 subunit of NFkB observed in HepG2-PKB-CA cells. Previously, Andjelkovic et al. [1997] have demonstrated that the transient expression of PKB-α with myristoylation/plamitylation signal, lead to its membrane targeting. In the present study, the stable expression of PKB-α with myristolation signal reveals that

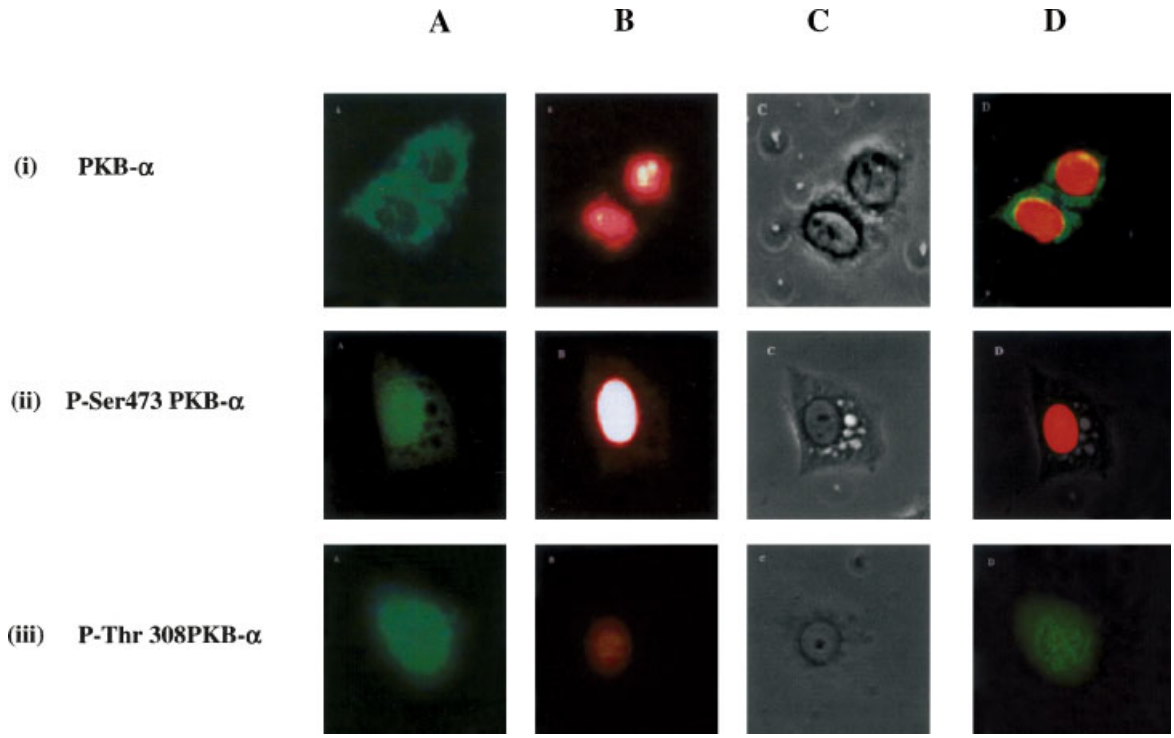

Fig. 8. Fluorescent microscopy and subcellular localization of PKB in HepG2-PKB-CA cells. Overnight starved cells were grown on glass cover slips and prepared for immuno florescence as described in ''Materials and Methods.'' The subcellular localization of PKB-α and phosphorylated PKB-α at Ser^{473} and Thr^{308} residues were carried out using primary anti-bodies against PKB-α and P-Ser^{473} and P-Thr^{308} PKB-α probed with FITC-conjugated secondary anti-body. Propidium iodide was used to visualize the nucleus. Images in **panels** (**i–iii**) represent (**A**) as a green fluorescence, (**B**) as a red fluorescence, (**C**) as a phasecontrast, (**D**) as a overlay of image (A), (B), and (C). Panels represent, (i) subcellular localization of PKB-α, (ii) subcellular localization of P-Ser^{473} PKB-α, and (iii) subcellular localization of P-Thr^{308} PKB-α. The images presented are representative of rest of the cells grown on cover slips. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

phosphorylated (active) form of PKB-α is also present inside the nucleus. In fact, Ahmed et al. [1993] have demonstrated myristoylation of v-Akt/PKB and subcellular localization showed a dispersed presence (approximately, 40% on plasma membrane, 30% in the nucleus, and 30% in cytosol) of v-Akt/PKB. The nuclear presence of active PKB-α in HepG2-PKB-CA cells raises the possibility of presence of demyristoylation mechanism, which is still unexplored.

Overall, the current study reflects phosphorylation patterns, activation, and translocation of PKB-α in HepG2-PKB-CA cells. It also supports the view that PKB-α plays a critical role in cell proliferation and survival. The nuclear localization of active PKB-α in HepG2-PKB-CA cells is associated with activation of p65 subunit of transcription factor NFkB with plausible constitutive activation of PI 3-kinase pathway inside the nucleus. It will be interesting to further delineate the interaction of PKB-α with other nuclear targets.

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