Journal of Cellular Biochemistry 86:118–127 (2002)

Different Cellular Localization, Translocation, and Insulin-Induced Phosphorylation of PKBα in HepG2 Cells and Hepatocytes

Noor Afshan Syed,[1] Kyla Nadine Horner,[1] Vikram Misra,[2] and Ramji Lal Khandelwal[1]*

[1]Department of Biochemistry, University of Saskatchewan, 107 Wiggins Road, Saskatoon, SK S7N 5E5, Canada
[2]Department of Veterinary Microbiology, University of Saskatchewan, Veterinary Road, Saskatoon, SK Canada

Abstract Protein kinase B (PKB), a serine/threonine protein kinase, prevents apoptosis and promotes cellular transformation. PKB activity is stimulated by insulin. In this report, we examined the relative amounts of expression, location, and translocation upon insulin stimulation of PKBα in normal primary hepatocytes and carcinoma cells, HepG2 cells. Non-phosphorylated PKBα was present in both types of unstimulated cells. The phosphorylated form of the enzyme was present in the nucleus of unstimulated HepG2 cells but not in normal hepatocytes. In the cytoplasm, PKBα was found in greater abundance in the hepatocytes as compared in HepG2 cells. Insulin induced the translocation of phosphorylated PKBα from the nucleus to the nuclear membrane in HepG2 cells. In contrast, insulin caused translocation and phosphorylation of PKBα from the cytosol to the plasma membrane in normal hepatocytes. In addition, there is a higher expression of PKBα in the HepG2 cells as compared to normal primary hepatocytes. These findings provide an important distinction between hepatocellular HepG2 cells and normal liver cells and suggest that the presence of constitutively active nuclear PKB in the transformed cells might be an important contributor in cell transformation and immortality of hepatoma cells. J. Cell. Biochem. 86: 118–127, 2002.

Key words: liver cells; cell transformation; phosphorylation; translocation; confocal microscopy; insulin signaling

Protein kinase B (PKB) is a serine/threonine protein kinase of molecular weight 63 kDa [Coffer et al., 1998; Meier and Hemmings, 1999]. PKB exists as three isoforms, PKBα, PKBβ, and PKBγ. The primary structure of the PKB enzymes is homologous to that of both protein kinase A and PKB, and therefore, PKB is also known as RAC kinase (Related to A and C Kinase). PKB is the cellular homologous of the transforming retrovirus protein called v-Akt, which contains a pleckstrin homology (PH) domain at the amino terminal. The catalytic domain of v-Akt has been shown to be identical to PKB. The PH domains are considered to be necessary for protein–protein and protein–lipid interactions. Phosphorylation of both a threonine (Thr308) and a serine (Ser473) residue is required for activation of PKBα and dephosphorylation abolishes this activation [Alessi and Cohen, 1998; Kandel and Hay, 1999; Meier and Hemmings, 1999].

Unstimulated wild-type human PKBα was found to be in the cytoplasm when transiently expressed into monkey kidney COS-1 and human embryonic kidney 293 (HEK 293) cells. The PKBα in these cells could be activated within 2 min by insulin, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) [Andjelkovic et al., 1997]. Recruitment of the cytoplasmic enzyme to the plasma membrane is proposed to be the main effect of these effectors. Previously, we have shown that in HepG2 cells, endogenous PKB is phosphorylated and activated by insulin and this insulin-induced activation and phosphorylation of PKBα is

Grant sponsor: CIHR–regional partnership program.

*Correspondence to: Dr. Ramji Lal Khandelwal, Department of Biochemistry, University of Saskatchewan, 107 Wiggins Road, Saskatoon, SK S7N 5E5, Canada.
E-mail: ramji.khandelwal@usask.ca

Received 14 February 2002; Accepted 7 March 2002
DOI 10.1002/jcb.10189

prevented by wortmannin (an inhibitor of phosphatidylinositol-3 kinase (PI-3 kinase)) [Syed and Khandelwal, 2000]. This suggests that PI-3 kinase phosphorylated lipid products, phosphatidylinositol-3,4-bisphosphate (PtdIns-(3,4)P_2), and/or PtdIns(4,5)P_2, are necessary for the activity of PKB in the nucleus and cytoplasm [Martelli et al., 1999; Vanhaesebroeck and Alessi, 2000]. PtdIns(3,4)P_2 and/or PtdIns-(4,5)P_2 are thought to interact with the PH domain of PKB and cause translocation to the membrane or to lipid vesicles and furthermore, make Thr308 of PKBα more accessible for phosphorylation by other protein kinases. It is suggested that 3-phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylates Thr308. The PH domain of PDK1 is necessary for its actions on PKB. This provides evidence that it is necessary for PDK1 to be in the same vicinity of lipid vesicles as PKB in order for phosphorylation to take place. PDK2 is an unidentified protein kinase, which is thought to phosphorylate the Ser473 residue of PKBα [Alessi and Cohen, 1998; Kandel and Hay, 1999; Vanhaesebroeck and Alessi, 2000].

In vivo, three downstream substrates for PKB are, GSK-3, 6-phosphofructo 2- kinase, and Bcl-2 family member, BAD [Kandel and Hay, 1999]. These substrates are phosphorylated in vivo in response to insulin and growth factors. In vitro, it has been shown that PKB is able to phosphorylate $p70^{s6k}$, PHAS-1, human caspase-9 and nuclear factors including CREB and Forkhead transcriptional factors [Kandel and Hay, 1999]. PKB is also linked to the phosphorylation of BRCAI, which is a breast cancer susceptible gene [Altiok et al., 1999]. Previously, it was shown that in transfected human HEK 293 and COS-1 cells, PKBα is translocated from the cytoplasm to the nucleus, after stimulation with insulin [Andjelkovic et al., 1997; Meier et al., 1997]. In recent years, PKB has been shown to inhibit apoptosis and promote transformation in cells [Kandel and Hay, 1999; Meier and Hemmings, 1999; Sabbatini and McCormick, 1999; Pugazhenthi et al., 2000; Tang et al., 2000; Chen et al., 2001]. It was, therefore, interesting to compare the cellular location, expression, and insulin-induced translocation of PKBα in normal hepatocytes and hepatocellular carcinoma HepG2 cells. HepG2 cells have proven to be valuable in studying liver derived functions [Aden et al., 1979; Javitt, 1990]. They maintain most functions of the liver and are stable through many passages. We have utilized these qualities of HepG2 cells as a source of in vitro model to study the differences in PKBα in these cells as compared to normal hepatocytes.

MATERIALS AND METHODS

Cell Culture

HepG2 cell were a gift from Dr. W. J. Roesler (Department of Biochemistry, University of Saskatchewan). The reagents for culturing of HepG2 cells were purchased from Life-Technologies (Burlington, Ontario, Canada). HepG2 cells were grown in 100 mm tissue culture dishes (VWR Canlab, Mississauga, Ontario, Canada) and maintained in DMEM/F-12 (1:1) media supplemented with 10% fetal calf serum. Cells were incubated with 5% CO_2 at 37°C.

Hepatocytes were prepared from male Sprague–Dawley rats (150–200 g). Animals were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). Perfusion methods were as described by Seglen [1976] and Berry et al. [1991].

Immunofluorescence

For immunofluorescent studies, HepG2 cells and hepatocytes were grown on round 18 mm cover slips. Cells were treated with insulin or left untreated. 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 Chrompure IgG sheep whole molecule (negative control) (Transduction Labs, Bio/Can Scientific, Mississauga, Ontario, Canada), or anti-phospho-rat PKBα (Ser473) (sheep polyclonal IgG), or antiphospho-rat PKBα (Thr308) (sheep polyclonal IgG) or anti-rat PKBα (sheep polyclonal IgG) (UBI, Lake Placid, NY) plasma membrane specific antibody (MAb 7A8) (generously donated by Dr. S. Laferte) was used as a positive control for detection of plasma membrane of hepatocytes. The cells were then incubated for 45 min with FITC-labeled secondary antibody (1:200 dilution) (Transducstion Labs, Bio/Can Scientific, Mississauga, Ontario, Canada). These samples were then mounted onto glass slides using mounting media Citifluor™. Confocal images were collected using a Molecular Dynamic (Sunnyvale, CA) Multiprobe 2000 argon/krypton laser scanning confocal apparatus equipped with a Nikon Diaphot inverted epifluroscence

microscope. Indirect immunofluorescence was performed using Azeiss Axiskop Routine microscope for transmitted and incident-light fluorescence equipped with a Northern Eclipse image analysis software, by Empix Imaging, Inc. (Mississauga, Ontario, Canada).

Preparation of Nuclear and Cytoplasmic Extracts From HepG2 Cells and Primary Hepatocytes

Serum starved HepG2 cells and hepatocytes were treated with insulin for varying incubation times. The medium was aspirated and plates were washed with 10 ml tris buffered saline (TBS) containing 8 g/L NaCl, 0.2 g/L KCl, 3 g/L Tris-HCl (pH 7.4), or with phosphate buffered saline (PBS) solution. Cells were scraped into centrifuge tubes and centrifuged at 1,500*g* for 5 min. The pellet was re-suspended in 1 ml PBS. This solution was now transferred into Eppendorf tubes and a pellet was obtained by centrifugation for 15 s in microfuge. The supernatant was removed and the pellet was re-suspended in 400 μl ice-cold buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF by gentle pipetting. The cells were now allowed to swell on ice for 15 min. To this 25 μl of 10% solution of Nonidet NP-40 was added and vigorously vortexed for 10 s. This homogenate was then centrifuged for 30 s in microfuge. The supernatant was the cytoplasmic extract. Nuclear pellet was re-suspended in 50 μl ice-cold buffer containing 20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EGTA, 1mM EDTA, 1mM DTT, 1 mM PMSF and vigorously rocked for 15 min at 4°C on a shaking platform. The nuclear extract was centrifuged for 15 min in microfuge at 4°C. The supernatant (nuclear extract) were frozen for storage at −70°C. Western blot analysis was performed on these samples. Anti-Sp3 polyclonal antibody was purchased from UBI (Lake Placid, NY), other primary antibodies were the ones mentioned above.

Western Blot Analysis

Aliquots (20 μg of protein for HepG2 cells and 80 μg of protein for primary hepatocytes) were mixed with SDS–PAGE sample buffer and electrophoresed through 8–10% SDS–PAGE gels [Laemmli, 1970] and transferred to nitrocellulose membranes or PVDF membranes. PKBα was detected using polyclonal antibodies towards non-phosphorylated, Ser473, and Thr308 phosphorylated enzyme.

Protein concentrations were determined by the Bradford method [Bradford, 1976].

RESULTS

Immunofluorescent Microscopy

Previously, it has been shown with the aid of transient transfections of different constructs of PKB in HEK 293 cells, that the activation of PKB occurs by phosphorylation of Ser and Thr residues, and its recruitment via its PH domain to the plasma membrane [Andjelkovic et al., 1997; Meier et al., 1997; Kandel and Hay, 1999]. In the present study, we have examined the translocation of endogenous PKBα in unstimulated (control) and insulin stimulated HepG2 cells and normal hepatocytes by immunofluorescent microscopy technique. The location of PKBα was studied using primary antibodies towards non-phosphorylated and phosphorylated (Ser473) forms of the enzyme. Although the identity of PKD2 that phosphorylates Ser473 of PKBα is not known, the phosphorylation of this serine site is important for its translocation upon insulin stimulation. The effects of varying concentrations of insulin as well as incubation time were examined on these cells. Images were examined using a Zeiss Axioskop Routine microscope for transmitted and incident light fluorescence and also using confocal microscope. For negative controls, cells were incubated with Chrompure IgG sheep whole molecule followed by various dilutions of the FITC-conjugated secondary antibody until negligible signal was detected. This dilution (1:200) of secondary antibody was used for subsequent experiments. Antibodies that generated signals above the control were taken as positive signal.

In non-insulin stimulated HepG2 cells, PKBα was mostly found in the nucleus and small amounts in the cytoplasm (Fig. 1A). As a function of insulin concentrations, it was observed that PKBα initially migrated to the nuclear membrane upon stimulation with as little as 0.5 nM insulin. At higher insulin concentrations, PKBα accumulated in the nucleus (data not shown). As a function of incubation time, PKBα was initially translocated to the cytoplasm and then was distributed in the nucleus and the cytoplasm (see 2 and 5 min in Fig. 1A). Under similar conditions using anti-phospho

PKB (Ser473) antibody, we detected minute quantities of phosphorylated PKBα in the nucleus of unstimulated cells (Fig. 1B). After insulin stimulation, the phosphorylated enzyme was initially localized on the nuclear membrane (0.5 min incubation data not shown) followed by its scattering and accumulating within the nucleus (2 and 5 min data shown in Fig. 1B). Some phosphorylated enzymes initially also migrated to the cytoplasm up to 2 min (see 2 min data in Fig. 1B) but after 5 min of incubation with insulin, no phosphorylated enzyme was detected in the cytoplasm (Fig. 1B). These results suggest that in HepG2 cells, upon stimulation with as low as 0.5 nM insulin, PKBα was phosphorylated on Ser473 residue within 0.5 min on the nuclear membrane. After 2 min stimulation with insulin, small amounts of the phosphorylated PKBα is translocated to the cytoplasm (Fig. 1B), but the majority of the phosphorylated enzyme remains within the nucleus after 4–5 min (only 5 min data shown). These observations were confirmed with the aid of confocal microscopy in which a cross section of the cell and clearly defined boundaries of the cytoplasm and the nucleus are visible. As shown in Figure 2A, the phosphorylated (Ser473) PKBα was within the nucleus after 5 min incubation with insulin; results similar to previously seen in Figure 1B. The nucleus of HepG2 cells is quite large as compared to normal hepatocytes, therefore, at 5 μm most of the nucleus is clearly visible.

The effects of insulin on phosphorylation and translocation of PKBα in normal hepatocytes is shown in Figures 3A,B. In contrast to

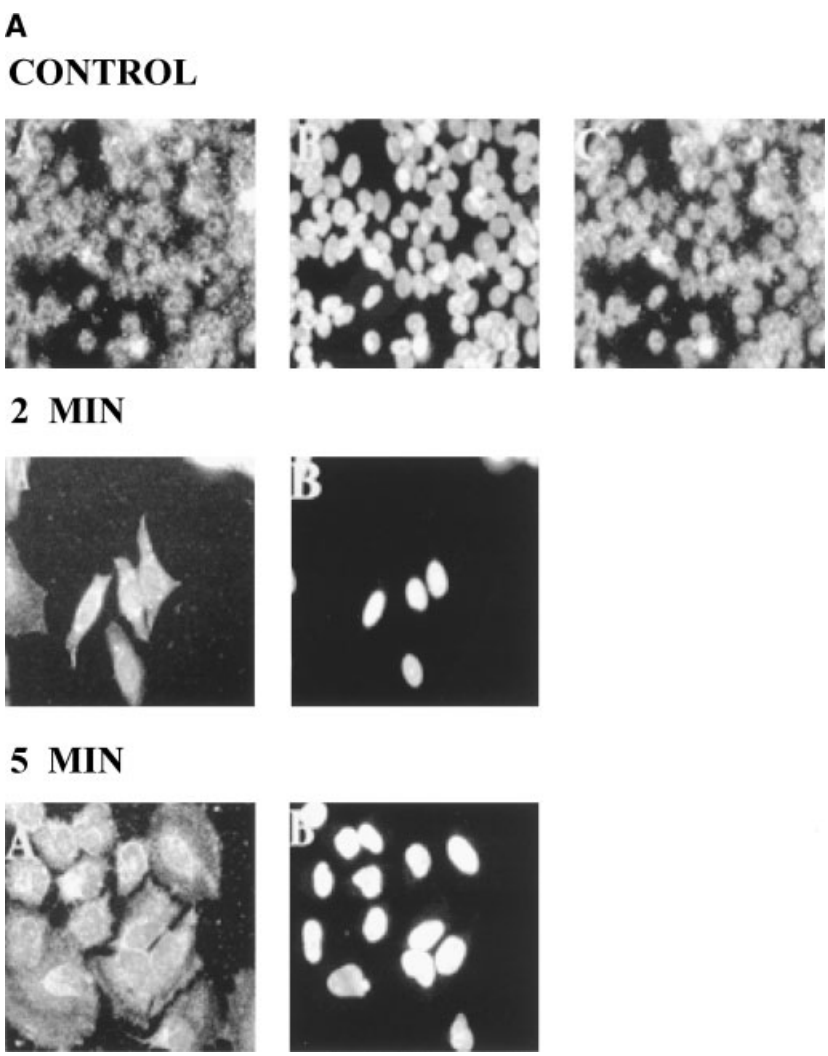

Fig. 1. **A**: Effect of insulin on the subcellular localization of PKBα in HepG2 Cells. Overnight starved HepG2 cells were grown on glass cover slips and treated for 0, 2, and 5 min with 100 nM insulin and prepared for immunofluorescence as described in Materials and Methods section. The subcellular localization of PKBα was visualized by anti-PKBα primary antibody and FITC conjugated secondary antibody (A panels). Propidium iodide or DAPI stain was used to visualize the nuclei of the cells (B panels). Top panel C is a combination of A and B. **B**: Effect of insulin on the subcellular localization of phosphorylated (SERINE 473) PKBα in HepG2 Cells. Overnight starved HepG2 cells were grown on glass cover slips and treated for 0, 2, and 5 min with 100 nM of insulin and prepared for immunofluorescence as described in Materials and Methods section. The subcellular localization of phosphorylated (serine 473) PKBα was visualized by anti-phospho PKBα primary antibody and FITC conjugated secondary antibody (A panels). Propidium iodide or DAPI was used to visualize the nuclei of the cells (B panels).

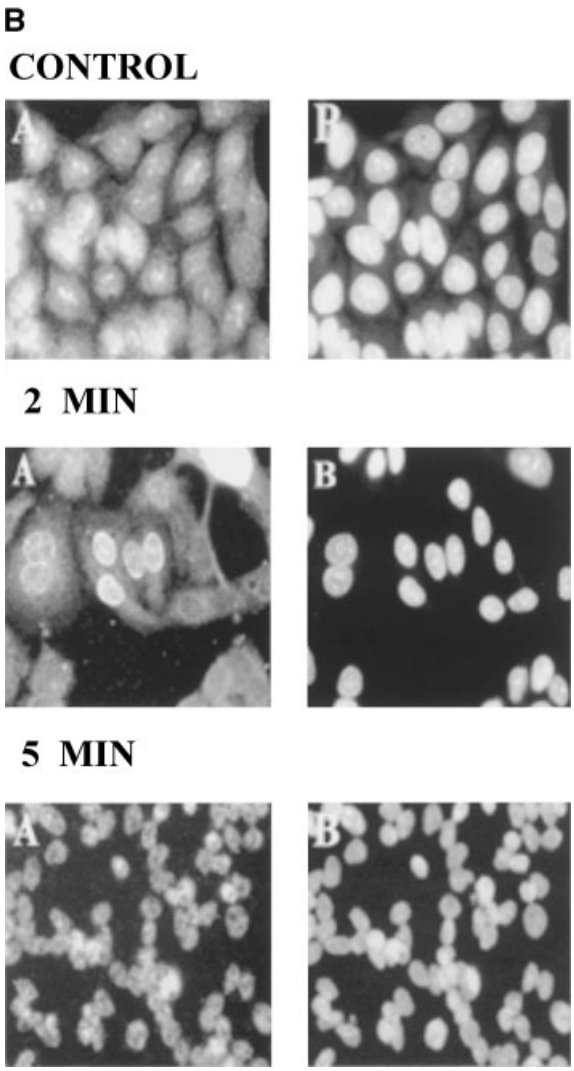

Fig. 1. (*Continued*)

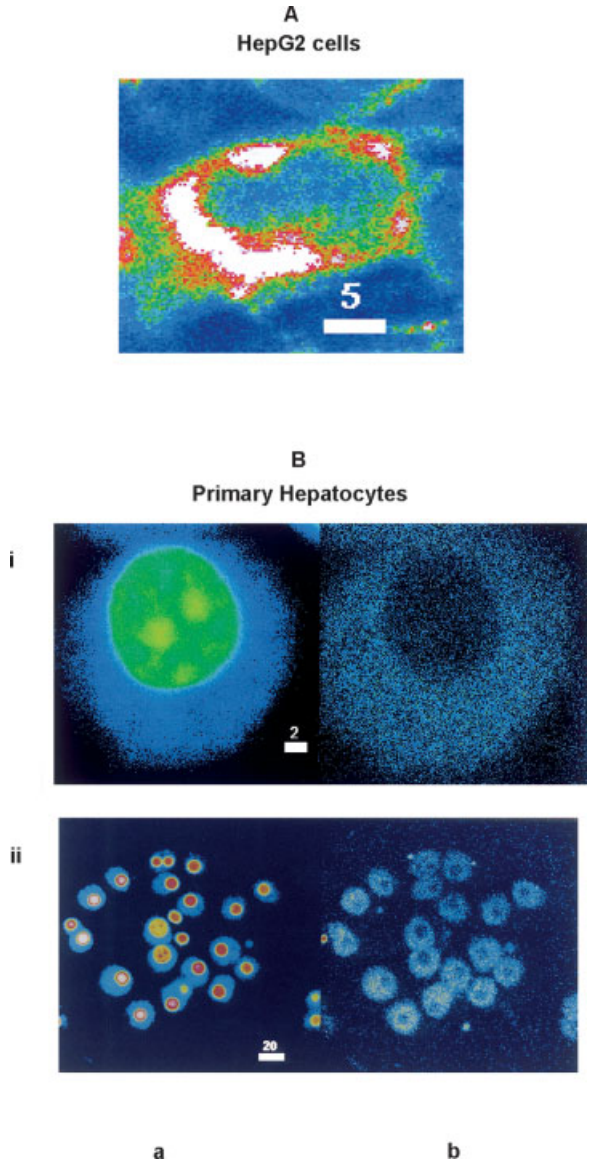

Fig. 2. **A**: FITC confocal image of phosphorylated (serine 473) PKBα in insulin stimulated HepG2 cells. Overnight starved HepG2 cells were treated with insulin for 5 min and prepared for confocal microscopy as described under Materials and Methods. Propidium iodide was excluded in order to avoid interference of this dye. The primary antibody used was anti-phospho-rat PKBα (serine 473). This is one representative photograph of a nucleus. Scale shown is 5 μm. Results show complete nuclear localization of phosphorylated PKB after 5 min stimulation with insulin in HepG2 cells. **B**: FITC confocal image of phosphorylated (serine 473) PKBα in primary hepatocytes. Hepatocytes were isolated and treated with insulin for 5 min and prepared for confocal microscopy as described in Materials and Methods. Panel a shows hepatocytes with propidium iodide and panel b shows FITC stain. i is an expanded and horizontal section of ii. Results showed there is no translocation of phosphorylated PKB into the hepatocyte nucleus.

the HepG2 cells, primary hepatocytes cytoplasmic and nuclear immunostaining demonstrated that non-phosphorylated PKBα was located in the cytoplasmic region in unstimulated cells (Fig. 3A). Upon insulin stimulation, there was an increase in abundance of enzyme at the nuclear and plasma membranes but no translocation into the nucleus was evident. In order to confirm the cytoplasmic/plasma membrane localization of PKBα in hepatocytes, a plasma membrane specific antibody (MAb 7A8) [Prokopishyn et al., 1999] was applied to the hepatocytes and detected with FITC-labeled secondary antibody (Fig. 3B). Similarly, nuclei localization was identified by nuclear staining with propidium iodide (Fig. 3B). Under our experimental conditions, no phosphorylated

PKBα was detected in unstimulated cells, but within 0.5 min of insulin stimulation, phosphorylated PKBα was seen in the cytoplasmic/plasma membrane region up to 3 min (see 0.5 and 2 min images in Fig. 3C). After 2 min of insulin stimulation, considerable amount of the phosphorylated enzyme appeared all over the cell and intensity increased (see 5 min image in Fig. 3C). The phosphorylated PKBα was seen in the nucleus after stimulation of cells with insulin for 5 min. In order to clarify these results seen after 5 min incubation, hepatocytes

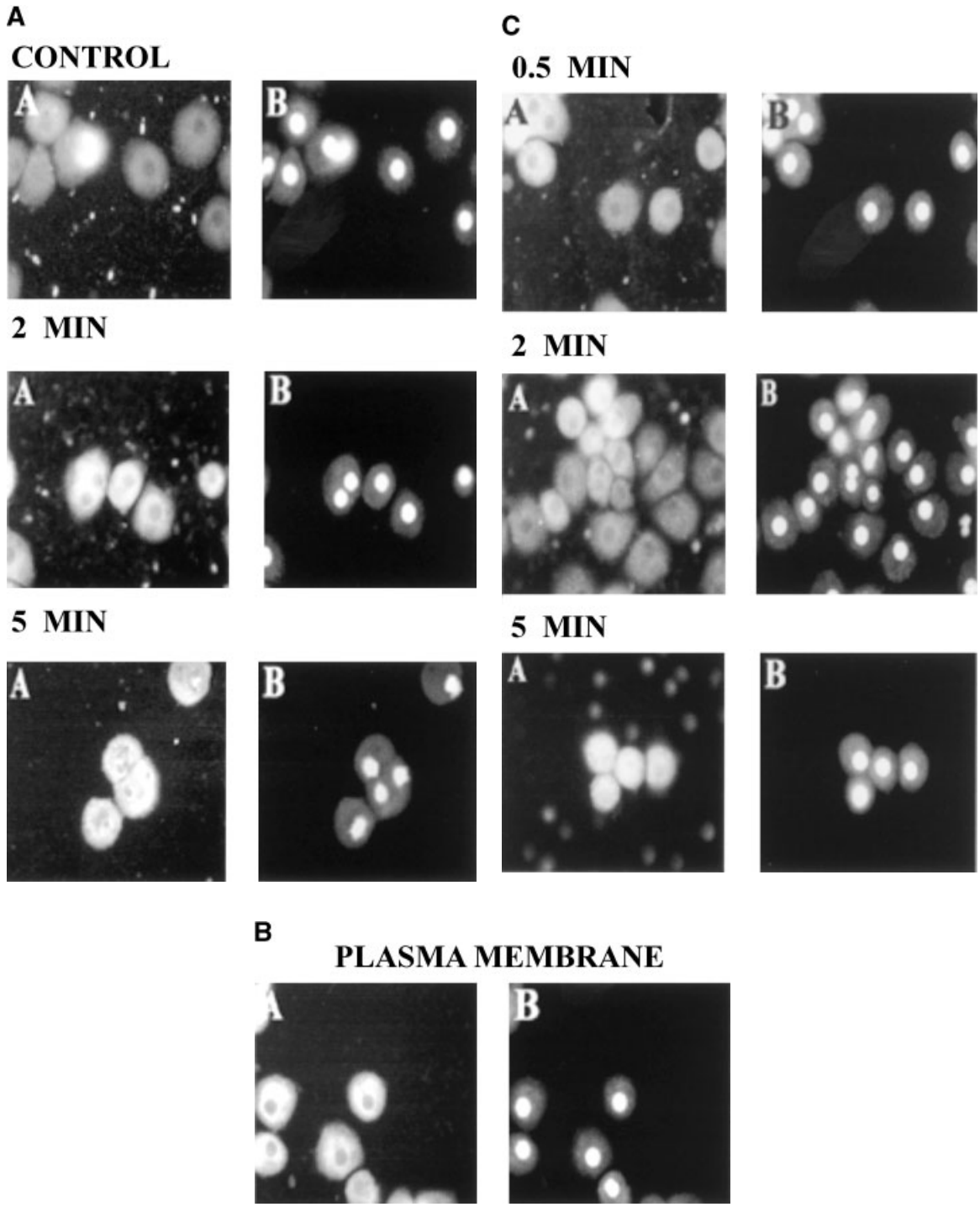

Fig. 3.

stimulated with insulin were examined with a confocal microscope (Fig. 2B). As shown in this figure, the enzyme was not detected in the nucleus but was present around the nuclear membrane.

Amount and Translocation of PKBα in the Cytoplasm and Nucleus in HepG2 Cells and Hepatocytes

The results observed with immunofluorescent microscopy technique were further confirmed with the isolation of cytoplasmic and nuclear fractions and ananlysis of total and phosphorylated form of PKBα by Western blots using anti-PKBα, anti-phospho-PKBα (Ser473), and anti-phospho-PKBα (Thr308) antibodies. As shown in Figure 4A, a specific nuclear protein Sp3 was present in all nuclear fractions but not in the cytoplasmic fractions indicating a clear separation of these two cellular fractions [Lania et al., 1997]. In control HepG2 cells (0 min samples), PKBα was mostly in the nuclear fraction; a small amount was visible in the cytoplasm (Fig. 4B). These results were similar to immunoflourescent studies where PKBα was mainly seen in the nucleus (Fig. 1A,B). After insulin stimulation, a decrease in nuclear fraction and a slight increase in enzymic protein were seen. Under similar conditions, the Ser473 phosphorylated form of the enzyme was only present in the nucleus; no protein was detected in the cytoplasm. In addition, the Thr308 phosphorylated form of PKB was present both in the nucleus and the cytoplasm in unstimulated cells (see 0 min, Fig. 4B); albeit it was more abundant in the nucleus. Upon insulin stimulation, the amount of the Thr308 phosphorylated form of the enzyme gradually increased in the cytoplasm (Fig. 4B).

Similar to the HepG2 cells, the Western blot analysis of total and phosphorylated enzymic-form in the cytoplasmic and nuclear fractions of hepatocytes was also carried out using anti-PKBα, anti-phospho-PKBα (Ser473), and anti-phospho-PKBα (Thr308) antibodies. In the non-stimulated state, PKBα was seen both in nucleus and cytoplasm (Fig. 4C). After stimulation with insulin, a decrease in protein was seen both in the nuclear and cytoplasmic fractions, suggesting that PKBα was translocated to other subcellular fraction(s) probably to the nuclear and plasma membranes. Using anti-phospho-PKBα (Ser473) and anti-phospho-PKBα (Thr 308), no protein could be detected in the nucleus or the cytoplasm. When whole cell lysate was subjected to Western blot analysis, an increase in Thr308 phosphorylated form of the enzyme was observed upon stimulation with insulin. These results in Figures 3A,C and 4C suggest that in normal hepatocytes, PKBα is phosphorylated (on Ser473 and Thr308) at plasma membrane after stimulation with insulin. The precise quantitation of PKBα was not possible but to perform Western blot analysis, 20 μg of cell lysate in HepG2 cells and 80 μg in primary hepatocytes was necessary to see significant results. These data indicate that the expression of PKBα protein in hepatocytes was significantly lower as compared to HepG2 cells.

Based on these data, a proposed scheme for phosphorylation and translocation of PKBα upon insulin stimulation in HepG2 cells and hepatocytes is summarized in Figure 5. In hepatocytes, the enzyme is localized in the cytoplasm and upon insulin stimulation, it is phosphorylated and translocated first to the plasma membrane and then to the nuclear membrane. On the other hand, the both forms

Fig. 3. **A**: Effect of insulin on the subcellular localization of PKBα in primary hepatocytes. Hepatocytes were isolated and grown on collagen coated glass cover slips, starved and treated with 100 nM insulin for 0, 2, and 5 min incubation times, and prepared for immunofluorescence as described in Materials and Methods section. The subcellular localization of PKBα was visualized by anti-PKBα primary antibody and FITC conjugated secondary antibody (A panels). Propidium iodide was used to visualize the nuclei of the cells (B panels). **B**: Localization of the plasma membrane in primary hepatocytes. Hepatocytes were isolated and grown on collagen coated glass cover slips, starved prepared for immunofluorescence using plasma membrane specific antibody (MAb 7A8) as the primary antibody as described in Materials and Methods section. The subcellular localization of α3 integrin subunit on cell surface was detected with FITC conjugated secondary antibody (A panel). Propidium iodide was used to visualize the nuclei of the cells (B panel). **C**: Effect of insulin on the subcelluar localization of phosphorylated (serine 473) PKBα in primary hepatocytes. Hepatocytes were isolated and grown on collagen coated glass cover slips, starved and treated with 100 nM insulin for 0, 2, and 5 min incubation times, and prepared for immunofluorescence as described in Materials and Methods section. The subcellular localization of phosphorylated (serine 473) PKBα was visualized by anti-phospho-PKBα primary antibody and FITC conjugated secondary antibody (A panels). Propidium iodide was used to visualize the nuclei of the cells (B panels). No signal was detected for 0 min incubation and therefore not shown in this figure.

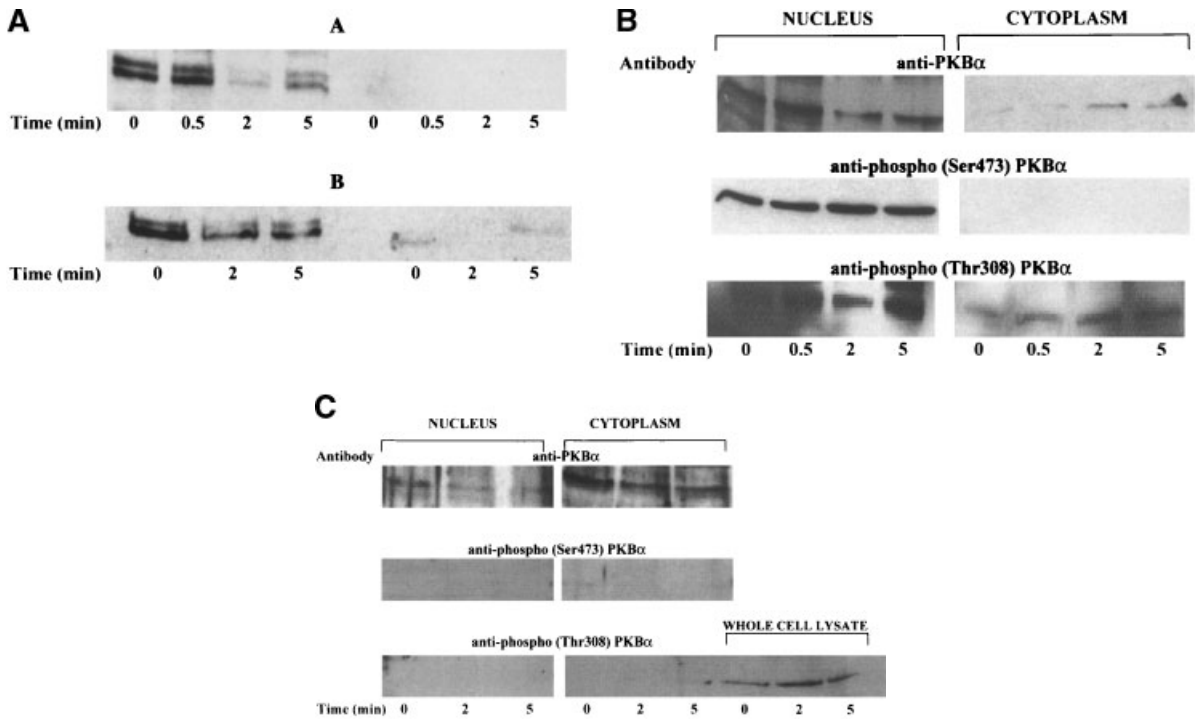

Fig. 4. **A**: Presence of a nucleus specific protein Sp3 in the nucleus and cytoplasm of HepG2 cells and primary hepatocytes. HepG2 cells (A) and hepatocytes (B) were incubated in the presence of 100 nM insulin for different incubation times. The harvested cells were homogenized followed by isolation of nucleus and cytoplasm. These samples were subjected to PAGE and Western blot analysis using anti-Sp3 (rabbit polyclonal IgG) antibody as described in Materials and Methods section. The 60- and 58-kDa bands are shown. **B**: Effect of insulin on PKBα in the nucleus and cytoplasm of HepG2 cells. Overnight-starved HepG2 cells were incubated in the presence of 100 nM insulin for different incubation times. The harvested cells were homogenized followed by isolation of nucleus and cytoplasm. These samples were subjected to PAGE and Western blot analysis using the indicated antibodies as described in Materials and Methods section. **C**: Effect of insulin on PKBα in the nucleus, cytoplasm, and whole cell lysate of primary hepatocytes. Hepatocytes were prepared, starved, and incubated in the presence of 100 nM insulin for different incubation times. The harvested cells were homogenized followed by nucleus and cytoplasm isolation. These samples were subjected to PAGE and Western blot analysis was carried out using the indicated antibodies as described in Materials and Methods section.

(non-phosphorylated and phosphorylated) of the enzyme are localized in the nucleus as well as in cytoplasm. Upon insulin stimulation, the enzyme is further phosphorylated and eventually accumulates in the nucleus. This basic difference in normal versus carcinoma cells might suggest an important role for PKBα in the immortality of cancer cells.

DISCUSSION

Previous in vitro studies have shown that in transfected HEK 293 and COS-1 cells, PKBα is located in the cytoplasm of unstimulated cells and upon stimulation with insulin and IGF-1, it is translocated to the plasma membrane [Andjelkovic et al., 1997]. Following translocation to the plasma membrane, PKB is activated by phosphorylation on Ser473 and Thr308 residues, and then it migrates to the nucleus [Andjelkovic et al., 1997; Alessi and Cohen, 1998; Coffer et al., 1998; Mitsuuchi et al., 1998]. The protein kinases responsible for phosphorylating these residues are thought to be PDK1 and PDK2. PDK1 phosphorylates the Thr308 residue, but the identity of PDK2 that is thought to phosphorylate Ser473 on PKBα is not yet known [Vanhaesebroeck and Alessi, 2000]. Our results with primary hepatocytes show that in these cells, endogenous PKBα exists in the cytoplasm in unstimulated condition, and upon stimulation with insulin it is phosphorylated on Ser473 residue on the plasma membrane. In the absence of insulin, the phosphorylated form of PKBα was not detectable in normal hepatocytes. These results are in agreement with other cells [Ahmed et al., 1993; Meier et al., 1997; Alessi and Cohen, 1998; Coffer et al., 1998; Mitsuuchi et al., 1998], except that we did not

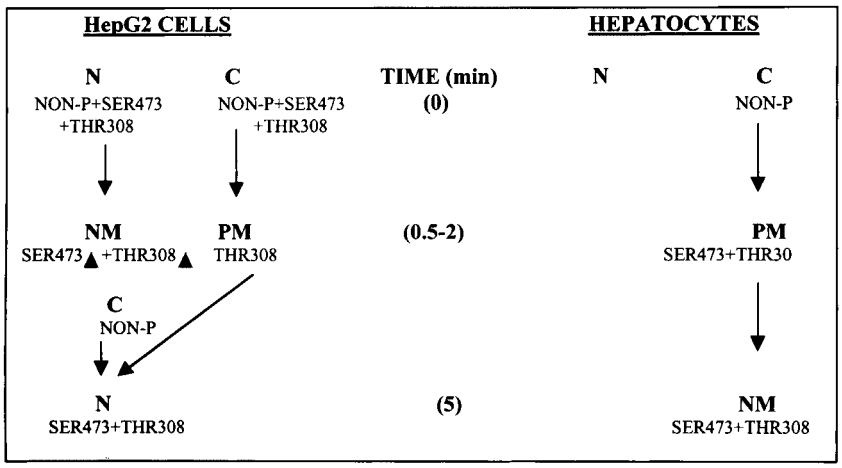

Fig. 5. Proposed mechanism by which insulin effects subcellular localization of PKBα in HepG2 cells and primary hepatocytes. Abbreviations: C, cytoplasm; N, nucleus; NM, nuclear membrane; PM, plasma membrane; ▲, increase in phosphorylation.

observe any translocation of this enzyme to the nucleus within 5 min of incubation time with insulin (Figs. 2C, 3A,C, and 4C). Under similar experimental conditions, PKBα was mainly located in the nucleus and in the phosphorylated form (Ser473) in HepG2 cells (Figs. 1A, B, 2A, and 4B). Furthermore, upon stimulation with insulin, PKBα was translocated and intensity of phosphorylation was increased on the nuclear membrane within 0.5 min (data not shown). The enzyme becomes concentrated in the nucleus after 1 min and the phosphorylated PKBα was predominantly localized within the nucleus after 5 min (Figs. 1B and 2A).

The notable difference in the localization of PKB in the normal hepatocytes and hepatoma cells could partially explain the immortality and transformation state maintained by cancer cells. The presence of phosphorylated PKBα in the nucleus in HepG2 cells indicated that it is constitutively active in cancer cells and may affect transcriptional factors regulating cell survival and proliferation. In our study, PKBα was predominantly localized in nucleus in HepG2 cells. In a previous report in v-akt-transformed mink lung and PA317 cells, c-akt protein product was found to be primarily located in the cytoplasm (90%) and v-akt protein product (myristolated) was dispersed approximately equally in plasma membrane (40%), nucleus (30%), and the cytoplasm (30%) [Ahmed et al., 1993].

In addition to its role in oncogenicity, recently, PKB has been described as an anti-apoptotic kinase and has been shown to be over-expressed in various human cancers [Alessi and Cohen, 1998; Kandel and Hay, 1999; Martelli et al., 1999]. Furthermore, PKB has been labeled as a proto-oncogene because ectopic expression of activated PKB promotes cell survival and transformation [Kandel and Hay, 1999]. Recently it is shown that disruption of PKBα (akt1) causes growth retardation and increased apoptosis in mice [Chen et al., 2001]. Our work further substantiates previous studies that nuclear localized activated PKB in HepG2 cells elicits anti-apoptotic effects.

We have previously shown that insulin phosphorylates and deactivates GSK-3β, and this process is reversed in the presence of wortmannin, an inhibitor of PI-3 kinase [Syed and Khandelwal, 2000]. Previously, it has been suggested that inactivation of GSK-3β by PKB leads to an increase in E2F activity and accumulation of cyclin D1 in the nucleus [Kandel and Hay, 1999]. Activated PKB has also been shown to induce c-*Myc* transcription [Kandel and Hay, 1999]. All of these studies provide further evidence that constitutively activated intranuclear PKB may play an important role in cell cycle progression and prolonged cell survival. In addition, the identity of PDK2 is still unknown, but if PDK2 is the kinase responsible for Ser473 phosphorylation on PKBα, it is possible that an altered and modified form of PDK1 might be present in the nucleus of HepG2 cells [Martelli et al., 1999; Borgatti et al., 2000]. Our results provide an important distinction between normal and cancer cells and a possible mechanism through which transformed hepatoma cells maintain their immortality as compared to normal hepatocytes. The mechanism

for these differences between normal and transformed cells has to be further addressed.

ACKNOWLEDGMENT

This work was supported by an operating grant from CIHR–regional partnership program.

REFERENCES

Aden DP, Fogel A, Plotkin S, Damjanov I, Knowles BB. 1979. Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature 282: 615–616.

Ahmed NN, Franke TF, Bellacosa A, Datta K, Gonzalez-Portal ME, Taguchi T, Testa JR, Tsichlis PN. 1993. The proteins encoded by c-akt and v-ack differ in post-translational modification, subcellular localization and oncogenic potential. Oncogene 8(7):1957–1963.

Alessi DR, Cohen P. 1998. Mechanism of activation and function of protein kinase B. Curr Opin Genet Dev 8:55–62.

Altiok S, Batt D, Altiok N, Papautsky A, Downward J, Roberts TM, Avraham H. 1999. Heregulin induces phosphorylation of BRCA1 through phosphatidylinositol 3-kinase/AKT in breast cancer cells. J Biol Chem 274: 32274–32278.

Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJC, Frech M, Cron P, Cohen P, Lucocq JM, Hemmings BA. 1997. Role of translocation in the activation and function of protein kinase B. J Biol Chem 272:31515–31524.

Berry MN, Edwards AM, Barritt GJ. 1991. Isolated hepatocytes preparation, properties and applications. Amsterdam: Elsevier.

Borgatti P, Martelli AM, Bellacosa A, Casto R, Massari L, Capitani S, Neri LM. 2000. Translocation of Akt/PKB to the nucleus of osteoblast-like MC3T3-E1 cells exposed to proliferative growth factors. FEBS Lett 477(1-2):27–32.

Bradford MM. 1976. A rapid and sensitive method for the quantiation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Chen WS, Xu P, Gottlob K, Chen M, Sokol K, Shiyanova T, Roninson I, Weng W, Suzuki R, Tobe K, Kadowaki T, Hay N. 2001. Growth retardation and increased apoptosis in mice with homozygous disruption of the *akt1* gene. Genes Dev 15:2203–2208.

Coffer PJ, Jin J, Woodgett JR. 1998. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. Biochem J 335:1–13.

Javitt NB. 1990. Hep G2 cells as a source for metabolic studies: lipoprotein, cholesterol and bile acids. FASEB J 4:161–168.

Kandel ES, Hay N. 1999. The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. Exp Cell Res 253:210–229.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

Lania L, Majello B, De Luca P. 1997. Transcriptional regulation of the Sp family of proteins. Int J Biochem Cell Biol 29:1313–1323.

Loh LC, Keeler VD, Shanley JD. 1999. Sequence requirements for the nuclear localization of the murine cytomegalovirus M44 gene product pp50. Virology 259:43–59.

Martelli AM, Capitani S, Neri LM. 1999. The generation of lipid signalling molecules in the nucleus. Prog Lipid Res 38(4):278–308.

Meier H, Hemmings BA. 1999. Regulation of protein kinase B. J Recept Signal Transduct Res 19:121–128.

Meier R, Alessi DR, Cron P, Hemmings BA. 1997. Mitogen activation, phosphorylation, and nuclear translocation of protein kinase Bβ. J Biol Chem 272:30491–30497.

Mitsuuchi Y, Johnson SW, Moonblatt S, Testa JR. 1998. Translocation and activation of AKT2 in response to stimulation by insulin. J Cell Biochem 70:433–441.

Prokopishyn NL, Puzon-McLaughlin W, Takada Y, Laferte S. 1999. Integrin α3β1 expressed by human colon cancer cells is a major carrier of oncodevelopmental carbohydrate epitopes. J Cell Biochem 72:189–209.

Pugazhenthi S, Nesterova A, Sable C, Young P, Heidenreich KA, Boxer LM, Heasley LE, Reusch JE-B. 2000. Akt/Protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. J Biol Chem 275:10761–10766.

Sabbatini P, McCormick F. 1999. Phosphoinositide 3-OH kinase (PI3K) and PKB/Akt delay the onset of p53-mediated, transcriptionally dependent apoptosis. J Biol Chem 274:24263–24269.

Seglen PO. 1976. Preparation of isolated rat liver cells. Methods Cell Biol 13:29–83.

Syed NA, Khandelwal RL. 2000. Reciprocal regulation of glycogen phosphorylase and glycogen synthase by insulin involving phosphatidylinositol-3 kinase and protein phosphatase-1 in HepG2 cells. Mol Cell Biochem 211: 123–136.

Tang Y, Zhou H, Chen A, Pittman RN, Field J. 2000. The Akt proto-oncogene links Ras to Pak and cell survival signals. J Biol Chem 275:9106–9109.

Vanhaesebroeck B, Alessi DR. 2000. The PI3K-PDK1 connection: more than just a road to PKB. Biochem J 346:561–576.