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Overexpression of Akt1 Upregulates Glycogen Synthase Activity and Phosphorylation of mTOR in IRS-1 Knockdown HepG2 cells

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Abstract Insulin receptor substrate (IRS) proteins are important docking proteins in mediating the insulin signaling cascade. We have investigated the effect of short interfering RNA (siRNA) mediated knockdown of IRS-1 on insulin signaling cascade in primary human hepatocellular carcinoma HepG2 cell line and HepG2 cells overexpressing Akt1/PKB-α (HepG2-CA-Akt/PKB). IRS-1 knockdown in both cell lines resulted in reduction of insulin stimulated Akt1 phosphorylation at Ser 473. In parental HepG2 cells, IRS-1 knockdown resulted in reduction (ca. 50%) in the basal level of phosphorylated mTOR (Ser 2448) irrespective of insulin treatment. In contrast, HepG2-CA-Akt/PKB cells showed an upregulation in the basal level of phosphorylated mTOR (Ser 2448) (ca. 40%). Insulin mediated phosphorylation of mTOR was reduced. IRS-1 knockdown also reduced the cell proliferation of parental HepG2 cells by ca. 30% in the presence/absence of insulin, whereas in HepG2-CA-Akt/PKB the cell proliferation was reduced by 15% and treatment of insulin further reduced it to ca. 50% (vs. control). IRS-1 knockdown also reduced the glycogen synthase (GS) activity in parental HepG2 cells, however, it was upregulated in HepG2-CA-Akt/PKB cells. These results suggest that knockdown of IRS-1 abolished basal as well as insulin mediated phosphorylation/activity of proteins involved in cell proliferation or glycogen metabolism in the parental Hep2 cells. IRS-1 knockdown in cells overexpressing constitutively active Akt1/PKB-α either did not change or upregulated the basal levels of phosphorylated/active proteins. However, insulin mediated response was either not altered or downregulated in these cells. J. Cell. Biochem. 103: 1424–1437, 2008.

Key words: IRS-1 knockdown; Akt1 (Ser 473); mTOR (Ser 2448); $p70^{S6K}$ (Thr 389); glycogen synthase; HepG2

Insulin resistance is one of the major implications of type II diabetes. Several factors like TNF-α, serine phosphatase inhibitors, free fatty acids, etc., are known to contribute to insulin resistance in various types of cells [Hotamisligil et al., 1993; Roden et al., 1996; Santomauro et al., 1999; Gual et al., 2005]. Insulin regulates several metabolic processes including glucose transport in muscle and adipose tissue, glycogen synthesis in muscle and liver cells, protein synthesis and mitosis in several cell types. Insulin binds to the α-subunit of insulin receptor (IR) and activates its intrinsic tyrosine kinase activity associated with the β-subunit. IR-β subunit tyrosine kinase phosphorylates insulin receptor substrate (IRS) proteins that mediates signaling to Src-homology (SH2) domains of several proteins like regulatory subunit (p85) of phosphatidylinositol 3-kinase (PI-3 kinase), growth receptor binding protein-2 (Grb-2), Syp, SHC, Crk, and Nck [Ueno et al., 2000; Valverde et al., 2001; Hers et al., 2002; Asano et al., 2005]. Binding of IRS to regulatory subunit (p85) subunit of PI-3 kinase stimulates its catalytic subunit (p110) which catalyzes the phosphorylation of phosphatidylinositol 4,5-diphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 is the secondary messenger that binds to pleckstrin homology domain of Akt/PKB leading to change in its conformation [Alessi et al., 1997] and renders it active by facilitating phosphorylation at Thr 308 and Ser 473 through phosphatidylinositide dependent kinases (PDK) 1 and recently demonstrated mammalian target of rapamycin (mTOR) complex with rictor [Sarbassov et al., 2005], respectively. Akt/PKB regulates several important

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cellular activities like glucose homeostasis, protein synthesis, cell survival, etc.

Among four isoforms of IRS proteins that have been identified based on tissue distribution, subcellular localization and binding to SH2 domain, IRS-1 and IRS-2 proteins are most widely expressed. IRS-3 is restricted to adipose tissue and IRS-4 is expressed in thymus, brain, and kidney. Although IRS-1 and IRS-2 proteins have structural and functional similarities they activate distinct cellular responses. Using IRS-1 and IRS-2 knockout animal models, IRS-1 has been shown to play a major role in IGF-1 induced mitosis; whereas IRS-2 plays a major role in glucose homeostasis. Both IRS-1 and IRS-2 double knockout mice become insulin resistant and hyperinsulinemic. IRS-1 deficient mice are smaller (50–60%) in size and also display decreased glucose metabolism [Araki et al., 1994; Tamemoto et al., 1994; Patti et al., 1995; Kadowaki et al., 1996; Ma et al., 2006]. Although, these IRS-1 deficient mice develop insulin resistance, they do not develop diabetes as they have normal pancreatic β-cells. IRS-2 deficient mice are normal in size but they develop the phenotype of type II diabetes due to insulin resistance and β-cell failure along with defects in the brain [Withers et al., 1998].

IRS-1 is a docking protein that modulates the activities of Akt/PKB through the PI-3 kinase pathway. Our interest was to investigate the effect of IRS-1 knockdown on the insulin mediated phosphorylation of Akt/PKB, proteins regulating cell proliferation (mTOR and p70 S6 kinase ($p70^{S6K}$)) along with proteins regulating glycogen metabolism (glycogen synthase (GS) and glycogen synthase kinase-3β (GSK-3β)). IRS-1 was knocked down in both parental HepG2 as well as cells overexpressing constitutively active Akt/PKB (HepG2-CA-Akt/PKB) cells using IRS-1 specific short interference RNA (siRNA). IRS-1 knockdown resulted in reduction in the insulin mediated phosphorylation of Akt/PKB at Ser 473 in parental HepG2 as well as HepG2-CA-Akt/PKB cells. Phosphorylation of GSK-3β at Ser 9 and $p70^{S6K}$ at Thr 389 also showed insulin resistance in IRS-1 deficient parental HepG2. However, IRS-1 deficiency in cells overexpressing Akt/PKB showed an upregulation in the basal levels of phosphorylated mTOR (Ser 2448) but no alteration in its downstream target $p70^{S6K}$ (Thr 389). IRS-1 deficient HepG2-CA-Akt/PKB cells showed a decrease/inhibition in the insulin mediated stimulation of mTOR and $p70^{S6K}$ phosphorylation. On the contrary, the parental HepG2 cells showed loss in both basal as well as insulin mediated phosphorylation of mTOR and $p70^{S6K}$, enzymes important role for the regulation of protein synthesis and cell proliferation. Further, there was a decrease in the cell proliferation of both parental HepG2 as well as HepG2-CA-Akt/PKB cells; albeit there was relatively higher reduction in parental cells. In addition a differential behavior of IRS-1 knockdown in the glycogen synthase (GS) activity in parental HepG2 and HepG2-CA-Akt/PKB cells was observed. IRS-1 knockdown in parental HepG2 cells showed a ca. 50% decrease in basal GS activity as well as in its stimulation by insulin. In contrast, in IRS-1 knockdown HepG2-CA-Akt/PKB cells there was an upregulation of both basal as well insulin stimulated GS activities.

MATERIALS AND METHODS

Materials

DMEM/F-12, fetal bovine serum (FBS) and antibiotic–antimycotic, geneticine were procured from Gibco, Invitrogen (Ontario, Canada). IRS-1 specific siRNA, scrambled siRNA and DharmaFECT4 were obtained from Dharmacon, Inc., RNA Technologies (Lafayette, CO). Protease inhibitor cocktail for mammalian cell culture, human recombinant insulin, bovine serum albumin, rapamycin from *Streptomyces hygroscopicus* and thiazolyl blue tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Ontario, Canada). PVDF membrane was purchased from Bio-Rad Lab (Ontario, Canada). Antibodies against p-Akt1/PKB-α (Ser 473), Akt total, p-mTOR (Ser 2448), mTOR total and rictor were procured from Cell Signaling Technology (MA). IRS-1, IRS-2, p-$p70^{S6K}$ (Thr389), GSK-3β (Ser 9), GSK-3β total and goat anti rabbit IgG HRP were obtained from Santa Cruz, Biotechnology, Inc. (CA). Antibodies for carrying out immunofluorescence work, mouse monoclonal anti-p-Akt1/PKB-α (Ser 473) (clone 11E6)-Alexa Fluor 488 conjugate was procured from Upstate Cell Signaling Solutions (CA), rabbit monoclonal anti-p-mTOR (Ser 2448) (49F9) was obtained from Cell Signaling Technology and Alexa Fluor 594 conjugated goat anti-rabbit IgG was obtained from Invitrogen-Molecular Probes (Ontario, Canada). Fluorescence mounting media was

purchased from Dako (Denmark). Chemiluminescence reagent was obtained from Perkin–Elmer (MA). Uridine diphospho-D-[$U^{14}C$]glucose was purchased from Amersham Biosciences UK limited. All the other chemicals and reagents were of analytical grade obtained from Sigma-Aldrich.

Methods

Cell culture. HepG2 cells were cultured in DMEM (pH 7.4) supplemented with FBS (10%) and antibiotic–antimycotic (1%). Cells were incubated in a CO_2 incubator (Fischer Scientific, Ottawa, Ontario, Canada) maintained at 37°C with humidified air and 5% CO_2. HepG2 cells overexpressing constitutively active Akt1/PKB-α (HepG2-CA-Akt/PKB) were generated as reported elsewhere [Gupta and Khandelwal, 2004]. HepG2-CA-Akt/PKB were grown in DMEM supplemented with 10% FBS and 1% antibiotic–antimycotic under the selection pressure of 0.1 mg/ml geneticine.

Transfection. HepG2 cells and HepG2-CA-Akt/PKB were grown up to 80% confluency in 100 mm culture plates. Cells/ml (1×10^5) were counted and seeded in 60 mm tissue culture plates in DMEM and FBS (10%). Human IRS-1 (NM005544) specific siRNA was a mixture of four different RNA sequences (AAAGAGGUCUGGCAAGUGA; GAACCUGAUUGGUAUCUAC; CCACGGCGAUCUAGUGCUU; GUCAGUCUGUCGUCCAGUA). siRNA transfection was carried out using DharmaFECT4 as per supplier's instructions. Control cells were transfected with scrambled siRNA. In tube 1 siRNA (2 μM) was diluted with advanced MEM and tube 2 dharmaFECT4 was diluted with advanced MEM and both the tubes were incubated for 5 min at room temperature. The contents of tubes 1 and 2 were mixed and incubated for 20 min at room temperature. The cell plates were washed with PBS and the siRNA mixture was diluted with DMEM supplemented with 10% FBS. The cell plates were incubated at 37°C with humidified air and 5% CO_2 for 24, 48, and 72 h. At each time point the cells were treated with and without insulin (10 nM) for 10 min. The cells were next washed in cold phosphate buffered saline (PBS) and lysed in a 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 and freshly added 1 mM phenyl methyl sulphonyl fluoride, 1% protease inhibitor cocktail for mammalian cell culture for 10 min on ice bath. The cells were scraped from the plate and lysates were kept on ice bath for 30 min. The lysates were spun at 13,000 rpm for 10 min, the pellet was discarded and the supernatant was stored at −20°C for future use.

Western blot analysis. Western blots analysis were carried out according to method developed by Towbin et al. [1992]. Aliquots of protein (20 μg) were subjected to SDS–PAGE [Laemmli, 1970; Sambrook et al., 1989] and were transferred on blotting grade PVDF membrane. The membrane was incubated with 5% nonfat dry milk dissolved in 1X 0.02% PBS-Tween-20 (milk-PBST) for 1 h at room temperature in order to block the nonspecific sites on the membrane. Blots were probed with primary antibodies against IRS-1, IRS-2, p-Akt (Ser 473), p-GSK-3β (Ser 9), p-mTOR (Ser 2448) or p-$p70^{S6K}$ (Thr 389), in milk-PBST, for overnight at 4°C. The membrane was then washed in PBST three times for 10 min each followed by incubation with suitable secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. The membrane was washed in PBST three times for 10 min each; visualization of hybridization was carried out using chemiluminescence's reagent.

MTT assay. MTT assay was used to measure the cell proliferation [Mossman, 1983]. The assay provides a simple method of detecting growing and viable cells. Pale yellow colored MTT is converted to purple colored aqueous insoluble formazan crystals by active mitochondrial succinate dehydrogenase. Formazan crystals were solubilized in DMSO and the absorbance at 570 nm was measured. Equal numbers of cells were seeded in a flat bottomed 96-well plates. Transfection of IRS-1 and IRS-2 siRNA was preformed as described in Transfection Section. The plates were incubated for 48 h with/without insulin (10 nM). This was followed by the addition of MTT (5 mg/ml) 15 μL/well and the plate was incubated for 3 h at 37°C, 5% CO_2 and humidified air. The wells were decanted and the formazan crystals formed were dissolved in DMSO. The absorbance of the plate was taken at 570 nm in an ELISA plate reader (Spectra Max 340PC from Molecular Devices Corporation, CA).

Glycogen Synthase Assay. GS assay was carried out as described by Thomas et al. [1973]. [^{14}C]glucose incorporation from uridine diphospho-D-[U^{14}C]glucose into glycogen was measured. Cells/ml (1×10^5) were counted and seeded. Transfection of scrambled or IRS-1 specific siRNA were performed as described in the Transfection Section. The plates were incubated for 48 h in a CO_2 incubator maintained at 37°C with humidified air. Cells were treated with or without insulin (10 nM) for 10 min. After the treatments the cell plates were washed with cold 1X PBS and lysed in a GS lysis buffer comprising of 50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 100 mM NaCl, 50 mM NaF, 1 mM microcycstin-LR, 1% Nonidet P-40, and protease inhibitor cocktail. Cells were scraped and spun at 10,000 g for 10 min, the pellet was discarded and the supernatant was used for GS activity measurements. The assay mix for GS_{total} activity consisted of 50 mM Tris (pH 7.4), 10 mM UDPG, 2% glycogen, 5 mM EDTA, and 10 mM glucose-6-phosphate. The assay mix for GS_{active} activity, glucose-6-phosphate was replaced by 28 mM Na_2SO_4. The specific radioactivity of UDPG used in the reaction mixture was 400 cpm/nmol. GS assay was initiated by the addition of 50 μl assay mix into the 50 μl lysate (enzyme solution) at 30°C for 2 h. The enzyme reaction was terminated by spotting 75 μl of the reaction mixture on Whatmann 31ET paper (2 × 2 cm), the paper was allowed to dry and was washed in cold ethanol (66%) two times, 30 min each. The papers were dropped in acetone for 10 min followed by drying and counted in a toluene based scintillant containing 0.4% PPO and 0.02% POPOP. One unit of GS activity is defined as the amount of enzyme that incorporated 1 nmol of [^{14}C glucose] from UDP [^{14}C]glucose into glycogen min^{-1}.

Immunofluorescence. HepG2 and HepG2-CA-Akt/PKB cells were seeded in Falcon culture slides and transfected with scrambled or IRS-1 specific siRNA as described in the Transfection Section, the slides were incubated for 48 h followed by treatment without or with insulin (10 nM) for 10 min. The cells were washed in 1 ml PBS with 1 mM sodium azide (PBS-azide) three times followed by fixing in 0.5 ml 4% paraformaldehyde (pH 7.4) for 30 min at 25°C, this was followed by washing with PBS-azide three times. The cells were next permiablized using 0.5 ml of 0.05% saponin; the excess reagents were washed in PBS-azide three times. Primary antibodies against p-Akt1/PKB-α (Ser 473) conjugated with Alexa Fluor 488 and p-mTOR (Ser 2448) were incubated overnight followed by washing in PBS-azide supplemented with 1% BSA (PBS-BSA) five times, with the last wash for 20 min. All the subsequent steps were carried out in dark; the cells were incubated with Alexa Fluor 594 conjugated anti rabbit IgG secondary antibody for 2 h followed by washing in PBS-BSA three times. Coverslips were mounted using fluorescence mounting media. Carl-Zeiss LSM410, confocal microscope at Structural Sciences Center, University of Saskatchewan, Saskatoon, SK, Canada was used to take the immunofluorecence pictures. Confocal micrographs of p-Akt (Ser 73) were green (488 nm) and were taken using an emission filter of 510–560 nm and p-mTOR was red (594 nm) and the emission filter used was 609–662 nm.

Other methods. Proteins were estimated according to Bradford's [1976] method. NIH image software (version 1.62) was used to determine the band intensities of the Western blots. Statistical analysis was performed using Student's *t*-test.

RESULTS

Establishing IRS-1 Knockdown Cell Lines

HepG2 cells with constitutively active Akt/PKB (HepG2-CA-Akt/PKB) were generated as described elsewhere [Gupta et al., 2004]. Parental HepG2 as well as HepG2-CA-Akt/PKB cells were transfected either with IRS-1 specific or scrambled siRNA for 24, 48, and 72 h (Fig. 1). There was ca. 80% knockdown in the IRS-1 protein in both parental HepG2 and HepG2-CA-Akt/PKB cells at 24 h. More than 90% knockdown of IRS-1 was observed at 48 and 72 h in parental HepG2 cells (Fig. 1A,C) as well as in HepG2-CA-Akt/PKB cells (Fig. 1B,D). This treatment resulted in no significant change in IRS-2 levels in parental HepG2 cells (Fig. 1B,D) as well as HepG2-CA-Akt/PKB cells (Fig. 1B,D).

Effect of IRS-1 Knockdown on the Active/Phosphorylated Akt1 (Ser 473)

In control parental HepG2 cells, a time-dependent increase in the phosphorylated Akt (Ser 473) with a maximal peak at 48 h was observed. As expected, insulin caused an

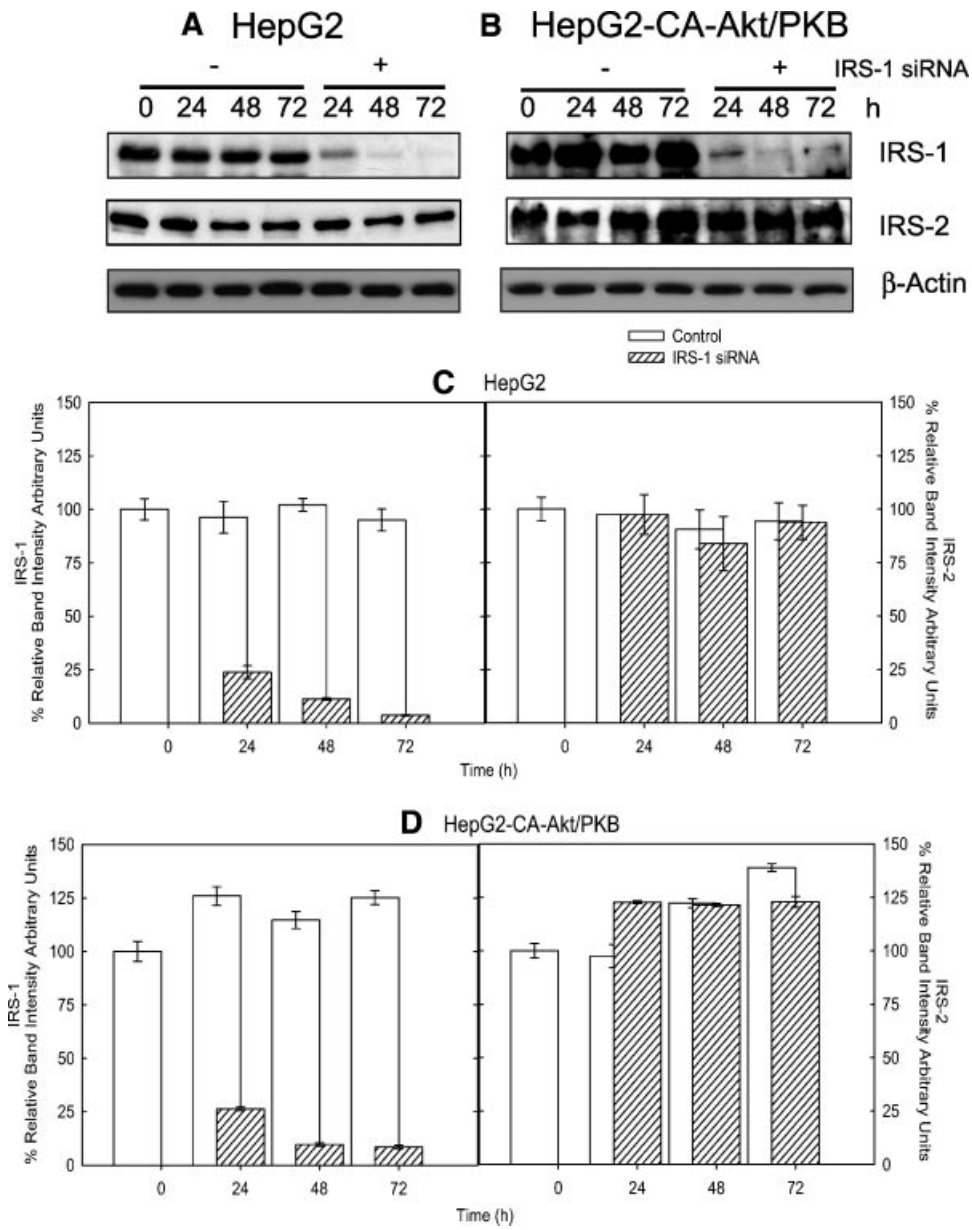

Fig. 1. IRS-1 knockdown in HepG2 and HepG2-CA-Akt/PKB cells using short interference RNA (siRNA). Cells were transfected with either scrambled or IRS-1 specific siRNA for 24, 48 and 72 h and lysates for Western blot analysis were prepared as described in the Materials and Methods Section. Aliquots of 20 μg protein were used for Western Blot analysis with IRS-1 and IRS-2 antibodies. A typical Western blots are shown in (**A**,**B**), β-actin shows the protein loading. Quantified relative band intensities are shown in (**C**,**D**), the bars represent an average of three experimental readings, the error bars represents ± standard deviation. In each case control cells at 0 h (no treatment) were taken as 100%.

increase in the phosphorylation of Akt1 at Ser 473 at all time points (Fig. 2A,C). The knockdown of IRS-1 in these cells almost abolished the insulin mediated increase in the phosphorylation of Akt1 at Ser 473.

In control HepG2-CA-Akt/PKB cells, the levels of phosphorylated Akt1 (Ser 473) were increased upon insulin treatment. The IRS-1 knockdown in HepG2-CA-Akt/PKB cells showed no significant change in the phosphorylated Akt1 levels. Insulin mediated phosphorylation of Akt1 in these cells was also abolished in IRS-1 siRNA transfected cells (Fig. 2B,D). No significant changes in the total Akt under all experimental conditions was observed in both the cell lines studied (Fig. 2).

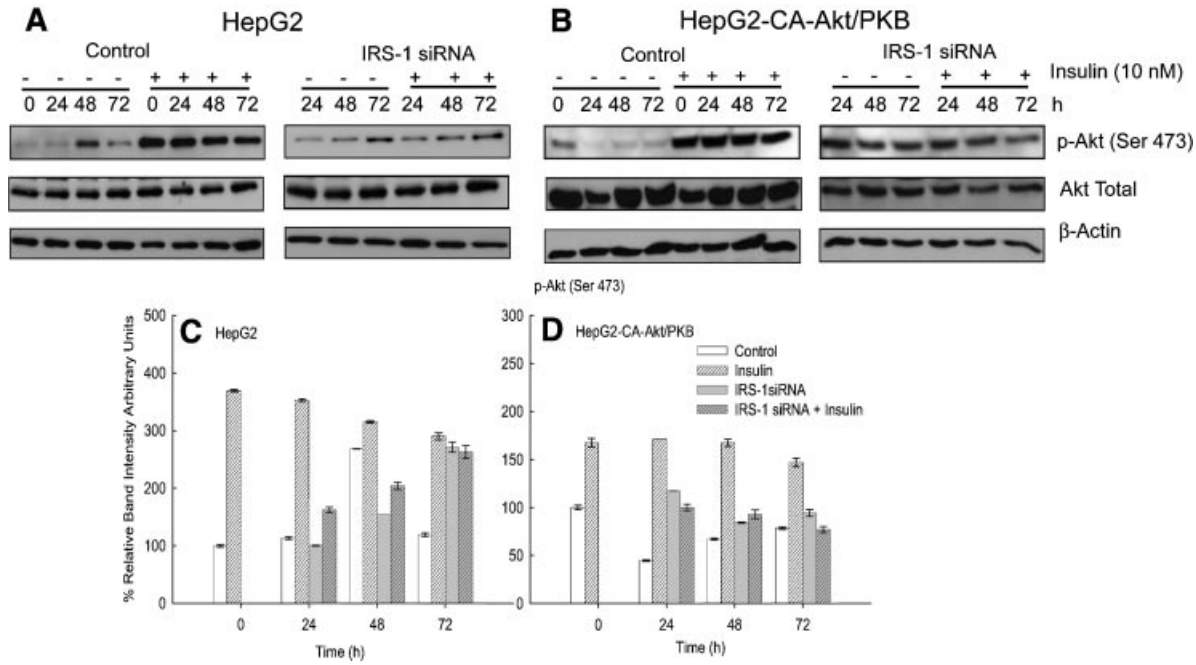

Fig. 2. IRS-1 knockdown results in decreased insulin stimulated Akt1 phosphorylation. Cells plates of 80% confluence were used, 1×10^5 cells/ml were counted and allowed to fix. These cells were used for transfection with IRS-1 specific siRNA for 24, 48, and 72 h with/without insulin (10 nM) treatment for 10 min. The cells were lysed and Western blot analysis was carried out as described in the Materials and Methods Section. The blots were probed with p-Akt (Ser 473), Akt total and β-actin. (**A**,**B**) Shows the Western blots, (**C**,**D**) shows the relative bands intensities of the Akt (Ser 473). The white bars represent the controls and gray bars represent the IRS-1 knockdown cell lines. The unfilled bars represent no insulin treatments and crossed bars represent the insulin treated samples. Each bar represents an average of three readings and error bars represents ± standard deviation. Controls at 0 h were taken as 100% in each case.

IRS-1 Knockdown in HepG2-CA-Akt/PKB Cells Causes Upregulation of p-mTOR (Ser 2448) and Rictor Without Affecting the Levels of p-p70^{S6K} (Thr 389)

mTOR and p70^{S6K} are protein kinases regulated by Akt involved in the regulation of cell proliferation/protein synthesis. It is also been demonstrated that mTOR complex with rictor causes the phosphorylation of Akt at Ser 473. Therefore, we investigated the effect of IRS-1 knockdown on the phosphorylated levels of these two protein kinases and rictor in parental HepG2 as well as HepG2-CA-Akt/PKB cells. For these experiments, a time point of 48 h IRS-1 siRNA transfection was chosen as almost complete knockdown of IRS-1 levels at this time point was observed in both types of cells.

In control parental HepG2 cells, there was an increase (ca. 20%) in the phosphorylation of mTOR (Ser 2448) in the presence of insulin. As seen in the case of phosphorylated Akt1, knockdown of IRS-1 in parental HepG2 cells also caused a decrease (ca. 50%) in the phosphorylated levels of mTOR and no stimulation by insulin was observed (Fig. 3A,C). Total mTOR levels remained unaltered under all experimental conditions. The levels of rictor, upon transfection with IRS-1 siRNA in parental HepG2 cells also showed a decrease both in the presence/absence of insulin. Control HepG2-CA-Akt/PKB cells showed an increase (ca. 70%) in the insulin mediated phosphorylation of mTOR (Ser 2448). The knockdown of IRS-1 in these cells slightly upregulated basal levels of mTOR phosphorylation (ca. 40%). Insulin caused an inhibition in the mTOR phosphorylation and a decrease in the levels of phosphorylated mTOR (Fig. 3B,C) in these cells; the total mTOR levels were unaltered. IRS-1 knockdown in HepG2-CA-Akt/PKB cells showed no significant changes in the levels of rictor, however, treatment of insulin resulted in a decrease.

Similar trends in the levels of phosphorylated p70^{S6K} (Thr 389) was also observed. In control parental HepG2 cells, there was an increase (ca. 30%) in the insulin mediated phosphorylation of p70^{S6K} at Thr 389 (Fig. 3A,D). IRS-1 knockdown in parental HepG2 cells caused a decrease in the

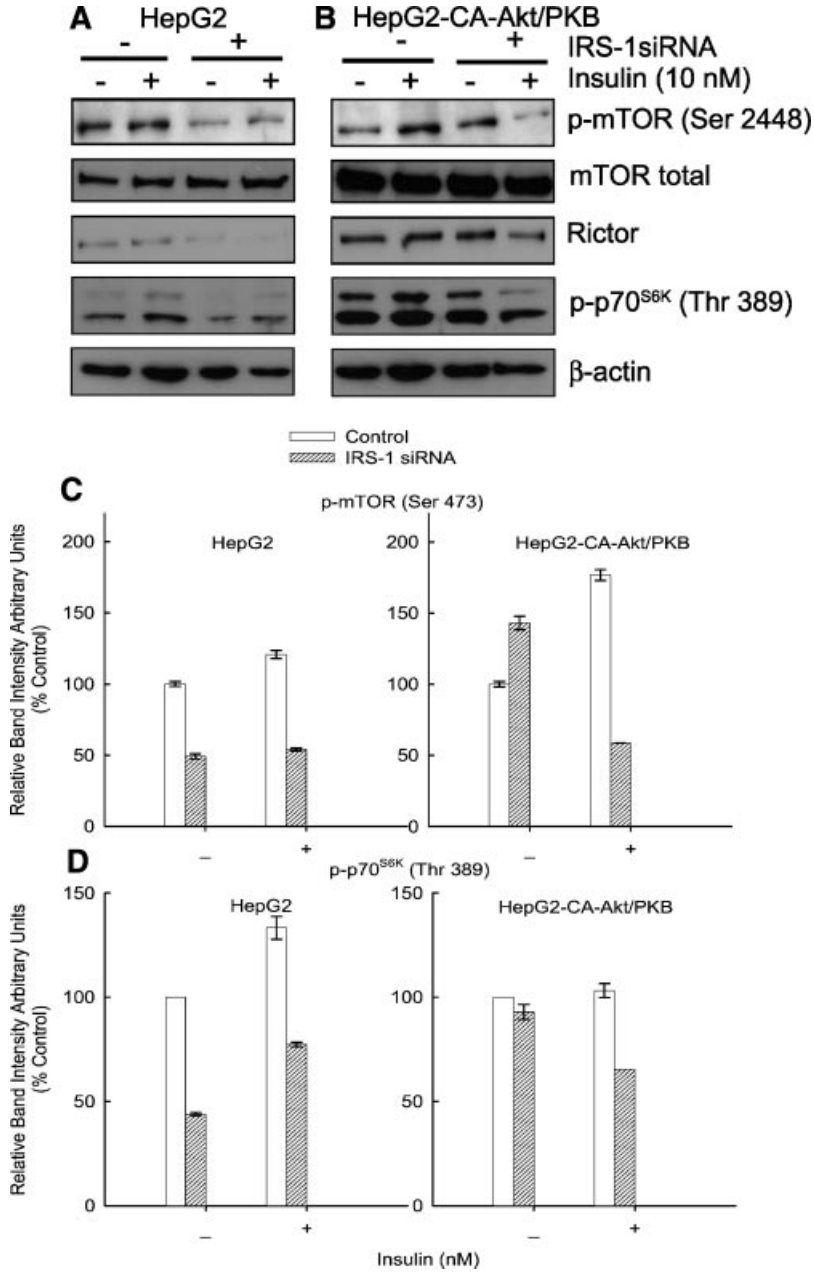

Fig. 3. Effects of IRS-1 knockdown on the phosphorylation of mTOR (Ser 2448) and p70^{S6K} (Thr 389): Parental HepG2 and HepG2-CA-Akt/PKB cells were transfected with IRS-1 specific siRNA for 48 h, lysates were made and Western blot analysis was carried out as described in the Materials and Methods Section. (**A**,**B**) Shows the Western blots probed against p-mTOR (Ser 2448), p-p70^{S6K} (Thr 389), rictor and β-actin. Figures (**C**,**D**) represent the relative band intensities of p-mTOR (Ser 2448) and p-p70^{S6K} (Thr 389). Each bar is an average of three readings and the error bars represents ± standard deviation. Controls were taken as 100%.

phosphorylation of p70^{S6K} but insulin mediated stimulation was maintained. On the other hand, HepG2-CA-Akt/PKB cells showed no significant change in the basal levels of phosphorylated p70^{S6K} upon IRS-1 knockdown, however, insulin caused a decrease (ca. 35%) in its levels (Fig. 3B,D).

IRS-1 Knockdown Causes Reduction in Cell Proliferation in Both the Cell Lines

Akt/mTOR/p70^{S6K} pathway regulates cell proliferation/survival pathway. Therefore, we investigated the effects of IRS-1 knockdown (48 h) on cell proliferation in both cell lines (Fig. 4).

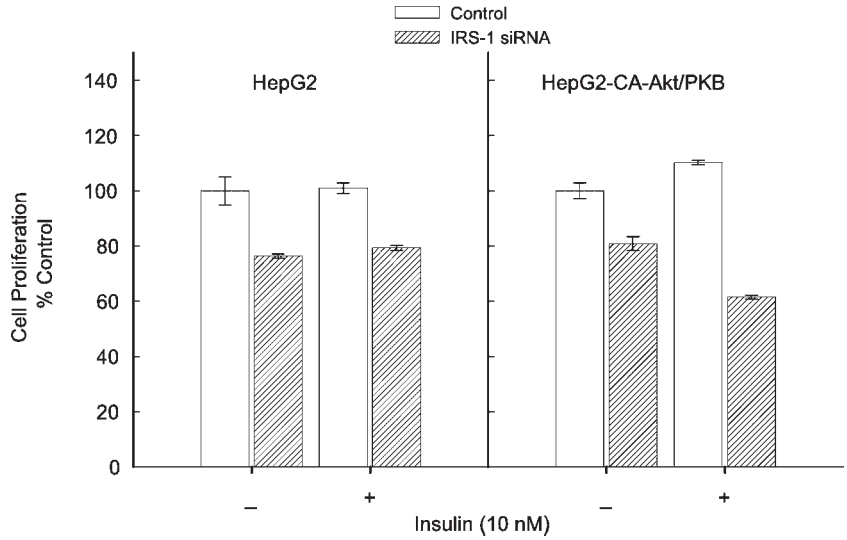

Fig. 4. Effect of IRS-1 knockdown on the cell proliferation of parental HepG2 and HepG2-CA-Akt/PKB cells. Cells were seeded in 96-well plate and siRNA (scrambled or IRS-1) was transfected for 48 h in the presence and absence of insulin (10 nM) and cell proliferation was measured by MTT assay as described in the Materials and Methods Section. Control cells, transfected with scrambled siRNA were taken as 100%. Each bar is an average of three experiments and the error bars represents ± standard deviation.

There was a ca. 30% decrease in cell proliferation upon IRS-1 knockdown in parental HepG2 cells. This decrease in cell proliferation was not altered by insulin treatment (Fig. 4A). IRS-1 knockdown in HepG2-CA-Akt/PKB cells showed a ca. 15% decrease in the cell proliferation and this was further decreased to ca. 50% in the presence of insulin (Fig. 4B). These results on cell proliferation correlated with the phosphorylated levels of mTOR and $p70^{S6K}$ in IRS-1 knockdown in both parental HepG2 and HepG2-CA-Akt/PKB cells.

IRS-1 Knockdown in Cells Expressing Varying Levels of Akt1 Leads to Differential Regulation of Glycogen Synthase Activity

Next we investigated the effects of IRS-1 knockdown on the GS activity. GS is a key enzyme for the synthesis of glycogen. Control parental HepG2 cells showed a two-fold increase in the insulin mediated stimulation of GS activity (Fig. 5A). IRS-1 knockdown caused a decrease (ca. 60–70%) in the GS activity, both in the absence/presence of insulin. GS activity in HepG2-CA-Akt/PKB cells was ca. 75% higher than the parental HepG2 cells and insulin only showed a slight activation (Fig. 5A). IRS-1 knockdown in these cells resulted in significant increase in GS activity but insulin mediated activation was abolished (Fig. 5A).

We further investigated whether changes in GS activity are mediated through alterations in GSK-3β activity. GSK-3β provides a link between Akt and GS activity. As shown in the Figure 5B, IRS-1 knockdown of parental HepG2 cells did not alter the levels of phosphorylated GSK-3β (Ser 9) but insulin mediated effects were abolished. This was in contrast with results obtained for GS activity. In HepG2-CA-Akt/PKB cells the knockdown of IRS-1 did not alter the GSK-3β phosphorylation. Insulin stimulated the phosphorylation of GSK-3β in control HepG2-CA-Akt/PKB cells but a decrease in the phosphorylated levels of GSK-3β (Ser 9) was observed in IRS-1 knockdown cells. In this case also no correlation between the GSK-3β phosphorylation and GS activity was observed. The levels of total GSK-3β were unaltered under all experimental conditions (Fig. 5B). This might indicate that there may be some other molecule(s)/pathway is/are involved in the regulation of GS in siRNA mediated knockdown cells.

Translocation of Phosphorylated Akt1 and mTOR in IRS-1 Knockdown Cells

Confocal micrographs of the parental HepG2 and HepG2-CA-Akt/PKB cells were further examined to study the localization in phosphorylated Akt1 and mTOR. Both p-Akt1 (Ser 473)

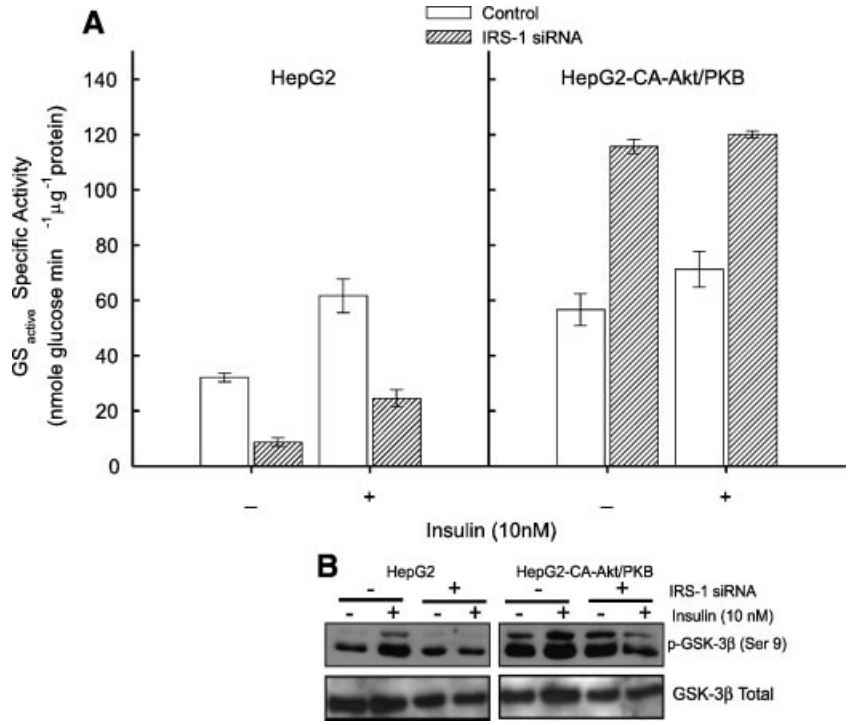

Fig. 5. Effect of IRS-1 knockdown in parental HepG2 and HepG2-CA-Akt/PKB cells on GS activity and phosphorylation of GSK-3β (Ser 9): Details of siRNA (scrambled and IRS-1) transfection, preparation of whole cell lysates for Western blot analysis and glycogen synthase (GS) assay are described in the Materials and Methods Section. Specific activity of GS_{active} is shown in (**A**). Each bar is an average of four assays and the error bars represents the ±standard deviation. A representative Western Blot probed against p-GSK-3β (Ser 9) and GSK-3β total are shown in (**B**).

and p-mTOR (Ser 2448) were predominantly cytoplasmic (although some small amounts were present in the nucleus), in parental HepG2 cells (Fig. 6A). IRS-1 knockdown in parental HepG2 cells could inhibit p-mTOR (Ser 2448) as shown in the Figure 3A,C. This is further supported by immunofluorescence study wherein mTOR levels were decreased as seen in the overlapped image (Fig. 6A) confirming that IRS-1 knockdown in parental HepG2 cells decreases the levels of phosphorylated mTOR (Ser 2448). HepG2-CA-Akt/PKB cells also had p-Akt1 and p-mTOR predominantly in the cytoplasm with some fractions in the nucleus. Interestingly, IRS-1 knockdown in these cells rendered both the phosphorylated proteins predominantly in the nucleus with some fraction in cytoplasm. The mTOR levels were upregulated as observed in the overlapped image (Fig. 6B) confirming the results shown in the Figure 3B,C.

DISCUSSION

PI-3 kinase/Akt mediated signal transduction is one of the signaling cascades known to play an important role in the development and progression of several types of cancers and metabolic disorders. Many proteins involved in this cascade are abrogated in these diseases. IRS proteins are important cytoplasmic-docking protein that relays the signal from the IR to PI-3 kinase and Akt. IRS-1 and IRS-2 have been reported to be overexpressed in several types of tumors [Nagle et al., 2004; Dearth et al., 2006]. In this study, we knocked down IRS-1 protein in parental HepG2 and HepG2-CA-Akt/PKB cells using siRNA (Fig. 1) and this resulted in decrease in the insulin mediated phosphorylation of Akt1 (Ser 473) in both types of cells (Fig. 2).

The effects of IRS-1 knockdown on the levels of mTOR and rictor along with downstream target $p70^{S6K}$ were also investigated. IRS-1 knockdown in parental HepG2 cells resulted in decrease in the basal and insulin mediated phosphorylation of both mTOR (Ser 2448) and $p70^{S6K}$ (Thr 389) along with the levels of rictor (Fig. 3A,B). A complete abolition was not observed in IRS-1 knockdown cells and this could be due to the presence of IRS-2 that can

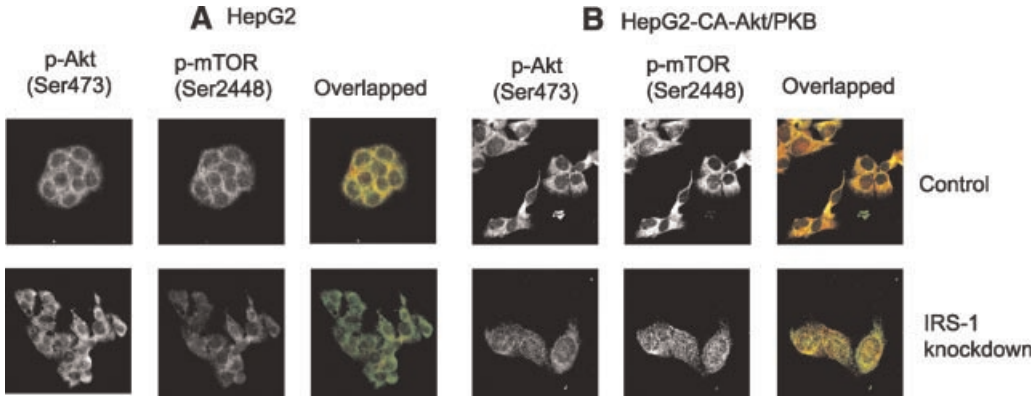

Fig. 6. Effect of IRS-1 knockdown on the localization of p-Akt (Ser 473) and p-mTOR (Ser 2448) in HepG2 and HepG2-CA-Akt/PKB cells. Cells were seeded and transfected with IRS-1 siRNA for 48 h at 37°C with CO_2 (5%) and immunofluorescence was performed as described in the Materials and Methods Section. The subcellular localization of p-Akt was analyzed using a Alexa Fluor 488 conjugated p-Akt (Ser 483)-green color and p-mTOR (Ser 2448) was analyzed using a primary antibody and Alexa Fluor 594 conjugated secondary antibody-red color. Confocal images of the individual proteins are shown in gray for (**A**) parental HepG2 and (**B**) HepG2-CA-Akt/PKB cells and the overlapped images of both the proteins are shown in color. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

contribute to the activation of these signaling proteins. As noted previously, knockdown of IRS-1 did not alter the IRS-2 levels (Fig. 1A,C) under all experimental conditions. In IRS-1 knockdown HepG2-CA-Akt/PKB cells, there was an upregulation of basal levels of phosphorylated mTOR but no significant change in the basal levels of phosphorylated $p70^{S6K}$ (Fig. 3A,B). However, the insulin mediated phosphorylation of both the proteins was inhibited (Fig. 3B,D). In this case also levels of rictor showed the same trend as phosphorylated mTOR (Ser 2448). In our earlier studies, we have demonstrated that the overexpression of constitutively active Akt1 in HepG2 leads to a small increase (24%) in the basal level of phosphorylated mTOR (Ser 2448) and a significant increase of (112%) in the phosphorylated $p70^{S6K}$ (Thr 389) as compared to parental HepG2 cells [Varma and Khandelwal, 2007]. In spite of the increase in phosphorylated mTOR levels in HepG2-CA-Akt/PKB, IRS-1 knockdown caused an upregulation. Earlier, it has been reported that the overexpression of constitutively active Akt1 in muscle cells causes a 30% decrease in the IRS-1 levels [Cleasby et al., 2007]. As a part of negative feedback loop, activation of Rheb/mTOR/S6K leads to insulin resistance due to reduction in IRS levels [Shah et al., 2004]. IRS-1 has multiple binding sites for PI-3 kinase, which can subsequently facilitate the activation of Akt and mTOR. Regulation of IRS activity occurs through phosphorylation at Tyr and Ser/Thr residues. A positive modulation occurs by phosphorylation on Tyr and some of the Ser residue and negative modulation occurs by phosphorylation at Ser/Thr residues [Danielsson et al., 2005; Gual et al., 2005; Greene et al., 2006; Scioscia et al., 2006; Yan et al., 2006]. Negative feedback effect due to chronic stimulation of Akt or mTOR or $p70^{S6K}$ on IRS-1 has been demonstrated in muscle insulin resistance. This effect is due to phosphorylation of IRS-1 at Ser residue which inhibits Tyr phosphorylation and targets IRS-1 to proteosomal degradation pathway [Carlson et al., 2004]. Homozygous IRS-$1^{(-/-)}$ mice induced with breast cancer were demonstrated to have elevated IRS-2 activity and enhanced PI-3 kinase/Akt/mTOR activities; these mice also showed high metastasis [Ma et al., 2006]. In NIH3T3 cells, it was demonstrated that mTOR phosphorylates IRS-1 (Ser 636/639) and this phosphorylation could be inhibited by rapamycin [Ozes et al., 2001]. Another study showed that the inhibition of IRS-1 by phosphorylation at Ser 312/161 residues did not require mTOR or $p70^{S6K}$ [Kido et al., 2000; Hers and Tavare, 2005]. IRS-1 knockdown, in our study showed an upregulation of basal mTOR phosphorylation, in HepG2-CA-Akt/PKB cells, this could be due to combination of both IRS-1 knockdown and overexpression of constitutively active Akt in these cells. The other feedback loops demonstrated in the regulation of IRS-1 is that of GSK-3β which also phosphorylates IRS-1 at Ser 332 thereby, inhibiting the insulin signaling cascade [Liberman and Eldar-Finkelman,

2005]. Therefore, the phosphorylation of these protein kinases is not decreased with the IRS-1 knockdown in cells overexpressing Akt (Figs. 3 and 5).

Akt regulates the activity/phosphorylation of mTOR and therefore its downstream target $p70^{S6K}$. These proteins are involved in the regulation of cell proliferation [Schmelzle and Hall, 2000; Rohde et al., 2001]. We have earlier reported a linear correlation between the phosphorylation of mTOR (Ser 2448) and the cell proliferation [Varma and Khandelwal, 2007]. In this study also, the effect of IRS-1 knockdown on phosphorylation of mTOR and cell proliferation data were in agreement (Figs. 3 and 4). IRS-1 knockdown showed a decrease (ca. 30%) in the cell proliferation of parental HepG2 cells and it was not altered with the insulin treatment (Fig. 4A). HepG2-CA-Akt/PKB cells also showed a small decrease (15%) in the cell proliferation, but this effect was further enhanced (ca. 50%) in the presence of insulin (Fig. 4B). In another study using breast cancer cell lines, BT-20 and BT-20 cells overexpressing IRS-1, it was observed that BT-20 cells overexpressing IRS-1 forms tumors twice as large as parental BT-20 cells [Dalmizrak et al., 2007]. In this study, nuclear translocation of IRS-1 was also demonstrated. IRS-1 is also known to play an important role in the regulation of cell size [Baserga, 2004]. Deletion of IRS-1 in mice or drosophila [Bohni et al., 1999] causes a 50% reduction in body sizes.

Cellular localization of phosphorylated Akt (Ser 473) and mTOR (Ser 2448) can provide insights into their functional roles. In several types of cells, it has been reported that Akt is a cytoplasmic protein that binds to the plasma membrane upon treatment with insulin [He et al., 2006]. We have previously shown the localization of p-Akt (Ser 473) into the nucleus upon insulin treatment in HepG2 cells [Syed et al., 2002]. Phosphorylated Akt (Ser 473) in IRS-1 knockdown parental HepG2 cells was predominantly cytoplasmic, whereas, in HepG2-CA-Akt/PKB cells it was predominantly nuclear. Many proteins involved in the PI-3 kinase /Akt pathways including IRS-1/-2 [Podlecki et al., 1987; Meier et al., 1997; Dalmizrak et al., 2007], PI3-kinase [Drakas et al., 2004], PDK1, etc. are known to shuttle between the nucleus and cytoplasm. Nuclear localization of these proteins suggests that they can act as co-transcriptional factors [Dalmizrak et al., 2007]. In different cell lines mTOR has been reported to be localized within the cytoplasm [Kim and Chen, 2000; Varma and Khandelwal, 2007], endoplasmic reticulum, golgi [Drenan et al., 2004] and mitochondria [Schieke and Finkel, 2006]. mTOR also shuttles between the nucleus and cytoplasm. In this study, IRS-1 knockdown in HepG2-CA-Akt/PKB cells, resulted in predominant localization of mTOR within the nucleus as compared to controls, unlike parental HepG2 cells where IRS-1 knockdown resulted in predominantly cytoplasmic localization of mTOR. Nuclear localization of mTOR could be involved in the activation of $p70^{S6K}$ and 4E-BP1 which also localize in the nucleus [Kim and Chen, 2000].

Akt is not only known to regulate the activities of protein kinases involved in cell proliferation such as mTOR and $p70^{S6K}$ but it also regulates protein kinase GSK-3β involved in the regulation of GS activity. Therefore, we also investigated the effects of IRS-1 knockdown on the levels of phosphorylated GSK-3β and GS activity. IRS-1 knockdown in parental HepG2 cells decreased GS activity by 50% only. A complete abolition of its activity was not observed probably due to the presence of IRS-2 proteins. In HepG2-CA-Akt/PKB cells the IRS-1 knockdown caused an upregulation in the GS activity. The reasons for differential behavior of parental HepG2 and HepG2-CA-Akt/PKB cell could be due to (i) that these HepG2-CA-Akt/PKB cells already have constitutively activated Akt and (ii) upregulation of mTOR in IRS-1 knockdown cells contribute to maintaining high levels of activated/phosphorylated Akt (Fig. 2), thereby high GS activity. It has been demonstrated that mTORC2, a complex of mTOR-GβL with rictor phosphorylates Akt at Ser 473 as a part of feedback mechanism [Sarbassov et al., 2005]. Taniguchi et al. [2005] have reported an increase in gluconeogenic enzymes phosphoenolpyruvate carboxykinase, glucose-6 phosphatase and fructose 1, 6 bisphosphatase levels and a decrease in glycolytic enzyme glucokinase levels in livers of IRS-1 knockdown mice.

In order to explain this differential behavior we investigated the phosphorylation of GSK-3β at Ser 9, however, there was no correlation between the levels of phosphorylated GSK-3β and GS activities both in parental HepG2 cells as well as the HepG2-CA-Akt/PKB cells. It is possible that under these experimental conditions GS is activated through some

other mechanism and this has to be further investigated.

Both IRS-1 and IRS-2 have been reported to be overexpressed in several types of tumors [Boissan et al., 2005; Dearth et al., 2006] and the evidence of distinct roles of IRS-1 and IRS-2 has been demonstrated in several tissue types such as liver, breast, etc. [Dearth et al., 2006; Ma et al., 2006]. In fact, IRS-$1^{(-/-)}$ and IRS-$2^{(-/-)}$ homozygous knockout mice induced with breast cancer show that IRS-1 homozygous knockouts had high rates of metastasis [Ma et al., 2006] in contrast to IRS-2 homozygous knockouts which lost the ability to metastasize [Nagle et al., 2004]. It would be worthwhile to study the effects of knockdown of IRS-2 alone and in combination with IRS-1 proteins. Downregulation of IRS-1 levels in liver cancers can reduce the cell proliferation and this effect is reduced in cells with constitutive overexpression of Akt1. GS levels were also upregulated in cells overexpressing constitutively active Akt1. Advanced stages of cancers (with overexpression of constitutive active Akt), IRS-1 inactivation coupled with therapeutic agents inhibiting mTOR (rapamycin) or glycogen metabolism could help in providing a better therapy and can inhibit tumor growth effectively.

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