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THE POTENTIAL ROLE OF PROTEIN KINASE B IN 3T3-L1 ADIPOCYTE DIFFERENTIATION

by

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ABSTRACT

The downstream targets of insulin/IGF-1 that lead to adipocyte differentiation appear to include the PI 3-kinase/p70 S6 kinase pathway. Recently several laboratories have shown that protein kinase B (PKB), an insulin-responsive serine/threonine kinase, is a direct target of PI 3-kinase and can activate p70 S6 kinase. The present study examines the potential role of PKB in insulin/IGF-1-dependent adipocyte differentiation. We have shown that PKB is expressed in 3T3-L1 preadipocytes, and that it is stimulated by insulin. The involvement of PI 3-kinase and p70 S6 kinase in adipocyte differentiation suggests that PKB may link PI 3-kinase to p70 S6 kinase signals. We have tested whether PKB is sufficient for 3T3-L1 preadipocyte differentiation. A version of PKB that is constitutively activated by linkage to the viral *gag* gene (Gag-PKB) was expressed in 3T3-L1 preadipocytes and shown to induce spontaneous differentiation in the absence of added insulin/IGF-1. The cells assumed a spherical shape and they acquired characteristic lipid droplets that stained positively for the neutral lipid stain, Oil Red O. Northern blot analysis demonstrated upregulation of LPL and aP2 mRNA, specific indicators of adipocyte differentiation. A plasmid vector, in which Gag-PKB was placed under the control of the glucocorticoid-responsive MMTV promoter, was then expressed in 3T3-L1 preadipocytes. When Gag-PKB expression was induced by dexamethasone, spontaneous differentiation followed, despite the absence of insulin/IGF-1. These results suggest that the genetic program of adipocyte differentiation is subject to regulation by PKB. Inducible expression of Gag-PKB will permit the generation of stable cell lines to further explore the role of PKB in adipocyte differentiation.

DEDICATION

This thesis is dedicated to my family for their love and support.

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INTRODUCTION

Adipose Tissue

The principle role of adipose tissue is to serve as a reservoir for energy storage. During periods of energy excess, adipose tissue is able to store energy from ingested calories as triacylglycerol. Conversely, during energy deprivation, triacylglycerol stores are able to provide energy as required through lipolysis. As the adipocyte develops, it is equipped with the enzymes or accessory proteins that are required for triacylglycerol synthesis or lipolysis to occur (MacDougald OA and Lane MD, 1995). The balance between lipolysis and triacylglycerol synthesis is tightly regulated by hormones such as insulin, which promotes triacylglycerol synthesis, and glucagon, which promotes lipolysis (MacDougald OA and Lane MD, 1995).

However, energy storage is not the sole function of the adipocyte, since it expresses and/or secretes several bioactive proteins such as tumor necrosis factor (TNF) α , leptin, adipsin, plasminogen activator inhibitor-1 (PAI-1), and angiotensinogen. These proteins influence a variety of cellular processes. TNF α is a cytokine which inhibits insulin-stimulated glucose uptake and is implicated in causing insulin resistance in human obesity (Hotamisligil et al, 1995). The adipocyte secretes leptin, a protein which is believed to be involved in a feedback mechanism to inform the hypothalamus to decrease food intake and increase energy expenditure (Zhang et al, 1994). The adipocyte also synthesizes a variety of immune complement factors (Choy et al, 1992) such as ASP, which stimulates

triacylglycerol synthesis (Baldo et al, 1993). Expression of PAI-1 by the adipocyte may be responsible for impaired fibrinolysis, possibly promoting atherosclerosis in obese patients (McGill et al, 1994; Samad et al, 1996; Shimomura et al, 1996). The production of angiotensinogen by the adipocyte suggests a link to obesity-related hypertension (Frederich et al, 1992). The expression of the proteins stated above, and possibly others yet to be identified, has therefore implicated the adipocyte in a variety of physiological processes other than energy homeostasis alone.

The origin of adipocytes is still unclear. Currently, it is believed that the adipocyte lineage is derived from a multipotent mesenchymal stem cell which has the capacity to differentiate into adipocytes, chondrocytes and myocytes (MacDougald OA and Lane MD, 1995). The initial event in the formation of the adipocyte is commitment of the multipotent stem cell to the adipocyte lineage. Although poorly understood, it has been hypothesized that a specific gene(s), yet to be identified, controls this initial step (MacDougald OA and Lane MD, 1995). Once committed, the cell is designated as a preadipocyte and has the ability to enter the subsequent differentiation steps leading to the terminally differentiated adipocyte. Preadipocytes are located within adipose tissue and form a reservoir from which adipocytes can form.

An increase in adipose tissue mass can arise from an increase in adipocyte size and/or cell number (Hirsch et al, 1989). Increase in cell size, due to increased intracellular triacylglycerol stores, is generally characteristic of individuals with mild obesity (Spiegelman BM and Flier JS, 1996). In more severe obesity or obesity arising in childhood, there is an increase in cell number as well as an increase in cell size (Julien et al, 1989). The potential to form new adipocytes continues not only through neonatal and early childhood years but well into the adult lifespan (Hauner et al, 1989)

Adipocyte differentiation (also known as adipogenesis) is influenced by a large number of growth factors (Smas CM and Sul HS, 1995). Insulin, IGF-1, fibroblast growth factor, platelet-derived growth factor, epidermal growth factor, and tumor promoters all promote preadipocyte proliferation (Camp et al, 1997) but only insulin or IGF-1 can induce adipocyte differentiation (Smith et al, 1988). Various cytokines, including TNF-α, interleukins (IL-1, IL-6), transforming growth factor β, and interferon-γ inhibit adipocyte differentiation (Hu et al, 1996).

In vitro model for adipocyte differentiation

Studies involving the molecular details of adipocyte differentiation were made experimentally feasible by the development of immortalized cell lines (Green H and Kehinde O, 1974a; Green H and Meuth M, 1974b). The 3T3-L1 preadipocytes are derived from the Swiss 3T3 cell line prepared from disaggregated 17-19 day old Swiss 3T3 mouse embryos. These cells are already committed to the adipocyte lineage, and were selected for their ability to differentiate into rounded lipid-laden adipocytes (Green H and Meuth M. 1974b).

Once these fibroblast-like 3T3-L1 preadipocytes reach confluence (growth arrest), they acquire the capacity to undergo a genetic program of differentiation upon induction by insulin or IGF-1. At the beginning of this 8 day differentiation program, the preadipocytes undergo at least one round of DNA replication and cell doubling which has been proposed to lead to the clonal amplification of committed cells. Dexamethasone and methylisobutylxanthine (MIX) are included, for the first 48 hours only, to accelerate the differentiation program (Rubin CS et al, 1978). The specific effects of MIX and dexamethasone may involve increasing the expression of two important transcription

factors involved in adipose differentiation, CCAAT/enhancer binding protein β (C/EBPβ) and C/EBPδ, respectively (Wu et al, 1996). Differentiation is characterized by changes in cell morphology, hormone sensitivity and gene expression. During the differentiation process there is also a decrease in the production of extracellular matrix (ECM) components secreted, allowing the cells to convert to a spherical shape (Franke et al, 1987; Spiegelman BM and Farmer SR, 1982). Upon induction of proteins that facilitate triglyceride synthesis such as lipoprotein lipase (LPL), adipocyte specific fatty-acid-binding protein aP2, and a lipid-droplet associated protein perilipin, the cells accumulate lipid droplets (Smas CM and Sul HS, 1995). During the early stages of differentiation, mRNAs for C/EBPβ and C/EBPδ appear transiently (Wu et al, 1996). Expression of C/EBPβ and to a lesser extent C/EBPδ results in the induction of two other transcription factors, the peroxisome proliferator activated-receptor (PPARγ) and C/EBPα (Mandrup S and Lane D, 1997; Spiegelman BM and Flier JS, 1996). PPARγ and C/EBPα then act through a synergistic mechanism to induce several proteins that are essential in forming the terminally differentiated adipocyte phenotype (Brun et al, 1996; Lin FT and Lane MD, 1994; Tontonoz et al, 1994). The expression levels of proteins such as glycerol-3-phosphate dehydrogenase, malic enzyme, acetyl-CoA carboxylase, stearoyl-CoA desaturase, glycerol-phosphate acyltransferase, ATP citrate lyase, and fatty acid synthase increase 10-100 fold during differentiation (Smas CM and Sul HS, 1995). The number of glucose transporters and insulin receptors also increase, as does insulin sensitivity. A protein which protects against apoptosis, Bcl-2, (Magun et al, 1997) is also upregulated.

Terminally differentiated adipocytes produced by this *in vitro* differentiation protocol share many characteristics with adipocytes *in vivo*. Their sensitivity to insulin is similar to that of adipose tissue. Experiments involving subcutaneous implantation of

these preadipocytes in nude mice, at a site normally lacking adipose tissue, produced adipocytes that are histologically indistinguishable from those found in normal white adipose tissue (Green H and Kehinde O, 1979; Mandrup et al, 1997). One major advantage of using these cell lines is the ease of transfection so that genes of interest can be manipulated to study their effects on adipocyte differentiation.

As stated earlier, insulin or IGF-1 is required for differentiation of 3T3-L1 preadipocytes. At pre-confluence, insulin or IGF-1 stimulate mitogenesis only. 3T3-L1 preadipocytes possess twice the number of IGF-1 receptors as compared to insulin receptors (Smith et al, 1988). Experiments have shown that IGF-1 induces 3T3-L1 adipocyte differentiation at 30-70 fold lower concentration than insulin and it was concluded that IGF-1 and insulin both act through the IGF-1 receptor (Smith et al, 1988). Subsequently, a study which involved deletion of one of the 3T3-L1 preadipocyte insulin receptor genes by homologous recombination indicated that the insulin receptor was in fact required for the full differentiation response (Accili et al, 1991). The insulin receptor has also been shown to be competent to induce differentiation in a recent study using a CSF-IR/IR chimeric construct (Chaika et al, 1997). Insulin is readily available, and at higher concentrations it can bind not only its own receptor but the IGF-1 receptor as well (Cheatham et al, 1995). For these reasons, we, along with many investigators in the field, have used insulin in our studies of adipocyte differentiation.

Insulin Signalling

Insulin transmits its signal intracellularly by binding and thus activating its specific receptor, a transmembrane tyrosine kinase. The insulin receptor and related IGF-1 receptor are composed of two α-subunits and two β-subunits covalently linked through disulfide

bonds to form an α_2 β_2-heterotetramer. The α-subunit contains the insulin binding domain, whereas the β-subunit possesses the protein tyrosine kinase catalytic domain. Activation of the insulin receptor tyrosine kinase induces rapid autophosphorylation of the β-subunits. This is followed by activation of several proteins downstream of insulin to create a signalling cascade towards the nucleus.

The best characterized intracellular substrate of the insulin receptor tyrosine kinase is the insulin receptor substrate-1 (IRS-1). Upon ligand stimulation, IRS-1 becomes rapidly phosphorylated on multiple tyrosine residues that serve as docking sites for recruitment of several proteins that contain Src homology 2 (SH2) domains. SH2 domains are conserved sequences of approximately 100 amino acids that are able to bind tyrosine-phosphorylated proteins in a phosphorylation-dependent and sequence-specific manner (Koch et al, 1991; Pawson T, 1995). Several SH2 domain proteins are recruited by IRS-1 such as: the p85 subunit of PI 3-kinase, an upstream element in insulin-stimulated glucose transport and p70 S6 kinase activation; a complex consisting of growth factor binding protein Grb2 and SOS (Son Of Sevenless, a GDP exchange factor) which links IRS-1 to activation of the Ras-Raf-MAP kinase pathway; and the protein tyrosine phosphatase SHP2. This multimeric protein complex initiates an array of signalling pathways emanating from IRS-1 to mediate a variety of biological responses including mitogenesis, differentiation, and glucose transport. Our studies have focused on the PI 3-kinase pathway, its downstream targets, and their role in adipocyte differentiation.

Structural and Biochemical properties of PI 3-Kinase

One of the signalling proteins that binds to IRS-1 is the heterodimeric PI 3-kinase, which consists of 85 and 110-kDa subunits (p85 and p110). The p85 regulatory subunit

contains one Src homology 3 (SH3) domain, a breakpoint cluster region (BCR) domain and two Src homology 2 domains which bind to IRS-1 (Carpenter C and Cantley LC, 1996). SH3 domains are conserved sequences of approximately 50-100 amino acids which are involved in protein-protein interactions (Koch et al, 1991; Pawson T, 1995). SH3 domains bind to proline-rich sequences of approximately 10 amino acids with high affinity and specificity (Ren et al, 1993). At present, no function has clearly been attributed to the p85 BCR domain. However, since this region has considerable homology to the GTPase activating protein (GAP) domain of the proteins Rho and Bcr, it has been postulated that it might be involved in GTP hydrolysis (Kapeller R and Cantley LC, 1994; Zhang et al, 1993). At present, cDNA cloning has revealed two forms of the p85 regulatory subunit (p85α and p85β). Recently the gene for the p85α subunit has been shown to produce two alternatively spliced products (p55α and p50α) which may also bind to the p110 subunit (Inukai et al, 1997).

cDNA cloning has also identified five forms of the p110 catalytic subunit. The p110 catalytic subunit has both lipid kinase and protein kinase activity. This indicates that PI 3-kinase is a dual-specificity kinase, however little is known about its protein kinase domain. It has been postulated to be involved in negative-feedback regulation on insulin signalling through phosphorylation of serine residues on the p85 subunit and IRS-1 (Dhand et al, 1994; Kapeller R and Cantley LC, 1994; Lam et al, 1994). The lipid kinase domain of the p110 subunit phosphorylates PI(4,5)P_2 at the 3-position of the inositol ring to generate the PI (3,4,5)P_3 . Unlike PI(4,5)P_2, it is not cleaved by phospholipase C. Several studies suggest that PI (3,4,5) P_3 is dephosphorylated to form PI(3,4)P_2 and PI(3)P (Kavanaugh et al, 1996; Stephens et al, 1993). PI (3,4,5) P_3 and PI(3,4)P_2 rapidly accumulate in the membrane upon growth factor stimulation and are thought to act as

second messengers. Although its exact function has yet to be determined, PI 3-kinase has been implicated in the regulation of many cellular processes including cellular growth and differentiation, glucose transport, re-organization of the cytoskeleton, receptor internalization, neutrophil activation, and vesicle sorting (Carpenter C and Cantley LC, 1996).

Due to the role of PI 3-kinase in several cellular processes, potential targets of PI 3-kinase are being sought and gradually identified. A growing body of evidence has shown that PKB is a direct target of PI 3-kinase. Recent reports propose that PKB is involved in several cellular processes controlled by PI 3-kinase including proliferation, differentiation, glucose transport, and cell survival (Hemmings BA, 1997; Franke et al, 1997b). Other targets of PI 3-kinase, identified mainly through *in vitro* studies, include the calcium-independent protein kinase C isoforms PKCζ, PKCε, PKCδ and PKCη. (Carpenter C and Cantley LC, 1996; Toker et al, 1994).

Structural Properties of Protein Kinase B (PKB)

Protein Kinase B (PKBα), also named Racα (Related to A and C protein kinase), is a 60 kDa serine/threonine kinase that has sequence homology with protein kinase C (PKC) and protein kinase A (PKA) (Coffer PJ and Woodgett JR, 1991; Jones et al, 1991). The same kinase is also recognized to be the product of c-*akt*, the cellular homolog of the viral oncogene, v-*akt* (Bellacosa et al, 1991). Although a majority of the studies have been performed on PKBα, two other isoforms of PKB termed PKBβ and PKBγ have been identified. The two human cellular homologs, AKT1 (also known as PKBα or Racα) and AKT2 (also known as PKBβ or Racβ) have been reported to be amplified in certain carcinomas indicating that PKB may be involved in a pathway leading to oncogenic

transformation. AKT1 has been shown to be amplified in gastric adenocarcinoma and breast cancer epithelial cells. AKT2 has been demonstrated to be overexpressed in human pancreatic, breast and ovarian carcinoma (Bellacosa et al, 1995; Cheng et al, 1992; Cheng et al, 1996).

PKB can be divided into three domains: an amino-terminal pleckstrin homology (PH) domain, a catalytic domain, and a serine threonine-rich carboxy-terminal regulatory domain. PH domains are regions of approximately 100 amino acids residues in length that mediate both phospholipid-protein and protein-protein interactions. (Lemmon et al, 1996; Shaw et al, 1996). The PH domain is located at the amino-terminus within a region responsible for homodimerization (designated as the AH domain) (Datta et al, 1995). The catalytic domain has 75% similarity to the kinase domain of PKC and 65% similarity to PKA (Coffer PJ and Woodgett JR, 1991). The carboxy-terminal domain contains a critical serine (Ser-473), which lies in a consensus sequence that is highly conserved in several protein kinases (Pearson et al, 1995) and is phosphorylated upon activation of PKB by insulin or IGF-1 (Alessi et al, 1996a). The mouse PKBα is composed of at least 13 exons located on mouse chromosome 12; it is 90% identical to its human homolog AKT1 at the nucleotide level and 98% identical at the amino acid level (Bellacosa et al, 1993; Cheng et al, 1992; Jones et al, 1991).

The v-*akt* viral oncogene is thought to have arisen from the recombination between part of the viral gag gene (consisting of the 785 nucleotides downstream from the ATG codon) and the 5' untranslated region of c-*akt*, 60 bp upstream from the c-*akt* ATG codon (Ahmed et al, 1993; Bellacosa et al, 1991). During this recombination three additional nucleotides were inserted at the junction between *gag* and c-*akt*. The result was the introduction of a 63 bp nucleotide sequence (known as region X) between *gag* and c-*akt*

which generates a fusion protein containing a portion of the viral Gag domain followed by 21 amino acids (encoded by X) and the entire c-Akt protein. The coding region of the cellularly derived portion of v-*akt* is identical to c-*akt* with the exception of five G to A transitions that do not alter the protein sequence (Bellacosa et al, 1991). The oncogenic potential of v-*akt* is most likely due to the myristolation of the Gag sequence at the amino-terminus (Glycine[1]) which leads to localization to the membrane and constitutive activation (Ahmed et al, 1993; Bellacosa et al, 1993; Kohn et al, 1996a, Weiss et al, 1985).

Biochemical Properties of PKB

PKB is activated by insulin, IGF-1 and certain other growth factors (Burgering BMT and Coffer PJ, 1995; Franke et al, 1995; Kohn et al 1995, Alessi et al, 1996a; Cross et al, 1995). The evidence for PKB as a downstream target of PI 3-kinase is the loss of PKB activation in the setting of PI 3-kinase inhibition, either by wortmannin or LY294002 (Burgering BMT and Coffer PJ, 1995; Franke et al 1995; 1997; Kohn et al 1995; Alessi et al, 1996), or by overexpression of a deletion mutant of the p85 subunit of PI 3-kinase which acts as a dominant-negative (Burgering BMT and Coffer PJ, 1995;). As well, platelet-derived growth factor receptor mutants that fail to activate PI 3-kinase also fail to activate PKB (Burgering BMT and Coffer PJ, 1995; Franke et al, 1995). Finally, expression of a constitutively activated form of PI 3-kinase has been shown to activate PKB (Datta et al, 1996; Didichenko et al, 1996; Franke et al, 1997; Klippel et al, 1996).

The mechanism of propagation of signals from PI 3-kinase to PKB appears to be through the interaction of the 3-phosphorylated lipid products of PI 3-kinase and the PH domain of PKB. Both $PI(3,4)P_2$ and $PI(3,4,5)P_3$ can form high-affinity association with the PH domain of PKB (Franke et al, 1997a; Franke et al 1997c; James et al, 1996; Klippel

et al, 1997). Thus, the significance of the PH domain may be to allow PKB to attach to the membrane where it is subsequently activated. Kohn et al (1996a) have shown that a version of PKB which lacks a PH domain but contains a newly engineered myristolation motif that directs it to the membrane can be activated to a similar extent as the native protein. The PH domain has been shown to be essential for activation, since stimulation of PKB by PDGF was eliminated with constructs lacking a functional PH domain (Franke et al, 1995; Andjelkovic et al, 1996). However, it should be noted that activation of PKB by insulin in certain circumstances has been shown to occur with constructs lacking a PH domain. (Kohn et al, 1996a). The reasons for these differences remain to be established but may be attributable to the cell type used or other experimental conditions.

The increase in PKB kinase activity due to activation by PI 3-kinase has been shown to be accompanied by an increase in serine/threonine phosphorylation (Burgering, BMT and Coffer PJ, 1995; Kohn et al, 1996a). Phosphorylation of PKB following insulin and IGF-1stimulation has been shown to occur at two sites: threonine-308 (located in the catalytic domain) and serine-473 (located in the carboxy-tail region). Alessi et al (1996a) showed that mutation of both these residues to alanines creates a version of PKB that is unresponsive to insulin, suggesting that activation of PKB is dependent on phosphorylation. Interestingly, Alessi et al (1996a) also showed that serine/threonine phosphorylation of PKB was not due to autophosphorylation and for this reason it is hypothesized that an unidentified upstream kinase(s) can phosphorylate PKB. It is believed that this upstream kinase is also a target of PI 3-kinase since PKB phosphorylation is sensitive to wortmannin (Alessi et al, 1996a). Franke et al (1997a) and Datta et al (1995) showed that homodimerization of PKB leads to autophosphorylation and an increase in

PKB activity. Whether PKB can undergo autophosphorylation and/or be phosphorylated by an upstream kinase is still under investigation.

Two insulin-responsive kinases, glycogen synthase kinase-3 (GSK3) and p70 S6 kinase, have been postulated as downstream targets for PKB. At present, glycogen synthase kinase-3 has been shown to be the only direct target that is phosphorylated by PKB (Cross et al, 1995). GSK3 is an enzyme which is involved in the regulation of several cellular processes, including glycogen synthesis (Cross et al, 1995) and protein synthesis (Welsh et al, 1994). Phosphorylation of serine residues on GSK3, upon insulin stimulation of PKB, results in inhibition of GSK3 and stimulation of glycogen synthesis and protein synthesis.

p70 S6 kinase can be activated by various growth factors including insulin through a PI 3-kinase dependent pathway (Chung et al, 1994; Cheatham B, 1994). It phosphorylates the S6 subunit of the 40S eukaryotic ribosome which may be involved in regulation of translation initiation. (Ferrari et al, 1991; Jeno et al, 1988). p70 S6 kinase may also regulate the eiF-4E-binding protein (4E-BP1) which is involved with preferential translation of mRNAs containing 5'polyprimidine tracts (Lin et al, 1995). p70 S6 kinase has been shown to regulate transcription as well, based on its ability to phosphorylate the transcription cyclic-AMP-responsive element modulator CREMτ (de Groot et al, 1994). On the basis of studies with rapamycin, a specific inhibitor of p70 S6 kinase, p70 S6 kinase has been proposed to lie downstream of PKB (Franke et al, 1995). Furthermore, a constitutively active form of PKB has recently been demonstrated to activate p70 S6 kinase (Burgering et al, 1995; Kohn et al, 1996a). However the connection between PKB and p70 S6 kinase is most likely indirect (Bos JL, 1995; Downward J, 1995) since it does not contain the recognition sequence for serine/threonine phosphorylation by PKB.

Evidence of PKB as a Component of Insulin-Dependent Adipogenesis

Several insulin/IGF-1 cytosolic signaling proteins have been implicated in adipocyte differentiation including Ras, Raf-1, and mitogen-activated protein kinase (MAPK). Ras has been implicated in adipogenesis based on a study by Benito et al (1991) who reported that expression of activated Ras constructs led to adipocyte differentiation in the absence of insulin or IGF-1. As well, transfection of a dominant-inhibitory Ras mutant resulted in the inhibition of insulin-induced differentiation. Porras et al (1994) reported that expression of oncogenic Raf-1 kinase also induced adipocyte differentiation and the insulin-induced differentiation could be blocked by expression of a dominant-negative Raf-1 mutant. MAP kinase has been shown to be essential for differentiation based on a study by Sale et al (1995) who showed that adipocyte differentiation could be prevented in the presence of insulin when expression of p42 and p44 MAP kinase was inhibited using an antisense oligonucleotide strategy. These observations provide evidence that insulin signalling through Ras, Raf-1, and MAP kinase is important to induce the adipocyte differentiation program.

The potential role of PKB in insulin-dependent cellular responses is still uncertain. However, studies using rather selective inhibitors have shown that PI 3-kinase and p70 S6 kinase are required for 3T3-L1 adipocyte differentiation. Yeh et al (1995) have shown that the p70 S6 kinase inhibitor rapamycin can inhibit C/EBPα expression and adipocyte differentiation. The PI 3-kinase inhibitor, wortmannin, has been shown to inhibit adipocyte differentiation (Magun et al, 1996; Tomiyama et al, 1995). These results indicate that the PI 3-kinase/p70 S6 kinase pathway may be important for mediating insulin signals to induce the adipocyte differentiation program.

Rationale and Objectives

Initial work in our laboratory determined the effects of wortmannin on adipocyte differentiation. We used a defined, serum-free differentiation medium, in which insulin was the sole growth factor. As was found in other work studying wortmannin inhibition of adipocyte differentiation using a serum-supplemented medium (Tomiyama et al, 1995), wortmannin inhibited the differentiation response. Differentiation was assessed morphologically, and quantitated by measurement of glycerol phosphate dehydrogenase (GPDH) activity (Wise et al, 1979). The insulin-stimulated accumulation of PI(3,4,5)P_3, the growth factor-responsive 3-phosphorylated inositol phospholipid in preadipocytes (Sorisky et al, 1996), was inhibited in parallel fashion. After confluent 3T3-L1 preadipocytes were exposed to 100 nM insulin for 5 min, PI(3,4,5)P_3 increased 4-fold, but pre-incubation with 100 nM wortmannin for 10 min prior to insulin stimulation completely abrogated this response. These data, together with those previously reported (Tomiyama et al, 1995), suggest that PI 3-kinase is required for differentiation. The involvement of PI 3-kinase and p70 S6 kinase in insulin/IGF-1-induced adipogenesis suggests that PKB may link PI 3-kinase to p70 S6 kinase signals.

Therefore in order to investigate the role of PKB in adipocyte differentiation the following objectives were defined:

1.a) To measure the expression of endogenous PKB in 3T3-L1 preadipocytes and differentiated adipocytes.

b) To determine the effects of insulin on the activation of endogenous PKB using an *in vitro* enzyme assay.

2.) To transfect a constitutively activated form of PKB (Gag-PKB) in 3T3-L1 preadipose cells, and study the effects of Gag-PKB expression on adipogenesis using lipid-specific Oil Red O staining and Northern blot analysis of differentiation-specific mRNA markers.

3.) To construct and transfect a plasmid in which Gag-PKB is placed under the control of an inducible promoter and determine if induction of Gag-PKB expression can induce adipocyte differentiation. Successful inducible expression of Gag-PKB will provide the basis for long-term studies to dissect the role of PKB in the differentiation response.

METHODS

Cell Culture

The 3T3-L1 preadipocyte line is derived from the Swiss 3T3 mouse (Green et al, 1974a; Green et al, 1974b) and was obtained from American Type Culture Collection (ATCC, catalogue number: CCL 92.1). 3T3-L1 preadipocytes were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% calf serum (CS) (Gibco BRL), 100 μg/L penicillin (Gibco BRL) and 100 mg/L streptomycin (Gibco BRL). All further references to DMEM include the supplementation of these antibiotics. Cells were maintained at 37°C in a humidified incubator with an atmosphere of 10% CO_2. New cultures were thawed every 3-4 weeks from frozen stocks stored in liquid nitrogen and maintained at low passage (<5). The culture medium was changed every 2-3 days. Preadipocytes were subpassaged with brief exposure to trypsin-EDTA (Gibco BRL).

Protocol for 3T3-L1 adipocyte differentiation

3T3-L1 preadipocytes were grown to 100 % confluence, left for two more days, and then incubated overnight in 5% CS DMEM. To initiate the 8-day differentiation program (Day O), the media was replaced with 10% CS DMEM supplemented with 100 nM insulin (Boehringer Mannheim). Dexamethasone (0.25 μM, Steraloids) and MIX (0.5 mM, Sigma) were added for the first two days only to compress the differentiation program (Rubin et al, 1978). For the next 4 days (Day 2 to 6) cells were maintained in

10%CS DMEM containing only insulin (100 nM). Finally, the media was changed to 10%CS DMEM for two days (Day 6 to 8), after which the cells were used as described. The percent of differentiated cells was determined by light microscopy (presence of lipid droplets). In other experiments, I have confirmed that these changes correlate with the induction of a specific marker of terminal adipocyte differentiation, GPDH.

PI 3-Kinase Assay

3T3-L1 preadipocytes were grown to 100% confluence in 10% CS DMEM and then left overnight in 2% CS DMEM. The cells were first rinsed twice with Krebs-Ringer-Hepes (KRH) buffer (Sorisky et al, 1996; Gomez et al, 1990) and then incubated in KRH buffer prior to insulin stimulation. The cells were lysed in 500 µl of immunoprecipitation (IP) lysis buffer (1% Triton X-100, 200 µM sodium orthovanadate, 0.1 mg/ml PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 4 µg/ml benzamidine, 50 mM NaF, 1 mM β–glycerophosphate) followed by scraping with a rubber policeman and transfer of the lysates to microfuge tubes. The tubes were incubated on ice for 15 min to allow lysis to occur. The samples were then mixed with a 1ml pipetman and spun at 15000 g for 15 min at 4°C to remove particulate matter. The supernatant was transferred to a chilled microfuge tube and stored at -80° C. For immunoprecipitations performed using rabbit polyclonal antibody directed against the C-terminal portion of IRS-1 (Upstate Biotechnology), 75 µl of protein A-sepharose was added to preclear the lysates for 1hr at 4°C. Lysates (500 µl) were inmunoprecipitated overnight at 4°C with 5 µg of primary Ab in combination with 75 µl protein A-sepharose. Each immunoprecipitate was washed three times with IP lysis buffer. The immunoprecipitation protocol was the same with the mouse monoclonal anti-phosphotyrosine antibody(PY20, Transduction Laboratories),

except protein A-sepharose was replaced with protein G-sepharose, since Protein A does not bind mouse IgG effectively.

After immunoprecipitation using anti-phosphotyrosine or anti-IRS-1 antibody the immunoprecipitate was washed once with each of the following buffers: phosphate buffered saline (PBS), buffer 1 (0.5 M LiCl, 0.1 M Tris pH 7.5), water, and buffer 2 (0.1 M NaCl,1 mM EDTA, 20 mM Tris pH 7.5). 100 µl of Assay Buffer (0.1 M NaCl, 0.5 mM EDTA, 20 mM Tris pH 7.5, 10 µM ATP, 0.2mg/ml phosphatidylinositol) was added to the pellet and the reaction was started with the addition of 10 mM $MgCl_2$, and 20µCi of [γ-^{32}P]ATP for 3 min at 25°C. The reaction was stopped by adding 300 µl of $CHCl_3$/MeOH/11.6 N HCl (100:200:2). 200 µl of $CHCl_3$ was subsequently added and the phospholipids were isolated by extracting and discarding the aqueous (upper) phase. The organic phase was washed 2 times with 300 µl of MeOH/1N HCl (1:1). The aqueous phase was discarded after each wash. The organic phase was dried under nitrogen (N_2). The dried samples were resuspended in 30 µl of $CHCl_3$ and spotted on a TLC plate (Silica gel G60, Alltech). PI-4P (Sigma) was spotted in one lane as a standard. The TLC plate was placed in a chromatography tank containing MeOH/$CHCl_3$/NH_4OH/H_2O (100:70:15:25). After running the plate for approximately 2 hr, the standard was identified by staining in iodine. The conversion of PI to PI 3-phosphate was visualized by autoradiography.

Protein Kinase B (PKB) assay

Insulin stimulation of 3T3-L1 preadipocytes and lysis of the cells was performed as described for the PI 3-kinase assay. Immunoprecipitation was performed using rabbit polyclonal antibody directed against the C-terminal portion of PKBα (c-akt) (UBI). Control and insulin-treated lysates were precleared with 75 ul of protein A-sepharose for

1hr at 4°C. Lysates (500ul) were inmunoprecipitated for 2 h at 4°C with 5 ug of primary Ab in combination with 75 ul protein A-sepharose. Samples were washed three times with IP lysis buffer and three times with kinase assay buffer (50mM Tris pH 7.5, 0.5M MgCl2, 1mM DTT in H_2O). Immunoprecipitates were preincubated at 30°C with 30 ul of kinase assay buffer containing 30 μM crosstide substrate (gift from Dr. P. Cohen) for 10 minutes. Crosstide is a synthetic peptide with the sequence, GRPRTSSFAEG, which corresponds to a sequence in each of the two isoforms of GSK3 which are phosphorylated by PKB. The serine in bold corresponds to serine 21 in GSK3-α or serine 9 in GSK3-β. Crosstide has been shown to be phosphorylated by PKB in kinase assays previously performed by Cross et al (1995). Although crosstide is more specific for PKB, it has been shown to be phosphorylated to a lesser extent by p70 S6 kinase and MAPKAP-1 kinase (Cross et al, 1995). The reaction was initiated by adding 10 ul of ATP cocktail (125 μM ATP/20 mM Mg/ 10μCi [γ-^{32}P]ATP). After five minutes, a quick spin was performed and the reaction was stopped by removing 25 ul of the reaction mixture and blotting onto P81 phosphocellulose paper (Upstate Biotechnology). Crosstide bound tightly to the phosphocellulose papers and unincorporated P^{32} was washed away by rinsing the phosphocellulose papers three times in 40 mls of 0.75% phosphoric acid (5 min) followed by 5 min in 20 ml of acetone. Samples were counted for 1 minute in 5 mls of Ecolite scintillation fluid (ICN) in a LKB Rac beta scintillation counter.

PKB has a threefold higher activity towards crosstide than histone H2B and 11 fold higher activity than its activity towards myelin basic protein (Cross et al, 1995). Histone H2B and myelin basic protein are two substrates previously used to assay PKB (Franke et al, 1995; Burgering BMTand Coffer PJ, 1995; Kohn et al, 1997b) as well as several other kinases (Burgering BMTand Coffer PJ, 1995).

Immunoblotting Assay

Cells were lysed in Laemmli sample buffer (Laemmli et al). Lysate protein were subjected to 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Hoefer Scientific Instruments) and electrophoretically transferred to nitrocellulose paper (Towbin et al, 1979) for 4 hours at 0.8 amps. Rainbow coloured protein high molecular weight markers (Amersham) were used to monitor the transfer. To block nonspecific binding sites, the nitrocellulose was incubated with 5% skim milk in PBS for 3 hours. The blot was probed with primary antibody overnight at 4°C (Stott et al, 1989). Detection was by incubation with secondary horseradish-peroxidase conjugated antibody (Amersham) using enhanced chemiluminescence (ECL) kit according to manufacturer's instructions (Amersham). The blot was exposed to Kodak X-AR film (Eastman Kodak, Rochester, NY).

The anti-phosphotyrosine antibody (PY20, Transduction Laboratories) was used at 1.0 μg/ml. The anti-PKB antibody (a gift from Dr. BMT Burgering and Dr. PJ Coffer) used for immunoblotting is directed against a C-terminal peptide of PKBα (c-Akt) as described (Burgering BMT and Coffer PJ, 1995). The monoclonal Gag antibody (a gift from Dr. BMT Burgering and Dr. PJ Coffer) was used as described (Burgering BMT and Coffer PJ, 1995).

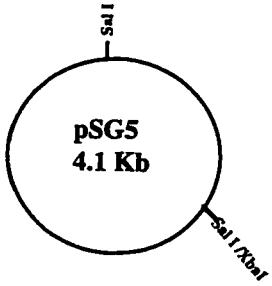
Plasmids Used in Transfection Experiments

Each pSG5 vector contains a simian virus (SV40) promoter to allow for stable *in vivo* expression in mammalian cells (Figure 1). Plasmid pSG5Gag and pSG5Gag-PKB were constructed as described previously (Burgering BMT and Coffer PJ, 1995). For the

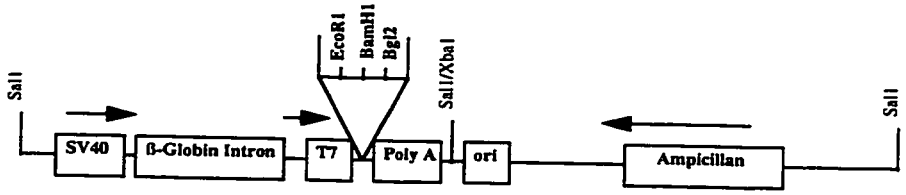
Figure 1 - pSG5, pSG5Gag, and pSG5Gag-PKB Expression Vectors

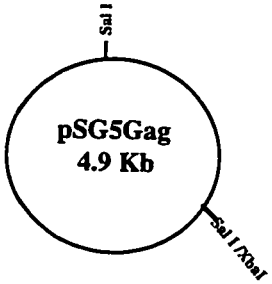
Illustration of pSG5, pSG5Gag and pSG5Gag-PKB plasmid vectors used in transfections of 3T3-L1 preadipocytes. For the pSG5Gag and pSG5Gag-PKB vector the cDNA for Gag and Gag-PKB are located downstream of the simian virus (SV40) promoter as indicated.

Figure 1

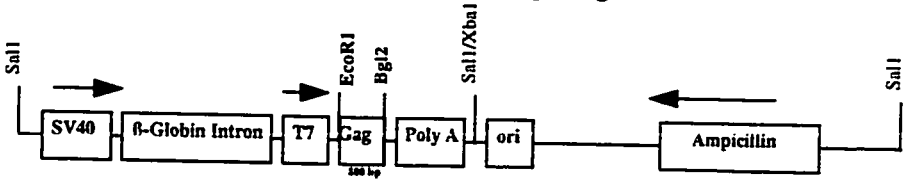


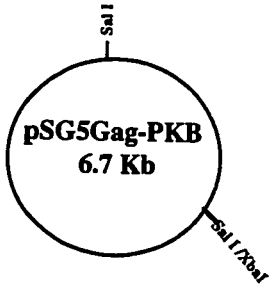
- Eukaryotic expression vector
- Early SV40 promoter allows *in vivo* expression
- T7 bacteriophage promoter allows *in vitro* transcription
- Intron II of rabbit ß-globin gene allows splicing of expressed transcript
- Polyadenylation signal greatly increases level of expression
- This construct was a gift from Dr. Burgering and Dr. Coffer



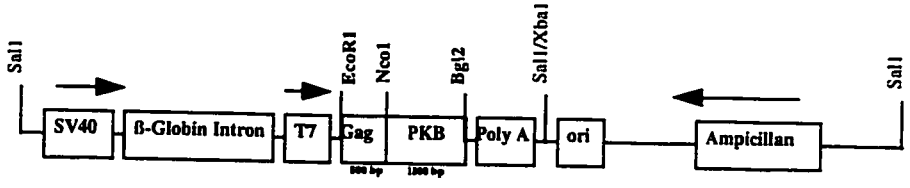


- pSG5 vector with the Gag insert subcloned into the BamH1 site using blunt end ligation
- The insert is 0.8 Kb
- This construct was a gift from Dr. Burgering and Dr. Coffer





- pSG5 vector with the GagPKB insert subcloned into the BamH1 site using blunt end ligation
- Gag-PKB was constructed by ligating the cDNA for the capsid protein, Gag, in frame with the initiation codon of PKB utilizing an engineered Nco1 site (Burgering et al).
- The insert is 2.6 Kb
- This construct was a gift from Dr. Burgering and Dr. Coffer



pSG5Gag-PKB vector, an 800 bp Moloney murine leukemia virus (MoMuLV) cDNA encoding for the p30 capsid protein, Gag, (Bellecosa et al, 1991) was ligated upstream in-frame with the full-length PKB cDNA. The cDNA for PKB was isolated from a bovine cDNA library as previously described (Coffer et al, 1991). Upon transfection of mammalian cells, it generates a fusion protein of 85 kDa (Burgering BMT and Coffer PJ, 1995) which is myristylated on its amino terminal glycine[1] residue (Bellacosa et al, 1993; Ahmed et al, 1993) and is constitutively active (Burgering BMT and Coffer PJ, 1995). All three pSG5 vectors were gifts from Dr. B. Burgering and Dr. PJ Coffer.

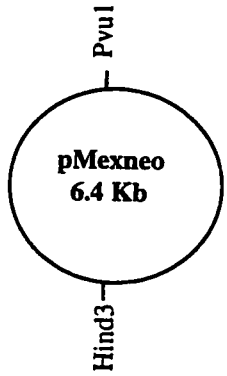
The pMMTV vector contains the mouse mammary tumour virus-long terminal repeat sequence (MMTV-LTR) which can initiate transcription in response to glucocorticoids such as dexamethasone both *in vivo* and *in vitro* (Lee et al, 1981; Payvar et al, 1983). The MMTV promoter is located upstream of a multiple cloning site (Figure 2). This vector was a gift from Dr. B. Burgering and Dr. P.J. Coffer.

The plasmid pMEXneo contains the cDNA for neomycin resistant gene (neo) under the control of the SV40 promoter (Figure 2). This gene confers resistance to antibiotics, geneticin (G418), neomycin and kanamycin . Since these antibiotics will inhibit protein synthesis and thus kill cells which do not express the neo resistant gene, they can be used as dominant markers to select cells which take up exogenous DNA (Jimenez et al, 1980; Colbere-Garapin et al, 1981). pMexneo was a gift from Dr. H. Hanafusa (Hempstead et al, 1994).

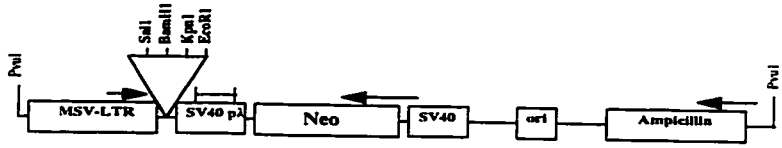
Figure 2 - pMexneo, pMMTV, and pMMTVGag-PKB Expression Vectors

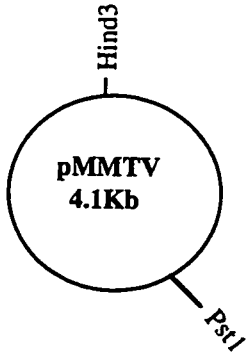
Illustration of pMexneo, pMMTV, and pMMTVGag-PKB plasmid vectors used in transfections of 3T3-L1 preadipocytes. pMexneo contains cDNA for the neomycin-resistant gene (neo) placed under the control of SV40 promoter as indicated. pMMTV contains the dexamethasone-responsive MMTV-LTR promoter located upstream of the multiple cloning site as indicated. pMMTVGag-PKB was constructed by subcloning the cDNA for Gag-PKB into the multiple cloning site of the pMMTV vector.

Figure 2

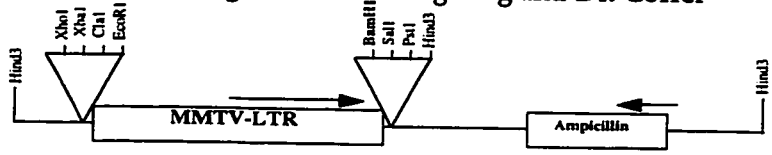


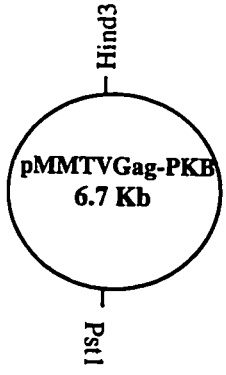
- Eukaryotic expression vector
- Early SV40 and MSV-LTR promoters allow for *in vivo* expression
- Neo is under the control of the SV40 promoter
- This construct was a gift from Dr. H Hanafusa



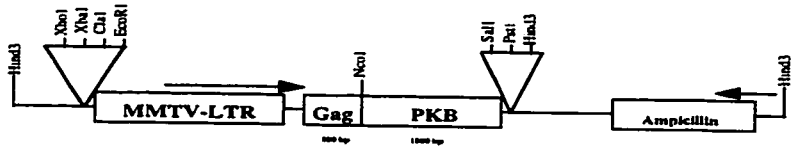


- Eukaryotic expression vector
- MMTV-LTR promoter allows *in vivo* expression
- BamH1 was the site chosen to insert the GagPKB sequence
- This construct was a gift from Dr. Burgering and Dr. Coffer





- pMMTVGag-PKB contains the GagPKB insert under the control of the dexamethasone-responsive MMTV-LTR promoter
- GagPKB was cut out of the pSG5Gag-PKB vector and sublconed into the BamH1 site of pMMTV using blunt-end ligation



Transformation and Isolation of Plasmids

In order to obtain suitable quantities of plasmid DNA for transfections and for the construction of the pMMTV-GagPKB vector, transformation by the heat-shock method into E.coli cells was performed. Competent E.coli cells DH5α (Gibco BRL) and aliquots were stored at -80°C . Aliquots were rapidly thawed immediately before use. Approximately 50 ng of plasmid DNA was added to 50 µl of competent cells and placed on ice for 30 minutes. Samples were heat-shocked for 2 min at 40°C and then placed on ice for a further 2 min. After adding 500 ul of LB medium, samples were placed in a shaking incubator for 1 hour at 37°C (225 rpm). 100 ul of each sample was plated on 100 mm LB agar plates containing ampicillin (100 ug/ml). Plates were incubated overnight at 37°C and single colonies were each innoculated into 3 mls of LB medium containing ampicillin (100 ug/ml). After placing each sample in a shaking incubator at 37°C (225 rpm), 1 ml of each culture was innoculated into 100 ml of LB medium containing ampicillin (100 ug/ml). The E. coli cells were allowed to grow overnight, shaking at 37°C (225 rpm) to an OD between 1.2 and 1.6 (12-16 hours). Plasmid DNA was then isolated from each culture using a Qiagen maxi-prep kit according to manufacturer's instructions. To assess purity and concentration of DNA, the absorption of each sample was measured at 260 nm (nucleic acids) and 280 nm (protein) using a spectrophotometer (Milton Roy Spectronic 601). The ratio (1.8-2.0) of absorbancy readings at 260 and 280 nm indicated nucleic acid purity. Approximately 300 ug of plasmid DNA was isolated from the maxi-preps. Each plasmid was visualized by agarose (1.0%) gel electrophoresis and quantitated by comparison of band intensities from λ Hind III markers (Gibco BRL). An absorption reading at 260 nm was also used to quantitate the plasmid DNA.

Construction of pMMTVGag-PKB plasmid

Gag-PKB cDNA was subcloned into the pMMTV vector using blunt ligation. Digestion of pSG5Gag-PKB vector was performed using both EcoR1and Bgl2 restriction endonucleases to obtain the Gag-PKB cDNA. All restriction endonucleases were obtained from Gibco BRL. This fragment was isolated using agarose gel electrophoresis and purified from the gel using the Geneclean II kit according to manufacturer's instructions (BIO 101 Inc.). A restriction digest of pMMTV plasmid vector was performed with BamH1 site. BamH1 cuts once in the polylinker site located downstream of the mouse mammary tumour virus promoter (MMTV). The linearized plasmid was separated by agarose gel electrophoresis and purified from the gel.

The linearized pMMTV was treated with calf intestine alkaline phosphatase (CIAP) (Gibco BRL) to prevent re-ligation. 1 unit of CIAP was used to dephosphorylate 2.5 µg of plasmid DNA. The reaction was performed for 20 minutes at 37°C. The reaction was stopped by heating for 10 minutes at 75°C. The plasmid was separated from CIAP by phenol-chloroform extraction. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Gibco BRL) was added and the sample was centrifuged for 2 minutes at 15,000 rpm at 4°C. The aqueous phase was extracted and transferred to a pre-chilled microfuge tube. One-tenth the volume of 3M sodium acetate (pH 5.2) was mixed into each sample. The DNA was precipitated at -80°C for 15 minutes with ice-cold 70% ethanol. After centrifuging at 15,000 rpm at 4°C the pellet was dried using a speed vac (Savant SC200) and resupended in 5µl of cold Tris-EDTA (pH 8.0).

Blunt ends were created using the Klenow fragment (Gibco BRL) to fill in the 5' overhangs, generated by the restriction endonucleases, on each end of the Gag-PKB

fragment and the linearized pMMTV vector. 1 unit of Klenow fragment was added per μg of DNA used. The 15 μl reaction mixture, containing1 μl of each dNTP (0.5mM each) and 1.5 μl of Klenow reaction buffer (Gibco BRL), was incubated for 10 minutes at 30°C. The reaction was stopped by heating for 10 minutes at 75°C. The DNA was isolated by phenol-chloroform extraction as described earlier.

T4 DNA ligase (Gibco BRL) was used to catalyze the formation of phosphodiester bonds between the Gag-PKB fragment and linearized pMMTV vector. 50 ng of pMMTV plasmid DNA and 500 ng of the Gag-PKB insert were used for the ligation reaction. The 5 μl reaction mixture containing 1 unit of T4 DNA ligase and 0.5 μl of 10x Reaction buffer was incubated overnight at room temperature. The DNA was isolated by phenol-chloroform extraction as described earlier. After the ligation reaction the resulting DNA was transformed into E.coli as described earlier and plasmid DNA was isolated using the Qiagen Mini-prep kit.

Positive colonies were screened by digestion with XhoI which cuts only in the Gag-PKB insert and not in the vector. To determine if the insert was in the proper orientation, Afl3 was used since it cleaves once in the vector and once in the insert. The Afl3 digest produces two fragments of different size (5.7 Kb and 1.1 Kb) if Gag-PKB is inserted in the correct direction. After identifying the colony containing the plasmid with Gag-PKB placed downstream of the MMTV promoter (pMMTVGag-PKB), a maxi-prep was performed to obtain the necessary quantities of the plasmid DNA (Qiagen Maxi-prep kit).

Transfection of 3T3-L1 preadipocytes

Transfections were performed by the $CaPO_4$ technique as described (Colbere-Garapin F and Garapin AC, 1983). A Stratagene mammalian transfection kit was used. 3T3-L1 preadipocytes of low passage number were plated 24 h before transfection (100,000 cells/60-mm plate) in 10%CS DMEM and grown for 24 h. The medium was changed to DMEM with no CS and the DNA was added as a coprecipitate with calcium phosphate for 4 h. For cotransfection, 2 μg of pMEXneo was mixed with 10 μg of pSG5Gag-PKB (Gag-PKB cells), pSG5Gag (Gag cells), pMMTV (MMTVcontrol cells), or pMMTVGag-PKB (MMTVGag-PKB cells). For the pMexneo control transfection, 4μg of pMEXneo plasmid DNA was used alone. Total DNA per plate was 20 μg, with addition of salmon sperm DNA (Gibco BRL), to optimize transfection efficiency. The cells were rinsed and incubated in 10 %CS DMEM. After subpassaging the cells once, the antibiotic, geneticin (G418) (Gibco BRL) was added to the medium at 400 μg/ml to select for colonies that had stably integrated the constructs. The cells were selected every 4 days. At 18 days representative plates were stained with Oil Red O. For colonies which were propogated to form stable cell lines, a cloning ring was used for the initial subpassage. Individual clones were stored in liquid N_2.

To induce Gag-PKB expression, MMTVGag-PKB cells were treated at 100% confluence with 1μM dexamethasone for 4 days. The effects of inhibitors, wortmannin (Kamiya) or rapamycin (Calbiochem), on dexamethasone induced adipocyte differentiation of MMTVGag-PKB(6) were tested by supplementing the medium with appropriate amounts of either one of these inhibitors to obtain a final concentration of 100 nM. Wortmannin is an non-competitive inhibitor of PI 3-kinase (Ui et al, 1995). It binds covalently to the p110 catalytic subunit of PI 3-kinase. Rapamycin inhibits the activation of p70 S6 kinase by binding to the upstream activating protein, TOR (Chung et al, 1994) . It

is believed to very specific for p70 S6 kinase since it does not inhibit the closely related p90 S6 kinase protein (Chou MM and Blenis J, 1995). The inhibitors were added at the start of dexamethasone treatment and the same concentrations of drugs were supplemented everyday for 8 days.

Oil Red O Staining

Oil Red O stains neutral lipids (Fried B and Butler MS, 1977; Horobin RW, 1981). Cells were washed with PBS and fixed for 2 h in 10% formalin. A 0.35% Oil Red O isopropanol solution was diluted with an equal volume of water, filtered and added to the fixed cells for 2 h. After washing, the cells were incubated overnight at 4°C with Giemsa stain (Gibco BRL), which serves to outline the general morphology of the cells. Cells were washed, and stained triglyceride droplets were visualized by light microscopy and then photographed.

Isolation of Total RNA

Total RNA was isolated by the guanidine thiocyanate method (Chomczynski P and Sacchi N, 1987). The cells were rinsed twice with cold PBS and lysed on ice for 5 min in 600ul of GTC solution (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7.0; 05% sarcosyl, 0.1M 2-mercaptoethanol). After scraping wth sterile rubber or disposable cell scraper, the cell lysates were transferred to a microfuge tube and snap-frozen in liquid nitrogen for storage at -80°C. Cell lysates were sheared through a 24 gauge needle 4-5 times, on ice. 60 ul of 2M sodium acetate (pH 4.0), 600 ul of phenol (water-saturated) and 120 ul of chloroform-isoamyl alcohol mixture (49:1) was added to each sample. The samples were vortexed thoroughly and incubated for 10 min on ice. After centrifuging at

10,500 rpm at 4°C for 30 min, the cellular DNA was separated into the acid phenol phase and the proteins were in the interphase layer. The upper aqueous layer containing the RNA was transferred to a clean eppendorf tube and mixed with an equal volume of isopropanol . RNA was precipitated overnight at -20°C. After centrifuging for 30 min at 15,000 rpm at 4°C, the resulting pellet was resuspended in 100 ul of GTC solution and precipitated with 300 ul of ethanol (3x volume), vortexed, and precipitated for 2 hours at -80°C. After centrifuging at 15,000 rpm, 4°C for 30 min., the pellet was washed with 70 % ethanol. The resulting RNA pellet was air-dried and dissolved in DEPC-treated H_2O. Samples were quantitated by a spectrophotometer (Milton Roy Spectronic 601) and only those with a A260/A280 ratio of > 1.8 were analysed further. An aliquot of each sample was resolved by agarose gel electrophoresis, and the intergrity of the purified RNA was confirmed by the presence of 28S and 18S rRNA bands, detected by ethidium bromide staining.

Plasmids Used as Probes

LPL and aP2 probes were kindly provided by M.C. Scholtz and H. Green, respectively. We thank B.M. Spiegelman for providing us with the 36B4 probe (Tontonoz et al, 1994). 36B4 serves as a control gene probe for RNA loading. It recognizes the acidic ribosomal phosphoprotein PO (Laborda J, 1991).

The cDNAs for LPL, aP2 and 36B4 were isolated by restriction endonuclease digestion and agarose gel electrophoresis. After purifying the insert from the gel, 25 ng of the cDNA was labeled with [α-^{32}P]dCTP (3000 Ci/mmol) by the random-priming method (Fineberg and Vogelstein, 1984) to a specific activity of at least 10^9 cpm/μg. The probes were labelled using the multiprime DNA labelling system (Amersham).

Northern Blotting Analysis

For Northern blot analysis, total RNA (17µg/lane) was denatured by heating to 55°C x 15 min in formaldehyde/formamide and resolved by electrophoresis on 1% agarose-formaldehyde gels. The RNA on the gel was visualized with ethidium bromide to verify equal loading before transferring to Hybond-N membrane in 20X SSC (3M NaCl and 0.3 M Na citrate). The membrane was baked for 2 h at 80°C and then hybridized in 5X SSC pH 7.0, 5X Denhardt's solution, 0.5% SDS with 32P-labeled probes overnight at 60°C. After several washes with a final stringency of 0.1X SSC at 60°C, the blot was exposed to Kodak X-AR film.

RESULTS

Effects of insulin on IRS-1, PI 3-kinase and PKB in 3T3-L1 preadipocytes

Before studying the effects of insulin on PKB activity in 3T3-L1 preadipocytes, we initially confirmed that IRS-1 was phosphorylated following insulin stimulation. 3T3-L1 preadipocytes were grown to confluence and were incubated with or without 100 nM insulin for 5 minutes as described in "Methods". Cellular proteins were then solubilized in Laemmli buffer (Laemmli UK, 1970). After separating the proteins on 7.5% polyacrylamide gels, and transferring to nitrocellulose, the blots were probed with anti-phosphotyrosine antibody (αPY). The results (Figure 3) demonstrate the expected increase in tyrosine phosphorylation of IRS-1 upon insulin stimulation.

An *in vitro* assay was performed to verify that insulin stimulated the association of IRS-1 with PI 3-kinase. 3T3-L1 preadipoctyes were grown to confluence and stimulated with or without 100 nM insulin for 5 minutes as described in "Methods". Preadipocytes were lysed, immunoprecipitated with anti-IRS-1 or anti-phosphotyrosine antibody, and immunoprecipitates were subjected to the PI 3-kinase assay as described in "Methods". In this *in vitro* assay, PI 3-kinase phosphorylates phosphatidylinositol (PI) at the 3 position of the inositol ring to produce PI 3-phosphate. The production of radiolabeled PI 3-phosphate was analyzed by thin-layer chromatography as seen in Figure 4. Insulin-stimulated association of PI 3-kinase with IRS-1 was observed in anti-phosphotyrosine and anti-IRS-1 immunoprecipitates.

Immunoblot analysis was performed to determine whether PKB was differentially expressed in 3T3-L1 preadipocytes versus adipocytes. 3T3-L1 preadipocytes were grown to confluence and lysed directly or differentiated over 8 days prior to lysis, using the differentiation protocol as described in "Methods". Proteins were separated by 10% polyacrylamide gels, and transferred to nitrocellulose, and the blot was probed with anti-PKB antibody. A band representing PKB was detected with equal intensity in both preadipocytes and adipocytes (Figure 5). This band migrated at 60 kDa as expected (Burgering et al, 1995). To account for the change in cell number during adipocyte differentiation, DNA was isolated from preadipocytes and adipocytes, and volumes representative of equal cell number based on the amount of DNA were used.

The activity of PKB in insulin-treated and untreated preadipocytes was measured using an *in vitro* assay. Confluent preadipocytes were stimulated with or without 100 nM insulin for 5 min and lysed as described in the "Methods". Samples were immunoprecipitated with anti-PKB antibody and then assayed for PKB activity. The assay measures the ability of PKB to phosphorylate the synthetic peptide, "crosstide" (Cross et al, 1995), which is based on the sequence surrounding its serine phosphorylation site in GSK-3 (serine-21 of GSK-3α and serine-9 of GSK-3β). Insulin induced a 4 fold increase in PKB activity as seen in Figure 6A . Immunoprecipitates from cell lysates were also taken for immunoblot analysis with anti-PKB antibody, which confirmed that equal amounts of PKB were immunoprecipitated (Figure 6B). A sample containing purified rabbit IgG antibody (5 μg) with no anti-PKB antibody was used as negative control for specificity of the PKB antibody.

Figure 3 - Effect of Insulin on Tyrosine Phosphorylation of IRS-1

Confluent 3T3-L1 preadipocytes were incubated with (+) or without (-) 100 nM insulin for 5 minutes. Proteins in cell lysates were resolved by SDS-PAGE (7.5% polyacrylamide) and electrophoretically transferred to a nitrocellulose membrane. Immunoblotting was performed using anti-phosphotyrosine (PY20) antibody followed by detection with peroxidase-linked secondary antibody and ECL. IRS-1 migrates at 185 kDa as indicated.

Figure 3

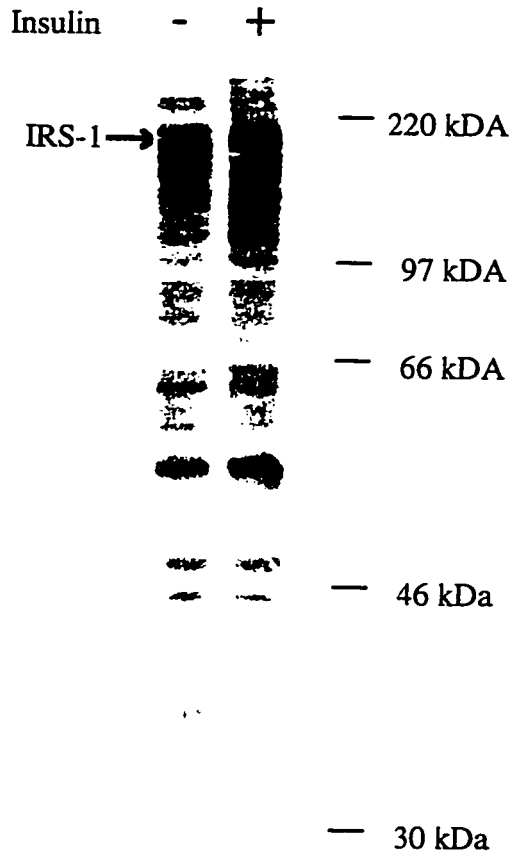


Figure 4 - Insulin-Stimulated Association of IRS-1 and PI 3-kinase

Confluent 3T3-L1 preadipocytes were incubated with (+) or without (-) 100nM insulin for 5 minutes. Proteins in cell lysates were immunoprecipitated with anti-phosphotyrosine (PY20) or anti-IRS-1 antibody. Immunoprecipitates were subjected to a PI 3-kinase assay as described in the "Methods". The production of radiolabeled PI 3-phosphate (PI-3-P) was analyzed by thin-layer chromatography. The origin of the chromatogram and the position of PI-3-P are indicated.

Figure 4

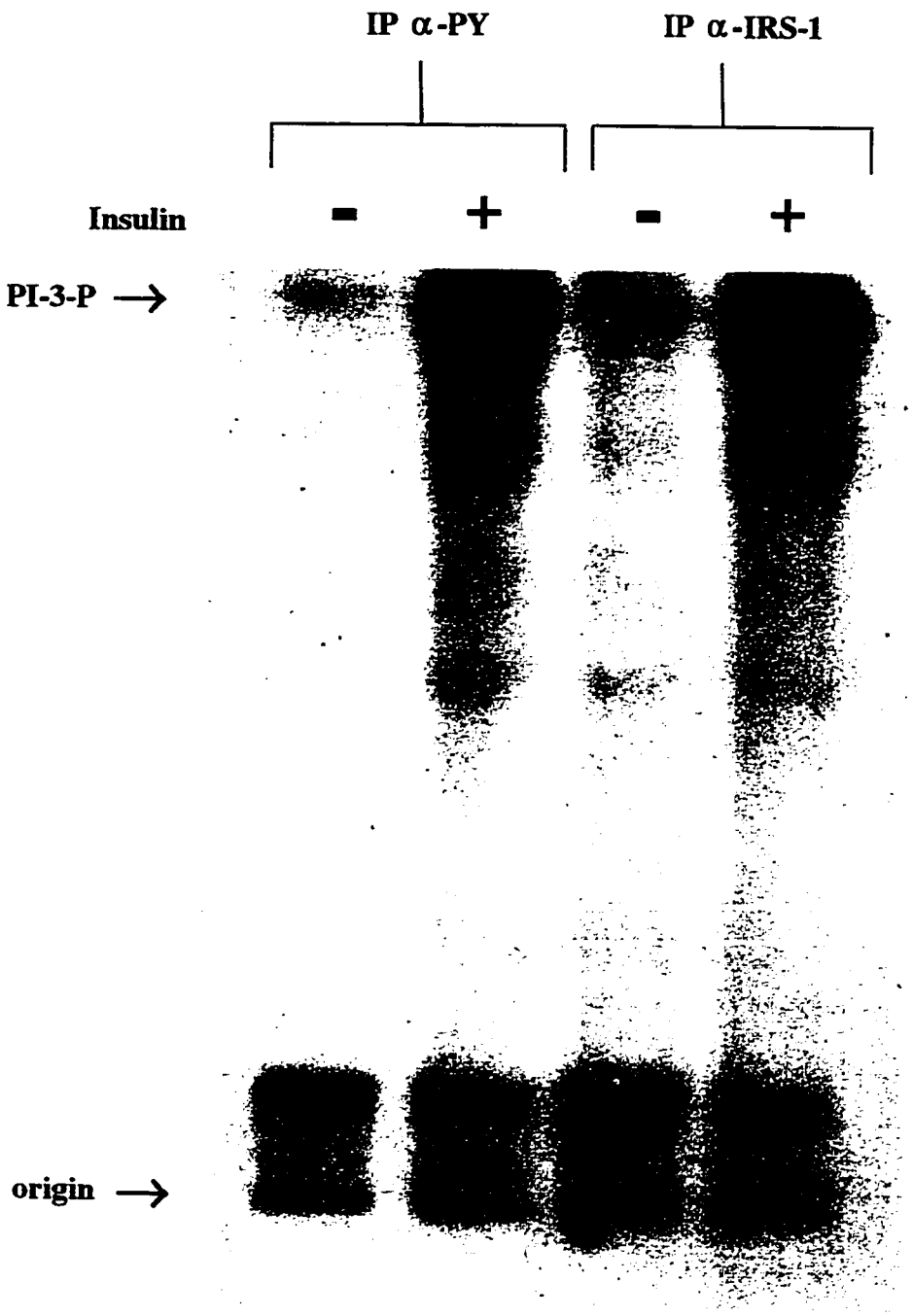


Figure 5 - Expression levels of PKB in 3T3-L1 Preadipocytes and Adipocytes

3T3-L1 cells were maintained as preadipocytes (1) or were differentiated over 8 days into adipocytes (2). Proteins in cell lysates were resolved by SDS-PAGE (10% polyacrylamide) and electrophoretically transferred to a nitrocellulose membrane. Expression of PKB was visualized by immunodetection (ECL) using anti-PKB antibody.

Figure 5

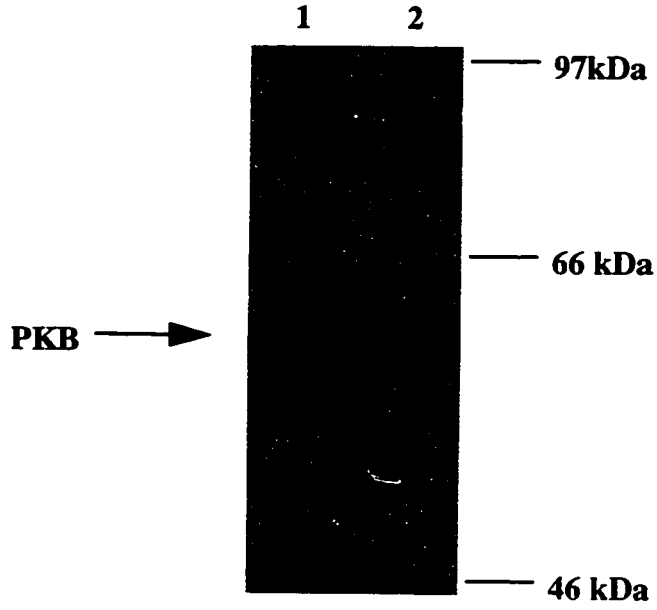
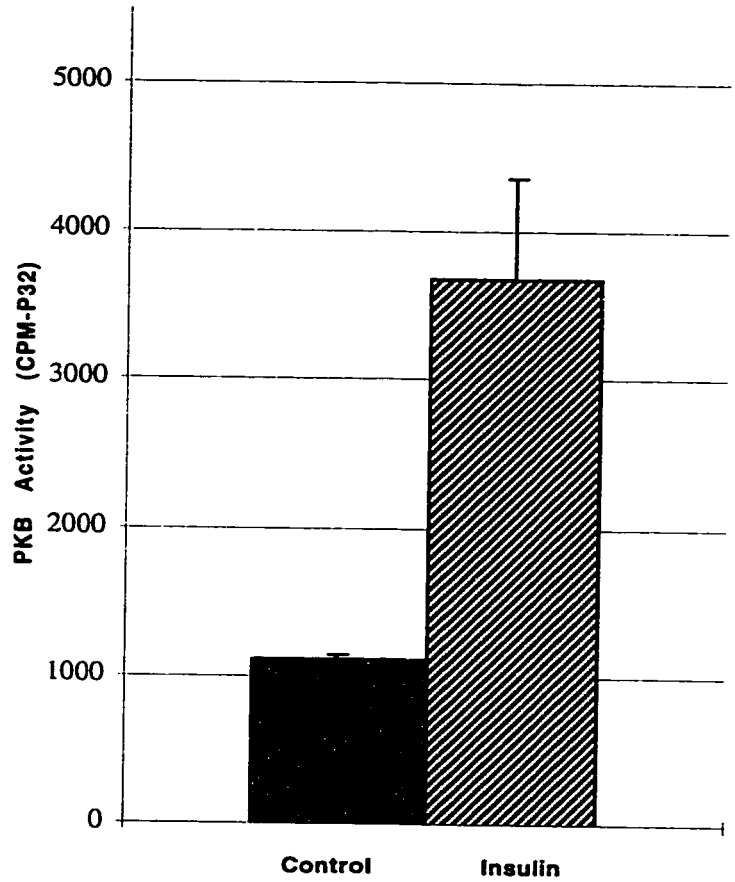


Figure 6 - Insulin Activation of Endogenous PKB in 3T3-L1 Preadipocytes

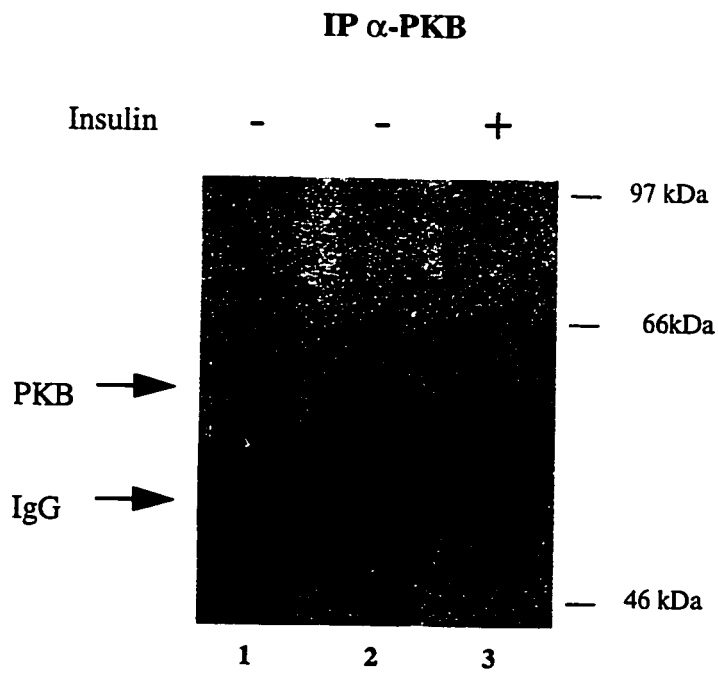
Confluent 3T3-L1 preadipocytes were incubated with or without (control) 100nM insulin for 5 minutes. PKB was immunoprecipitated from cell lysates with ant-PKB antibody and activity was measured as described in "Methods". The assay was performed for 10 minutes at 30 °C. Each bar represents the mean of two experiments ± range (A). Immunoprecipitates from cell lysates were subjected to immunoblot analysis (B) with anti-PKB antibody as described in the "Methods" to confirm equal amounts of immunoprecipitated PKB (Lane 2, 3). Lane 1 represents rabbit IgG antibody only as a negative control.

Figure 6

A


5000
4000
3000
2000
1000
0
PKB Activity (CPM-P32)
Control
Insulin

B


IP α-PKB
Insulin - - +
97 kDa
66kDa
PKB
IgG
46 kDa
1 2 3

Expression of Constitutively Activated PKB in 3T3-L1 Preadipocytes and Analysis of Colonies

To test whether a constitutively activated form of protein kinase B, Gag-PKB, is sufficient to induce 3T3-L1 adipocyte differentiation, the following strategy was used (Figure 7). Transfection of 3T3-L1 preadipocytes with plasmid vectors, pMexneo and co-transfection of pSG5Gag and pMexneo, served as controls for the co-transfection of pMexneo and pSG5Gag-PKB constructs. The plasmid pSG5Gag-PKB produces a fusion protein in which Gag is fused upstream of PKB, which is constitutively active (Burgering BMT and Coffer PJ, 1995). After geneticin (G418) selection, colonies were stained with Oil Red O lipid stain in order to assess differentiation. In addition, colonies were pooled and taken either for immunoblot analysis to confirm expression of Gag-PKB or Northern blot analysis to evaluate expression of the differentiation-specific mRNA markers, LPL and aP2.

During G418 selection, surviving colonies emerged one week after the initial transfection. Gag-PKB cells assumed the rounded morphology characteristic of adipocytes, and they accumulated lipid progressively over the next ten days. This was visualized by phase-contrast microscopy and by staining fixed cultures for lipid with Oil Red O (Figure 8). Figure 8A depicts a representative colony surviving G418 selection from the control transfection (pMexneo), which stained negatively for Oil Red O. In comparison, colonies surviving G418 selection from the Gag-PKB transfection (B) were observed to spontaneously differentiate without the addition of insulin. These cells stained positively for Oil Red O. Although control colonies could be successfully subpassaged, Gag-PKB colonies could not be propagated in this manner and therefore could not be rescued for further analysis. This suggests that Gag-PKB cells entered a terminal differentiation

program. Therefore, colonies that emerged from each transfection were lysed directly and pooled for both the immunoblot and Northern blot analyses.

Immunoblot analysis of total cell lysates taken from pooled colonies from the control versus Gag-PKB transfections was performed with anti-PKB antibody. A specific band at 85 kDa representing the Gag-PKB fusion protein was detected in Gag-PKB total cell lysates but not in control total cell lysates (Figure 9). An intense band at 60 kDa representing endogenous PKB was observed in both control and Gag-PKB lanes.

To substantiate further that Gag-PKB cells were undergoing adipogenesis, we isolated total RNA from control and Gag-PKB pooled colonies, and measured levels of mRNA expression for two specific markers of adipocyte differentiation, LPL and aP2. Figure 10A demonstrates equal loading of RNA in all lanes as assessed by 28S and 18S bands. Figure 10B shows the Northern blot after hybridization with probes for LPL and aP2, as indicated. Two separate pools of Gag-PKB colonies demonstrate induction of LPL (lane 1,2). aP2 induction is seen in one of the two pools (lane 2). No such induction of LPL or aP2 was observed for control cells (lane 3). 36B4 serves as a control gene probe for the amount of RNA loaded.

As stated earlier, colonies emerging from the pMexneo transfection were able to be subpassaged, which allowed for the formation of stable cell lines. These stable cell lines were completely capable of undergoing adipocyte differentiation when exposed to the complete differentiation protocol containing insulin . After 8 days, cells were stained with Oil red O lipid stain to reveal the lipid contents in the cells. The pMexneo stable cell lines were shown to differentiate to a similar extent as the 3T3-L1 untransfected wild-type cells, as seen in Figure 11A.

A co-transfection of pMexneo and an excess of pSG5-Gag (Gag cells) was also performed as a control. Surviving colonies did not spontaneously differentiate. Gag cells were able to be subpassaged which allowed for the formation of stable cell lines. Colonies were screened for expression of Gag-protein by immunoblot analysis using an anti-Gag antibody (Figure 11B). Clones 1, 2 and 3 were shown to express the highest level of Gag protein. Each of these clones differentiated to a similar extent as wild-type 3T3-L1 cells when subjected to the full differentiation protocol as seen by Oil Red O lipid stain. Differentiation of Gag clone 2 is shown in Figure 11A.

Therefore the lack of spontaneous differentiation by control and Gag cells was not because they lost their capacity to differentiate altogether, since they could still differentiate with the standard differentiation protocol.

Figure 7 - Transfection Strategy and Analysis of Colonies

The strategy illustrated in this figure was used to determine whether a constitutively activated form of protein kinase B, Gag-PKB, is sufficient to induce 3T3-L1 adipocyte differentiation. Transfection of pMexneo alone and co-transfection of pSG5Gag and pMexneo served as controls for the co-transfection of pSG5Gag-PKB and pMexneo. After G418 selection, surviving colonies were stained with Oil Red O lipid stain. Pooled colonies were taken either for immunoblot analysis with anti-PKB antibody or Northern blot analysis to evaluate the expression level of differentiation-specific markers, LPL and aP2.

Figure 7

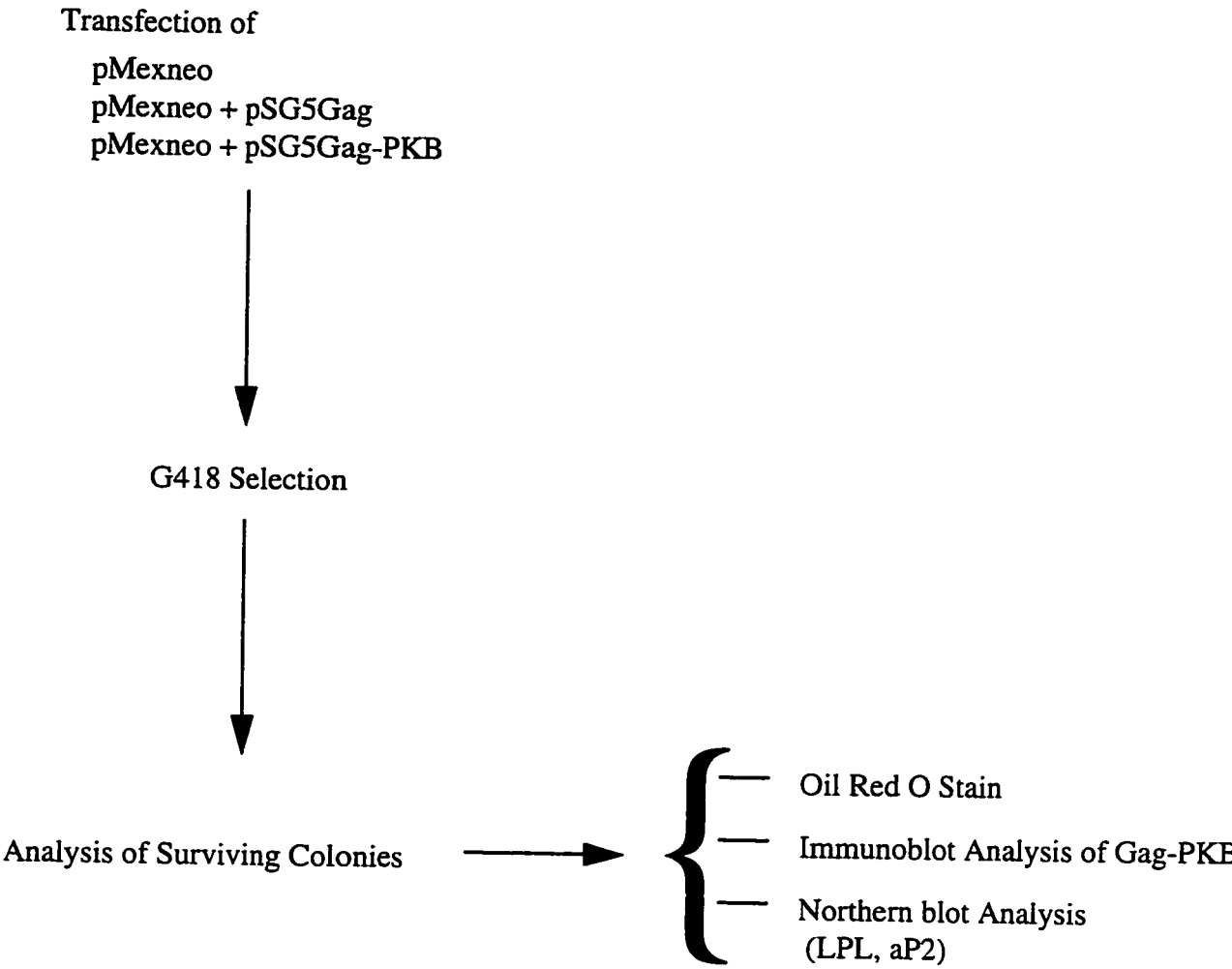
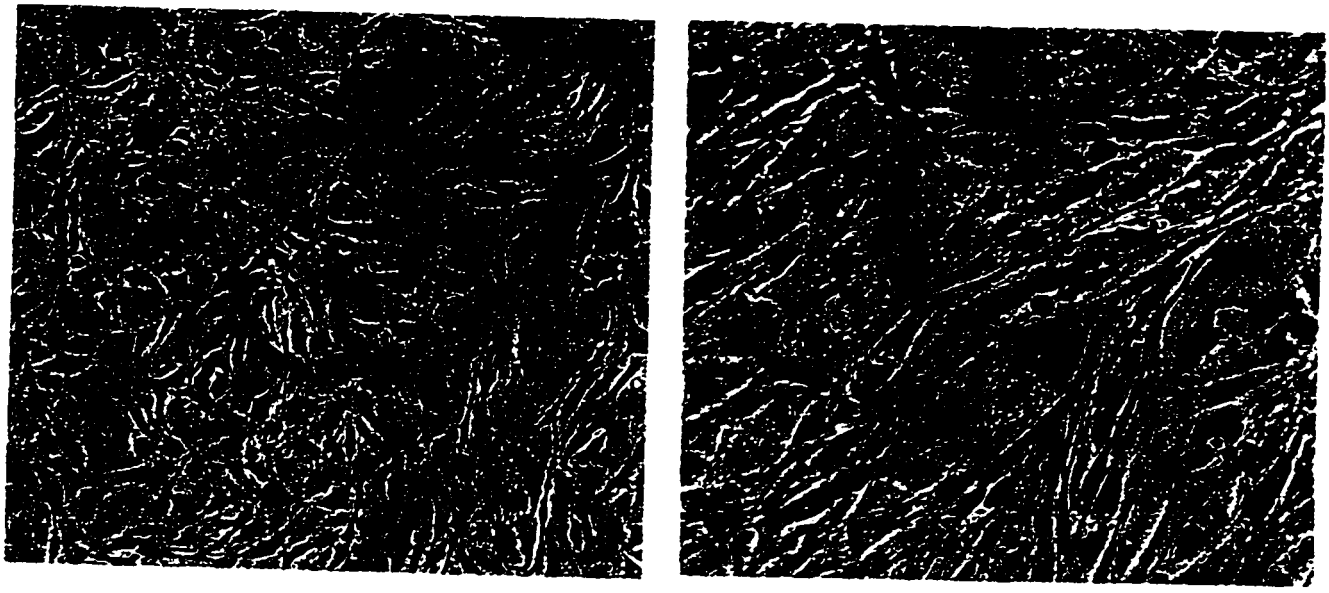

Transfection of
pMexneo
pMexneo + pSG5Gag
pMexneo + pSG5Gag-PKB
G418 Selection
Analysis of Surviving Colonies
Oil Red O Stain
Immunoblot Analysis of Gag-PKB
Northern blot Analysis
(LPL, aP2)

Figure 8 - Spontaneous Differentiation of 3T3-L1 Cells Transfected with Gag-PKB.

3T3-L1 preadipocytes were transfected as described in "Methods" and subjected to G418 selection: control cells (A), Gag-PKB cells (B). At 18 days post transfection, cells were stained with lipid-specific Oil Red O as described. Representative fields are shown here at 100x magnification. The results shown here are representative of 4 independent experiments.

Figure 8



A

B

Figure 9 - Gag-PKB Expression in Pooled Colonies from Control and Gag-PKB Transfections

Total cellular protein extracts from control (lane A) and Gag-PKB (lane B) cells (pooled colonies) were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-PKB antibody as described. The positions of the molecular weight markers, Gag-PKB and endogenous PKB are indicated.

Figure 9

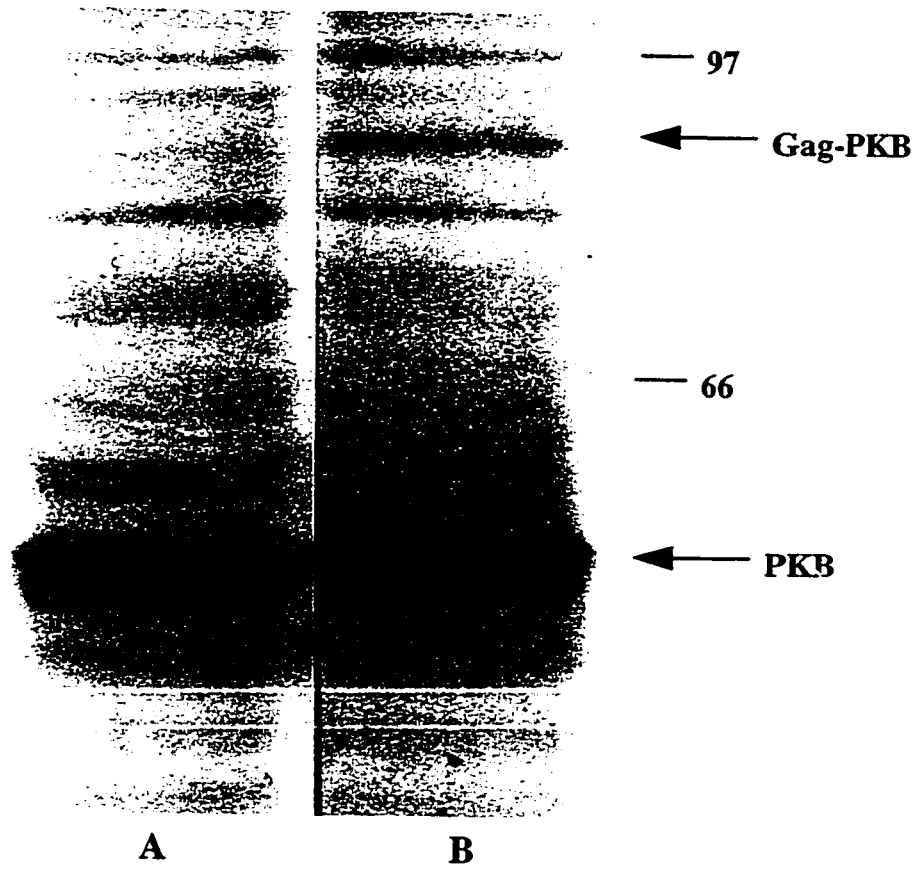


Figure 10 - Northern Blot Analysis Showing Induction of LPL and aP2 mRNA Expression in Gag-PKB cells

Total cellular RNA was isolated from control and Gag-PKB cells after 18 days of G418 selection. (A) 17 μg of RNA was resolved on a formaldehyde gel as described. Lane 1,2 - RNA from two separate pools of Gag-PKB cells, lane 3 - RNA from pooled control cells. Equivalent amounts of 28S and 18S RNA appear in each lane. (B) RNA was then transferred to Hybond nylon membrane, and hybridized with 32P-labelled probes for aP2, LPL, and 36B4 (a further control for amount of RNA), as described. Lane 1,2 and 3 designated as above.

Figure 10

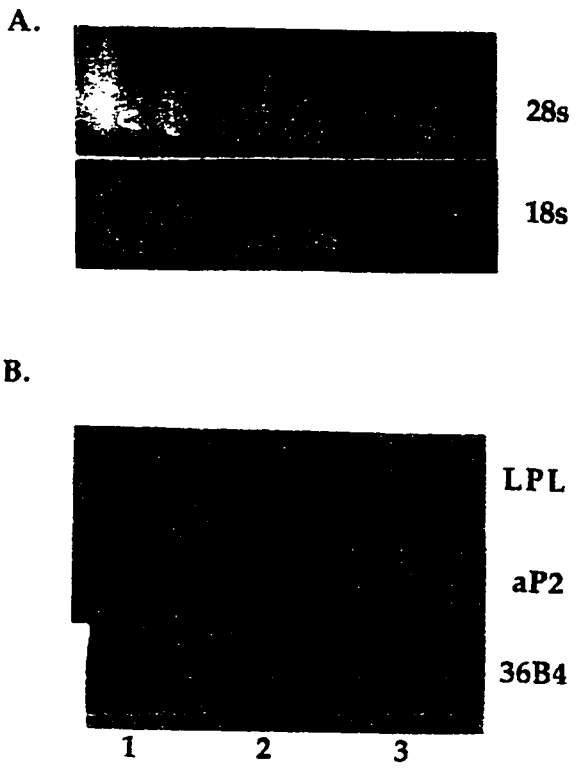
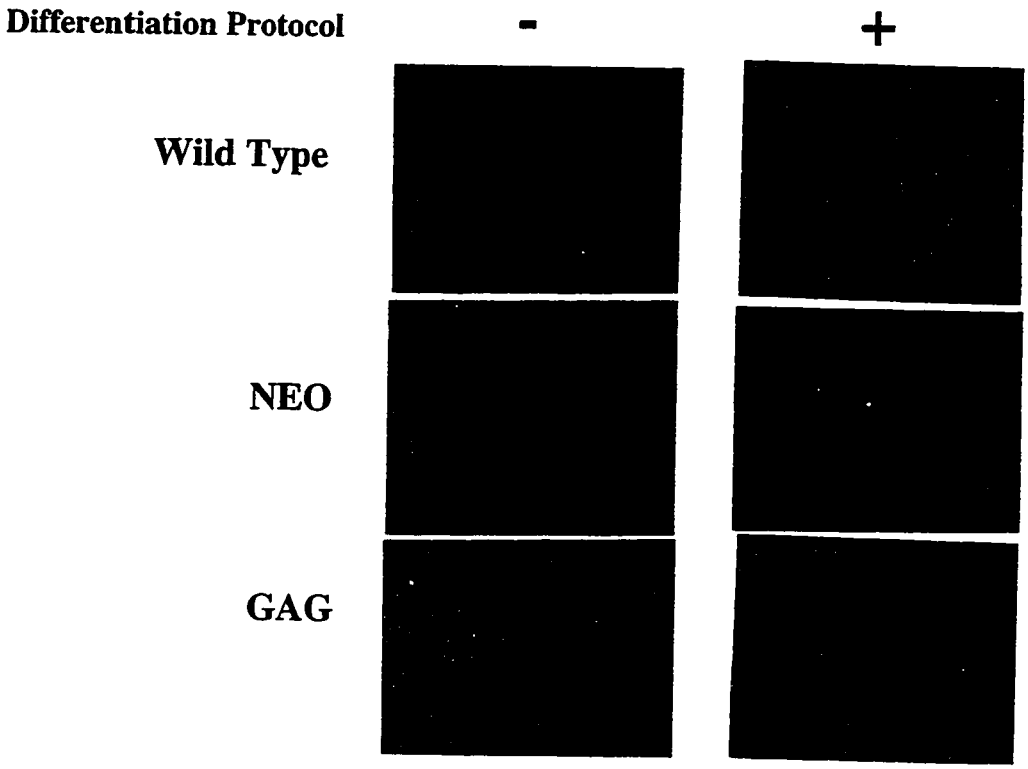


Figure 11 - Differentiation of 3T3-L1 pMexneo, pSG5Gag and Wild type Preadipocytes

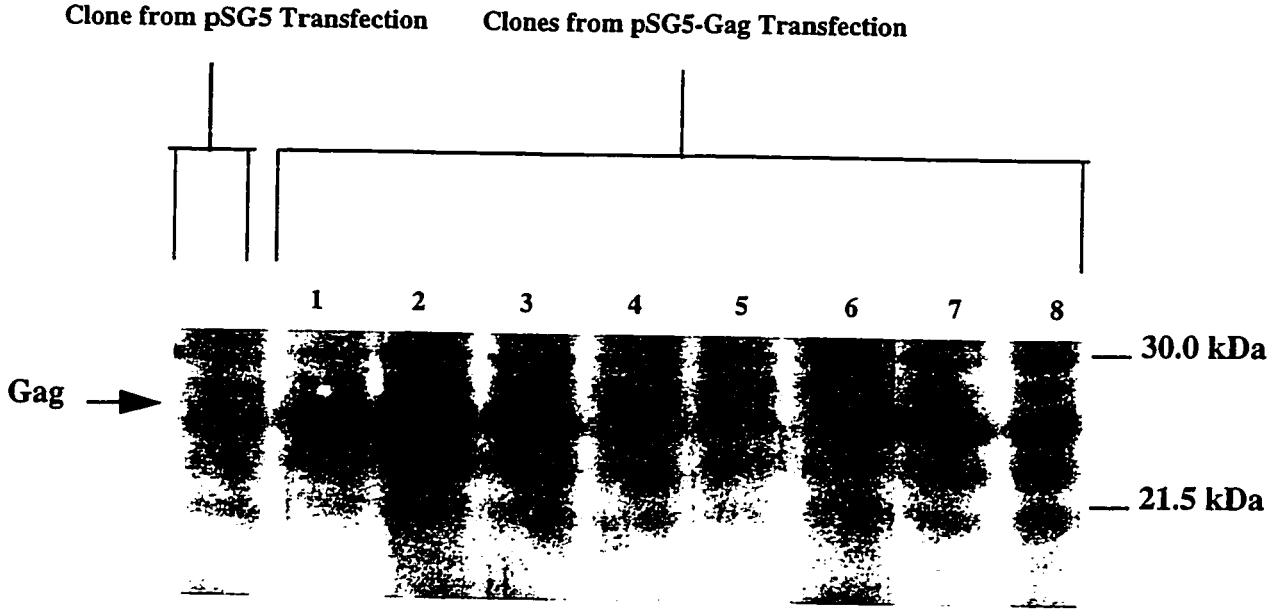
3T3-L1 preadipocytes were transfected as described in "Methods" and subjected to G418 selection. Stable lines were generated from the pMexneo transfection and the cotransfection of pSG5Gag and pMexneo. Transfected cells were then exposed to the standard differentiation protocol (+) as described in the "Methods" or cells were incubated with media containing 10% calf serum only (-) for the same period. After 8 days, cells were stained with Oil Red O to determine the lipid content of the cells (A). The images shown here are representative of 2 independent experiments (100x magnification). Colonies from the pSG5Gag transfection were screened for Gag protein expression levels (B). Proteins in cell lysates were fractionated by SDS-PAGE (15% polyacrylamide) and electrophoretically transferred to a nitrocellulose membrane. Immunodetection was performed using anti-Gag antibody followed by ECL. Gag protein migrates at approximately 26 kDa as indicated.

Figure 11

A


Differentiation Protocol
-
+
Wild Type
NEO
GAG

B


Clone from pSG5 Transfection
Clones from pSG5-Gag Transfection
1
2
3
4
5
6
7
8
Gag
30.0 kDa
21.5 kDa

Construction and Transfection of a Plasmid Vector in which Gag-PKB is under the Control of an Inducible Promoter.

Gag-PKB was placed downstream of the dexamethasone-responsive Mouse Mammary Tumour Virus-Long Terminal Repeat (MMTV-LTR) promoter. Since dexamethasone is normally added along with MIX and insulin to wild-type 3T3-L1 for the first two days of the differentiation protocol, we initially determined whether dexamethasone alone can induce adipocyte differentiation. Wild-type 3T3-L1 preadipocytes were grown to confluence and incubated with or without 1μM dexamethasone for 8 days and then stained with Oil Red O. No differentiation was observed either in the presence or the absence of dexamethasone (Figure 12).

Gag-PKB cDNA was subcloned successfully into the pMMTV vector using blunt-end ligation . Restriction digestion of the newly constructed pMMTVGag-PKB plasmid confirmed that Gag-PKB was placed in the correct orientation downstream of the MMTV-LTR promoter (Figure 13).

Following construction of the vector, pMMTVGag-PKB, a number of transfections were performed. Co-transfection of pMexneo and an excess of pMMTV vector with no insert (Control cells) served as controls for the co-transfection of pMexneo and an excess of pMMTVGag-PKB (MMTVGag-PKB cells) into 3T3-L1 preadipocytes. During G418 selection, surviving colonies emerging from each transfection were subpassaged before reaching confluence over the next 21 days to eventually form individual stable transfectants.

At this point, preliminary characterization of the clones was performed. Individual clones were grown to 100% confluence and screened for Gag-PKB expression by incubation with 1μM Dexamethasone for 4 days, followed by immunoblot analysis using

anti-PKB rabbit polyclonal antibody. Clone 6 had the highest level of Gag-PKB expression as indicated in Figure 14 and thus it was chosen for further studies (designated MMTVGag-PKB(6) cells). No expression of Gag-PKB was seen in the control cells. As expected, Gag-PKB and endogenous PKB were observed to be migrating at approximately 85 kDa and 60 kDa, respectively.

To determine whether we could control the expression of Gag-PKB, MMTVGag-PKB(6) cells were grown to 100% confluence and incubated with increasing concentrations of dexamethasone for a 4 day period. As seen in Figure 15, when the dexamethasone concentration was increased, the expression of Gag-PKB increased. Although there is some expression of Gag-PKB in the absence of dexamethasone, this level was not sufficient to induce adipocyte differentiation (addressed further in discussion).

MMTVGag-PKB(6) cells were grown to 100% confluence, incubated with or without dexamethasone (1μM) for 8 days, and stained with Oil Red O to determine if adipocyte differentiation can be induced. As seen in Figure 16, MMTVGag-PKB(6) cells differentiated into adipocytes in the presence of dexamethasone. In the absence of dexamethasone, the MMTVGag-PKB(6) cells did not differentiate . Control MMTV cells did not differentiate in the presence or absence of dexamethasone. MMTVGag-PKB(6) cells in the presence of dexamethasone as well as the PI 3-kinase inhibitor wortmannin, were still able to differentiate into adipocytes. However, when the p70 S6 kinase inhibitor rapamycin, was added with dexamethasone, there was a potent inhibition of adipocyte differentiation.

Figure 12 - Dexamethasone Is Not Able To Induce Adipocyte Differentiation of Wild-type 3T3-L1 Preadipocytes

3T3-L1 preadipocytes were grown to confluence and incubated with or without dexamethasone (1μM) for 8 days and stained with Oil Red O. No differentiation was observed in the presence (B) or absence of dexamethasone (A). However, as a positive control, 3T3-L1 preadipocytes were fully capable of differentiating in the presence of insulin (100 nM) (C). Representative fields are shown here at 100x magnification.

Figure 12

A

B

C

Figure 13 - Restriction Digest of plasmid vector containing Gag-PKB under the control of the Dexamethosone Inducible Mouse Mammary Tumour virus promoter

Gag-PKB cDNA was subcloned into a vector so that it is under the control of the dexamethasone-responsive mouse mammary tumour virus promoter (MMTV-LTR). This involved a blunt-end ligation strategy and restriction digests confirmed the proper orientation of the insert into the dexamethasone-inducible expression vector (pMMTVGag-PKB). Two bands of correct size from the Afl3 digest indicate the presence of Gag-PKB insert in the correct orientation (Lane 2). Lane 3 represents linearized plasmid of appropriate size from the Xho I digest indicating the presence of Gag-PKB insert (pMMTVGag-PKB). Lane 4 represents uncut pMMTVGag-PKB vector and Lane 1 represents Hind III λ markers. A 1.0% agarose gel was used.

Figure 13

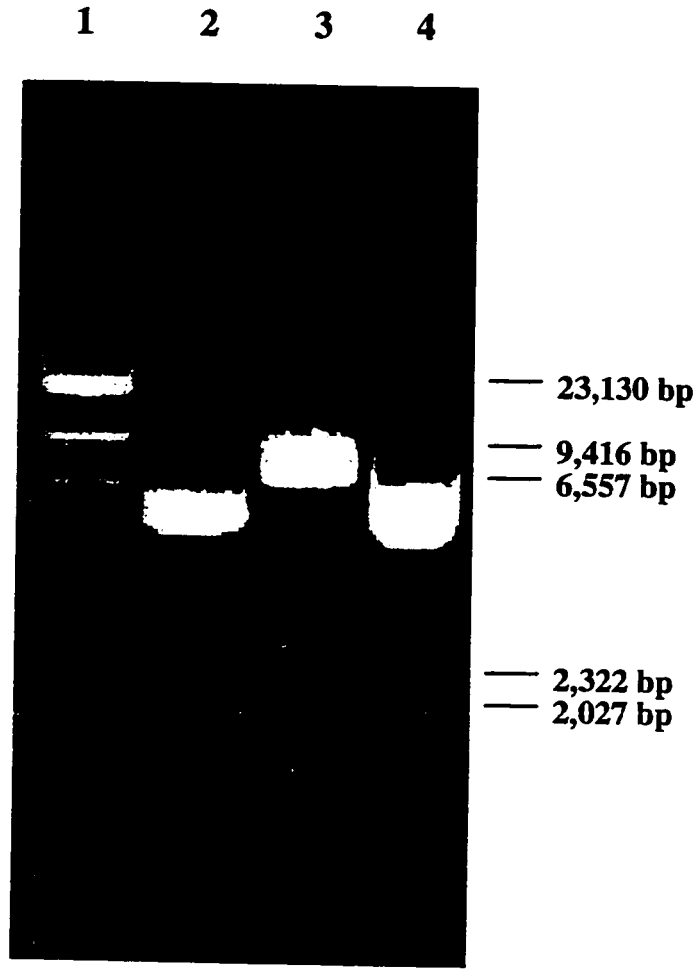


Figure 14 - Screening of Clones From the pMMTVGag-PKB transfection for Gag-PKB Protein Expression Levels

Clones from the pMMTVGag-PKB transfection (MMTV-Gag-PKB cells) and a clone from the pMMTV-no insert transfection (Control cells) were grown to confluence and incubated with 1μM Dexamethasone for 72 hours. Proteins in cell lysates were resolved by SDS-PAGE (10% polyacrylamide) and electrophoretically transferred to a nitrocellulose membrane. Immunodetection was performed using anti-PKB antibody followed by ECL. Gag-PKB protein migrates at approximately 85 kDa whereas endogenous PKB is shown to migrate at 60 kDa as indicated. Clone 6 was shown to have the highest Gag-PKB expression and was therefore used for further studies.

Figure 14

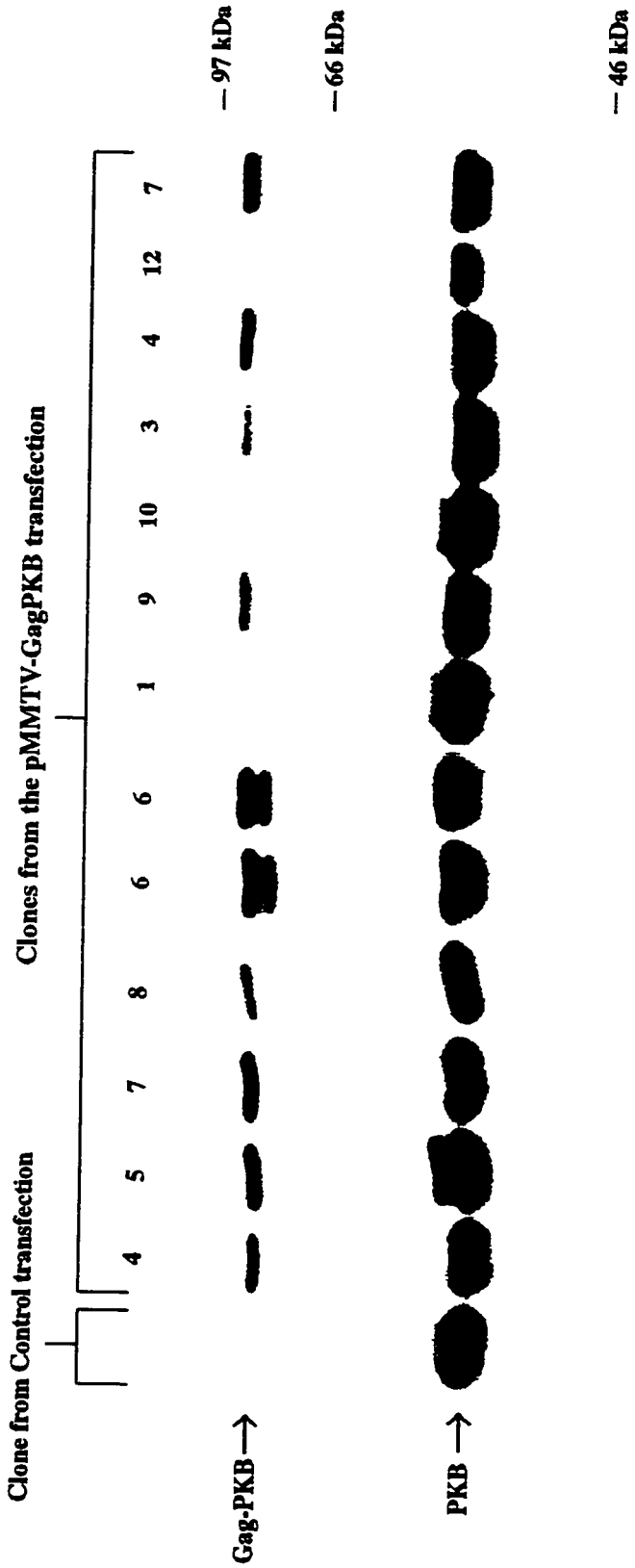

Clone from Control transfection
Clones from the pMMTV-GagPKB transfection
4
5
7
8
6
6
1
9
10
3
4
12
7
97 kDa
66 kDa
46 kDa
Gag-PKB
PKB

Figure 15 - Induction of Gag-PKB expression by Dexamethasone in MMTVGag-PKB(6) cells

MMTVGag-PKB(6) cells or Control cells were grown to confluence and incubated with or without dexamethasone for 4 days at various concentrations as indicated. Proteins in cell lysates were resolved by SDS-PAGE (10% polyacrylamide) and electrophoretically transferred to a nitrocellulose membrane. Immunodetection was performed using anti-PKB antibody. Gag-PKB and endogenous PKB migrate at approximately 85 kDa and 60 kDa, respectively.

Figure 15

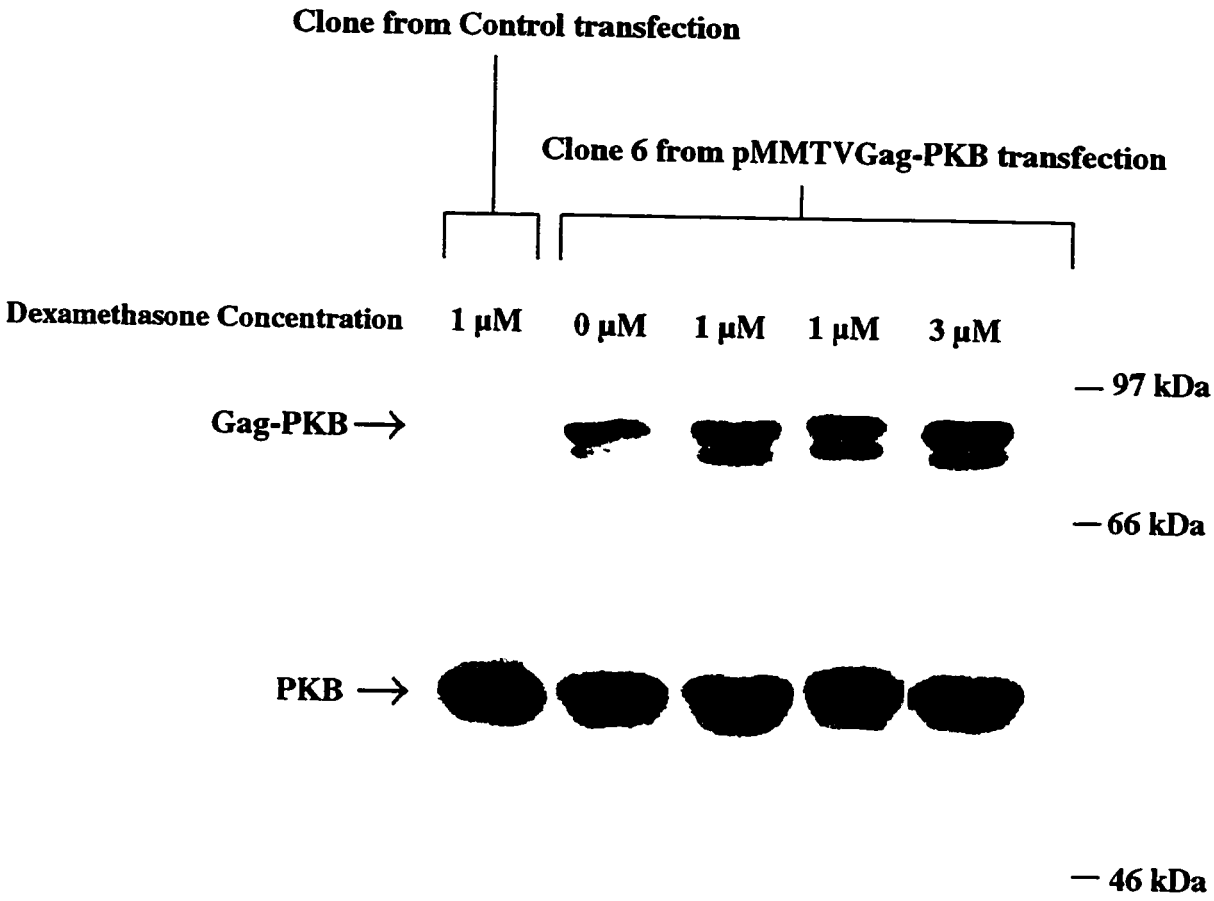
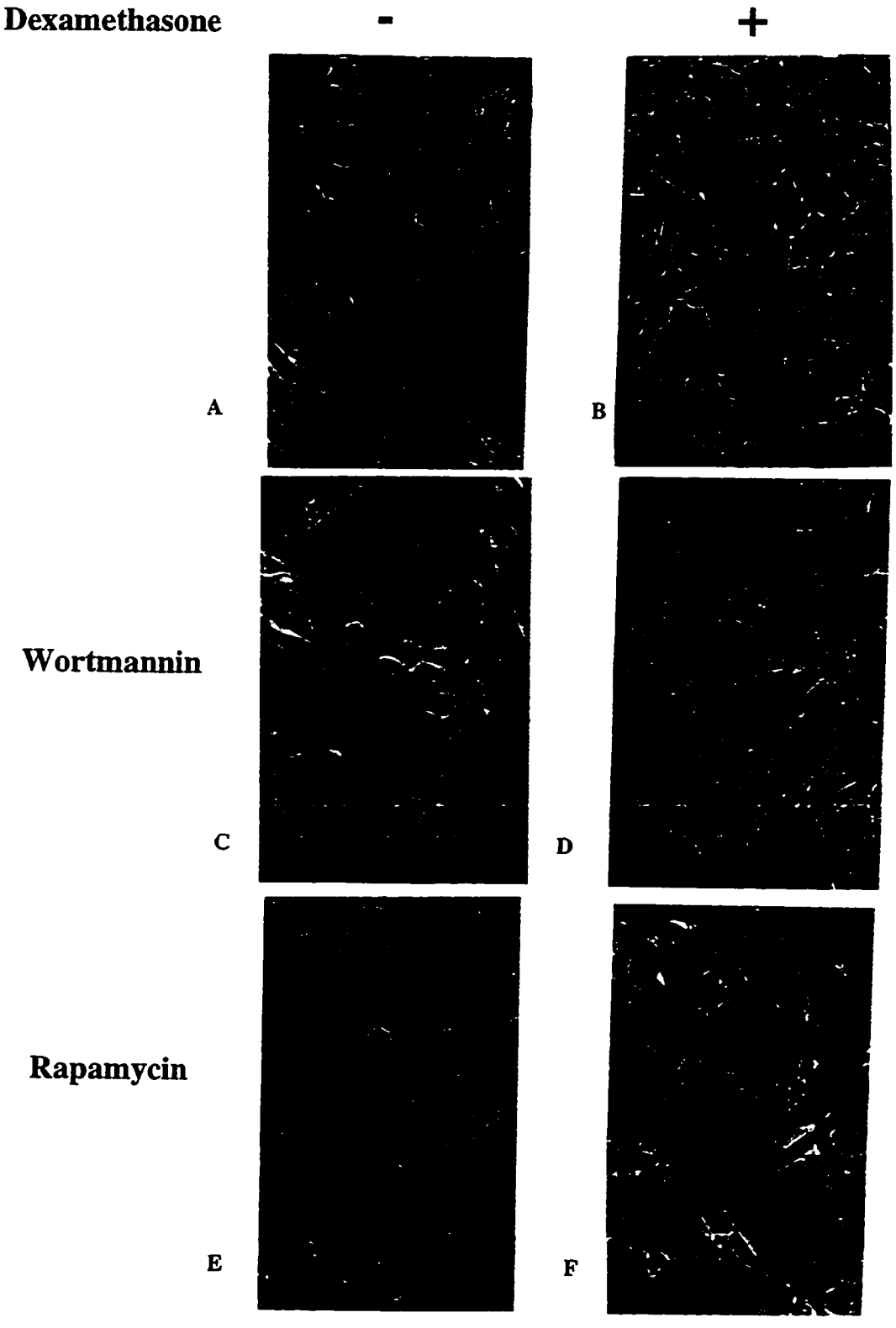

Clone from Control transfection
Clone 6 from pMMTVGag-PKB transfection
Dexamethasone Concentration
1 µM
0 µM
1 µM
1 µM
3 µM
— 97 kDa
Gag-PKB →
— 66 kDa
PKB →
— 46 kDa

Figure 16 - Dexamethasone Induces Adipocyte Differentiation of MMTVGag-PKB(6) cells

Confluent MMTVGag-PKB(6) were incubated with (B,D,F) or without (A,C,E) 1μM dexamethasone for 8 days, as indicated, in the presence of either 100 nM wortmannin (C,D) or 100 nM rapamycin (E,F). Representative fields are shown here at 200x magnification.

Figure 16



DISCUSSION

Our data demonstrate that PKB is expressed in preadipocytes and is responsive to insulin. Furthermore, expression of a constitutively activated form of PKB in 3T3-L1 preadipocytes induces spontaneous adipogenesis. The results demonstrate that the programmed induction of gene expression leading to adipocyte differentiation is subject to regulation by PKB, and suggest a potential role of PKB in insulin-induced adipogenesis.

Initially we determined whether IRS-1 and PI 3-kinase, two proteins which lie upstream in the insulin signalling cascade leading to PKB activation were present and functional in 3T3-L1 preadipose cells. Using immunoblot analysis with anti-phosphotyrosine antibody, we confirmed that insulin stimulated IRS-1 tyrosine phosphorylation. Using an *in vitro* kinase assay, we verified that the insulin-stimulated tyrosine phosphorylation of IRS-1 promoted its association with PI 3-kinase (Backer et al, 1992; Kelly et al, 1993; Myers et al, 1994). Insulin induces recruitment of PI 3-kinase to the membrane through IRS-1 which localizes PI 3-kinase closer to its membrane-bound substrate, PI (4,5) P_2, to produce the 3-phosphorylated lipid products. As previously mentioned, it is these 3-phosphorylated lipid products, PI (3,4) P_2 and PI (3,4,5) P_3, which act as second messengers to mediate PI 3-kinase downstream signalling in general. Insulin stimulation of 3T3-L1 preadipocytes increases the accumulation of PI (3,4,5)P_3, but not PI (3,4)P_2 (Sorisky et al, 1996). The reason for this is currently under investigation.

We determined whether PKB is present in 3T3-L1 cells, and whether its expression was regulated by adipocyte differentiation. PKB was clearly detectable and at the same level in preadipocytes and adipocytes. The equal expression of PKB in both stages of differentiation suggests it potentially plays different roles in both preadipocytes and

adipocytes. For example, our work suggests that PKB plays a role in adipogenesis, whereas other groups have recently suggested PKB can stimulate glucose transport in the mature adipocyte (Kohn et al, 1997b; Tanti et al, 1997)

To further elucidate the role of PKB in preadipocytes, *in vitro* assays were performed to determine if PKB is responsive to insulin. Insulin stimulation of 3T3-L1 preadipocytes produced a four-fold rise in PKB activity as compared to basal activity, which is comparable to the results by Kohn et al (1996b) who showed a six fold rise in the activity of a PKB construct overexpressed in 3T3-L1 preadipocytes. Using an *in vitro* assay similar to ours, Moule et al (1997) have observed a four to five fold stimulation of endogenous PKB in rat adipocytes. Therefore, despite the much lower levels of insulin receptor present in preadipocyte versus adipocyte (Rubin et al, 1978), insulin was capable of stimulating PKB.

Since PKB is responsive to insulin, and adipocyte differentiation is dependent on insulin, we investigated whether there was a role for PKB in 3T3-L1 adipocyte differentiation. As described earlier, PKB has been identified as a downstream target of PI 3-kinase and PI 3-kinase appears to play a role in differentiation of 3T3-L1 preadipose cells (Tomiyama et al, 1995). Previous work in our laboratory has also confirmed the effect of PI 3-kinase inhibitor, wortmannin, on differentiation and its effect on PI(3,4,5)P3 accumulation (Magun et al, 1996). Insulin-stimulated accumulation of PI(3,4,5)P3 was completely blocked by wortmannin. Since inhibition of PI 3-kinase reduced adipocyte differentiation, this provides indirect support that its downstream target, PKB, may be involved in this process. To test this hypothesis, a version of PKB which is constitutively active, Gag-PKB, was transfected into 3T3-L1 preadipocytes. Colonies surviving geneticin (G418) selection from the Gag-PKB transfection (Gag-PKB cells) were observed to spontaneously differentiate without the addition of insulin/IGF-1. Differentiation of these

cells was assessed by positive Oil Red O staining and by upregulation of the differentiation-specific mRNAs, LPL and aP2. The results of the immunoblot assay confirmed that Gag-PKB was expressed. Although, there appears to be more endogenous PKB expressed than Gag-PKB in Gag-PKB cell lysates, the spontaneous differentiation observed is due to the constitutive activation of Gag-PKB versus the presumably unstimulated endogenous PKB (Burgering BMT and Coffer PJ, 1995). Differences in antibody affinity to endogenous PKB versus Gag-PKB cannot be completely excluded, however. Control cells (empty vector with or without *gag* cDNA) did not spontaneously differentiate. However, these cells were able to be subpassaged to form stable cell lines and could differentiate when exposed to a standard differentiation protocol, indicating that transfection with the control constructs did not prohibit differentiation.

Both within as well as between individual Gag-PKB colonies, the pattern of adipose conversion was somewhat heterogeneous, in that each cell did not acquire lipid to the exact same extent. The differences in the degree of differentiation between Gag-PKB colonies might be accounted for by variations in the expression level of the transfected cDNA. The heterogeneity of adipogenesis amongst the cells of a single Gag-PKB colony could be due to particularities of the immediate cellular environment, such as whether the cell is at the growing edge of the colony as opposed to near the center in a zone of relative confluence. In addition, it is likely that some of the cells were not in the quiescent stage (G_0) of the cell cycle which may be required for differentiation to occur (MacDougald, OA and Lane MD, 1995).

While several of the colonies from the Gag-PKB transfection spontaneously differentiated, there was a subset of colonies that did not and this was observed in all four replicates of this transfection. Although this subset of cells most likely expressed little or no Gag-PKB protein, this was not confirmed. Since Gag-PKB cells could not be subpassaged

and stable colonies could not be formed, the relatively small colonies were lysed directly and pooled for immunoblot and Northern blot analysis. Therefore a mixture of cells expressing different levels of Gag-PKB were included in the analysis. Hence, the subset of colonies which expressed little or no Gag-PKB may have decreased the intensity of the band representing Gag-PKB expression in the immunoblot and presumably the LPL and aP2 mRNA signals in the Northern blot.

Our next step was to place the expression of Gag-PKB under the control of an inducible promoter. This strategy was chosen to permit colonies to be subpassaged and therefore to establish stable cell transfectants. Further studies addressing the level of Gag-PKB expression, and the effects of various inhibitors on adipogenesis, will require stable Gag-PKB clones.

The inducible promoter used was the glucocorticoid-responsive MMTV-LTR promoter. This promoter was chosen for the following reasons. First, the MMTV-LTR promoter has been used successfully by Benito et al to regulate the expression of activated ras constructs in 3T3-L1 preadipocytes, which also promote differentiation (Benito et al, 1991; Porras et al, 1994). In addition, it is more advantageous than bacterial origin regulatory systems such as the Escherichia coli tetracycline (tet) repressor system (Gossen et al, 1992). Initially stable cell lines transfected with a tetracycline-resistance gene would have to be established. Then a second set of transfections would have to be performed to obtain neomycin-resistant cell lines that express Gag-PKB under the control of the tet-resistant repressor regulatory system. Repeated passage of 3T3-L1 preadipocytes dramatically decreases their capacity to differentiate, and thus experimental procedures involving a large number of subpassages, as described with the tet regulatory system, are not desirable.

Before placing Gag-PKB under the control of the MMTV promoter, we confirmed that wild-type 3T3-L1 preadipocytes do not differentiate in the presence of dexamethasone. Upon co-transfection of pMMTVGag-PKB and pMexneo, surviving colonies were able to be subpassaged and form stable cell lines. We conducted initial studies to characterize one of these clones. After screening clones using immunoblot analysis with anti-PKB antibody, clone 6 (MMTVGag-PKB(6)) was shown to have the highest level of Gag-PKB expression in the presence of dexamethasone. When MMTVGag-PKB(6) preadipocytes were incubated in the absence of dexamethasone, the cells did not differentiate. However, in the presence of dexamethasone, MMTVGag-PKB(6) cells differentiated into adipocytes as shown by Oil Red O stain. Immunoblot analysis confirmed that treatment of MMTVGag-PKB(6) cells with dexamethasone increased the expression of Gag-PKB. As expected, preadipocytes from the pMMTV control transfection did not differentiate in the presence or absence of dexamethasone. The results suggest that the induced expression of Gag-PKB is responsible for the differentiation observed. We are currently obtaining more MMTVGag-PKB clones to confirm the results observed with clone 6. This will address the possibility of clonal variation, which has been described for 3T3-L1 clones (Tontonoz et al, 1994). In addition, it will be important to determine why the band representing Gag-PKB appeared as a doublet. The lower band may represent a version of Gag-PKB which has a different post-translational modification (i.e. myristolation or phosphorylation). Experiments will be required to determine whether this is the reason for the appearance of this band. Other clones will be required to further characterize the dose-response expression of Gag-PKB in the presence of dexamethasone. Since there was expression of Gag-PKB in the absence of dexamethasone, it will be important to determine whether there is a specific level of Gag-PKB expression required to induce differentiation. This could not be determined from the initial transfections (Figure 9) since colonies expressing different levels of Gag-PKB were

pooled. As well, a plasmid in which gag cDNA is placed downstream of the MMTV promoter is currently being constructed which will serve as even better control with complete certainty for random insertion of the MMTV-LTR in the genome.

Preliminary experiments to determine the effects of PI 3-kinase inhibitor, wortmannin and p70 S6 kinase inhibitor, rapamycin, were performed. MMTVGag-PKB(6) was able to differentiate in the presence of wortmannin indicating that Gag-PKB appears to be placed downstream of PI 3-kinase. Differentiation of MMTVGag-PKB(6) cells in the presence of rapamycin was potently inhibited, which suggests that Gag-PKB is positioned upstream of p70 S6 kinase. Further characterization of MMTVGag-PKB cells is currently being pursued in our laboratory.

Since all of our experiments were performed in serum-containing medium which contains cytokines and other growth factors, this may have some affect on adipose cell differentiation. For this reason, the same batch of serum used to culture Gag-PKB expressing cells was used for the control cells and therefore if serum induced adipose cell differentiation, it would have been seen in the control cells. However, a further experiment to eliminate this possibility, would be to confirm that expression of Gag-PKB induces adipose cell differentiation in serum-free medium.

In summary, observations from both the uncontrolled and controlled expression of Gag-PKB, both indicate that expression of a constitutively active form of PKB can substitute for exposure to insulin in the induction of 3T3-L1 adipocyte differentiation. Subsequent to our studies, a report by Kohn et al (1996b) showed that a constitutively active form of PKB, in which a *src* mysristoylation sequence was added to the amino terminus, also induced spontaneous differentiation of 3T3-L1 preadipocytes, which is in agreement with our findings. Figure 17 shows a model illustrating a possible role for PKB in the signalling pathway downstream of insulin leading to induction of adipogenesis. Future

experiments to further implicate PKB in this process might include determining if 3T3-L1 adipocyte differentiation can be blocked by the inhibition of endogenous PKB, either through pharmacologic means or via dominant-negative constructs as they become available and preliminary characterization of PKB's role in adipogenesis in human preadipocytes.

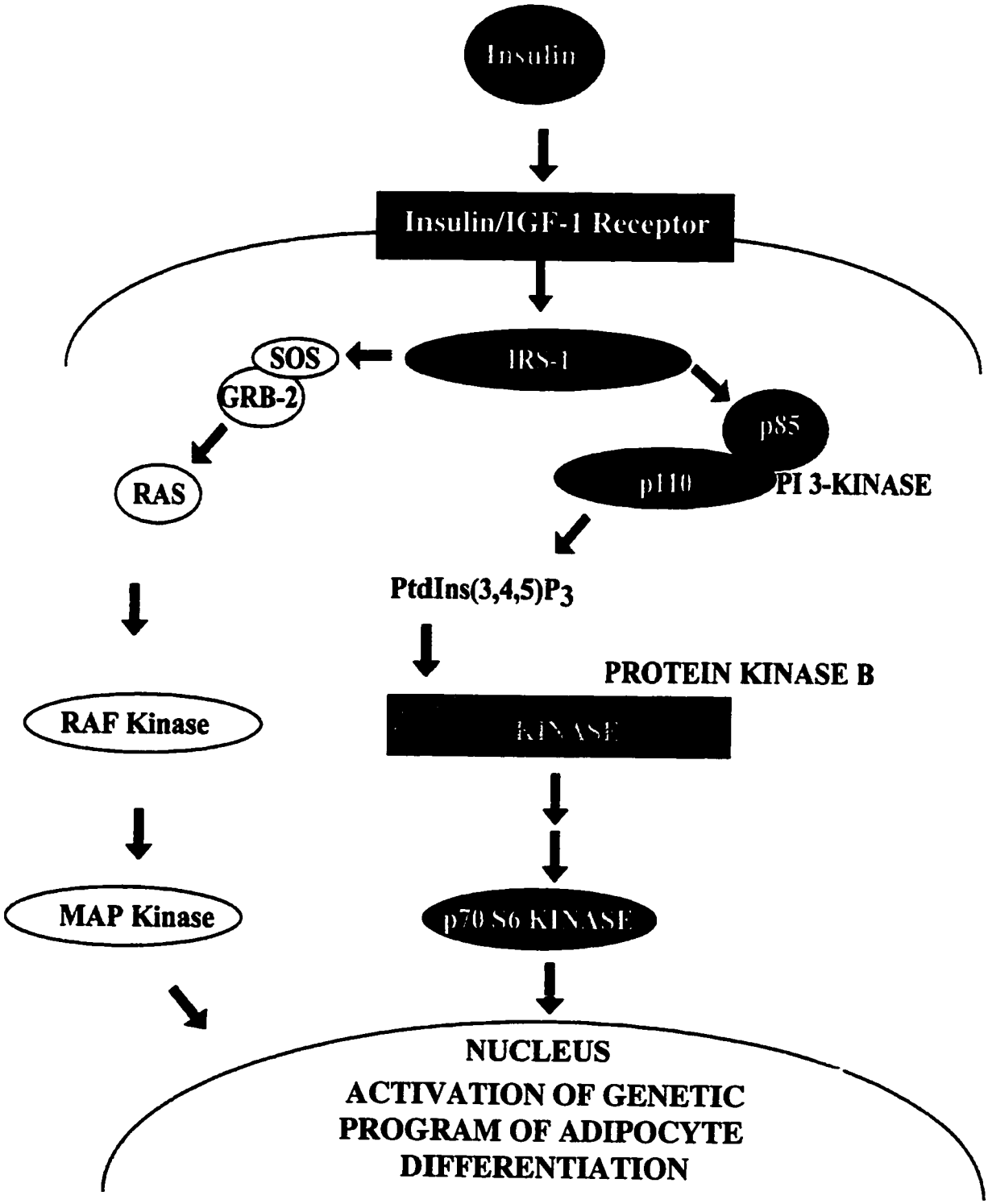
CONCLUSION

In this study, we have shown that PKB is expressed in 3T3-L1 preadipocytes and its activity is increased in response to insulin. Expression of a constitutively active form of PKB, Gag-PKB, was sufficient to induce spontaneous differentiation of 3T3-L1 preadipocytes in the absence of added insulin/IGF-1. The results demonstrate that the genetic program of adipocyte differentiation is subject to regulation by PKB, and suggest a potential role of PKB in insulin-induced adipogenesis. Further characterization of the PI 3-kinase-PKB-p70 S6 kinase pathway with the inducible expression of Gag-PKB described here may lead to a better understanding of the molecular mechanisms that regulate the adipocyte differentiation program.

Figure 17 - A Model Depicting the Potential Role of PKB in Insulin Signalling Leading to Adipocyte Differentiation

Upon ligand binding, activation of the insulin receptor tyrosine kinase results in autophosphorylation of itself as well as IRS-1. This allows docking of specific signalling proteins to specific phosphotyrosine residues on IRS-1. One of these proteins is PI 3-kinase, which can subsequently activate PKB and p70 S6 kinase. In this model, a PI 3-kinase/PKB/p70 S6 kinase signalling pathway is shown to activate the genetic program of adipocyte differentiation. The Ras/Raf/ Map kinase signalling cascade is depicted alongside this pathway.

Figure 17


Insulin
Insulin/IGF-1 Receptor
IRS-1
SOS
GRB-2
RAS
p85
p110
PI 3-KINASE
PtdIns(3,4,5)P3
PROTEIN KINASE B
RAF Kinase
MAP Kinase
p70 S6 KINASE
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APPENDIX A

List of Abbreviations

ASP	Acylation stimulating protein
ATP	Adenosine 5'-triphosphate
BCR	Breakpoint cluster region
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer binding protein
CoA	Coenzyme A
CREM	Cyclic-AMP-responsive element modulator
CS	Calf serum
CSF	Colony stimulating factor
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleicacid
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetate
G418	Geneticin
GAP	GTPase activating protein
GDP	Guanosine 5'-diphosphate
Grb	Growth factor binding protein
GSK	Glycogen Synthase Kinase
hr	hour(s)
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IL	Interleukin
IR	Insulin receptor
IRS-1	Insulin receptor substrate-1
LPL	Lipoprotein lipase
LTR	Long terminal repeat
MAPK	Mitogen-activated protein kinase
$MgCl_2$	Magnesium chloride
min	minute(s)
MIX	Methylisobutylxanthine
MMTV	Mouse mammary tumour virus
MOPS	3-(N-morpholino)propane sulfonic acid
mRNA	messenger Ribonucleic acid
NaCl	Sodium chloride
neo	neomycin
ori	origin of replication

PAGE	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PDGF	Platelet Derived Growth Factor
PH	Pleckstrin homology
PI	Phosphatidylinositol
PI (3)P	Phosphatidylinositol-3-phosphate
PI (3,4)P_2	Phosphatidylinositol-3,4-bisphosphate
PI (3,4,5)P_2	Phosphatidylinositol-3,4,5-trisphosphate
PI (4,5)P_2	Phosphatidylinositol-4,5-bisphosphate
PKA	Protein Kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PPAR	Peroxisome proliferator -activated receptor
PY	Phosphotyrosine
RAC	Related to A and C protein kinase
RNA	ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
SH	Src homology
SOS	Son of Sevenless
SSC	sodium chloride/ sodium citrate buffer
SV	Simion virus
TE	Tris/EDTA
tet	tetracycline
TNF	Tumor Necrosis Factor
TOR	Target of Rapamycin protein
TRIS	tris(hydroxymethyl)amino-methane

APPENDIX B

Curriculum Vitae

NAME:	RICK MAGUN
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1. Magun R, Burgering BMT, Coffer PJ, Pardasani D, Lin Y, Chabot J, Sorisky A (1996) Expression of a constitutively activated form of protein kinase B(c-Akt) in 3T3-L1 preadipose cells causes spontaneous differentiation. Endocrinology 137, 3590-3593.

2. Magun R, Boone DL, Tsang BK, and Sorisky A (1997) Apoptosis of 3T3-L1 cells is dependent on the stage of differentiation. Endocrinology (Submitted).

ABSTRACTS :

1. Magun R, Burgering BMT, Coffer PJ, Sorisky A . Expression of a constitutively activated form of PKB in 3T3-L1 preadipose cells causes spontaneous differentiation. 10th International Congress of Endocrinology (ICE'96) meeting in San Francisco, CA on June 12 ,1996. (Oral Presentation)

2. Magun R, Boone DL, Tsang BK, and Sorisky A . Apoptosis of 3T3-L1 cells is dependent on the stage of differentiation. 57th American Diabetes Association (ADA) Meeting in Boston, MA on June 21-24, 1997. (Poster Presentation)

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IMAGE EVALUATION
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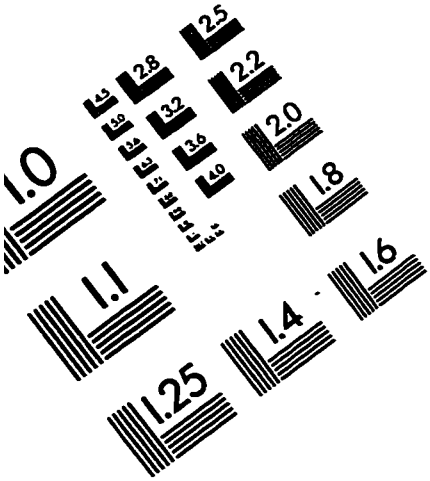

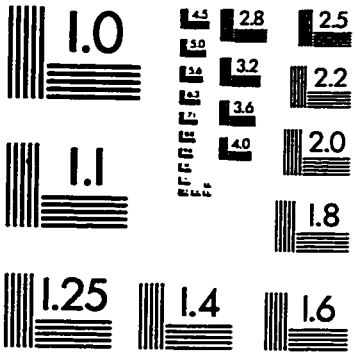

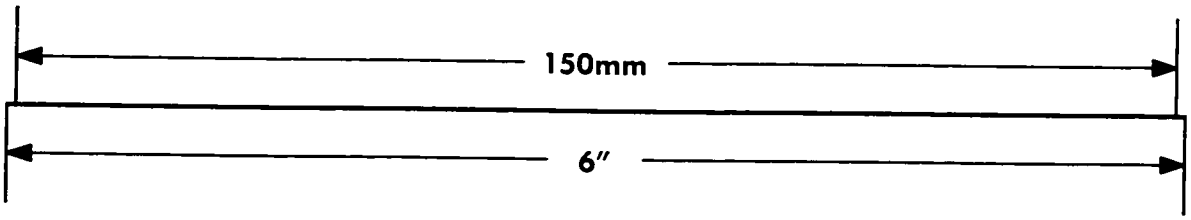

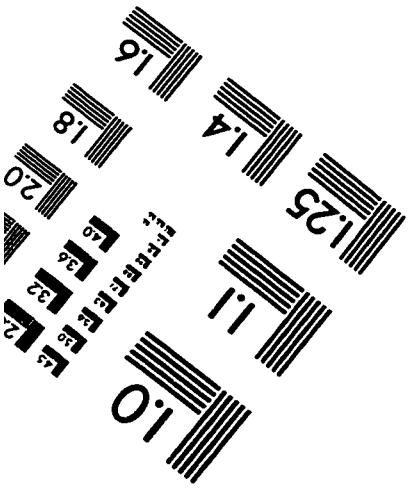

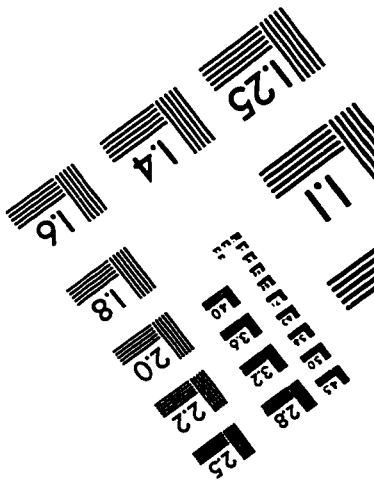

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